Precision medicine in chronic myeloid leukaemia by

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List of abbreviations

AGA	Additional genetic abnormalities
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
AP	Accelerated phase
ASO	Allele specific oligonucleotide
BP	Blast phase
CAST	Competitive allele-specific TaqMan
CCyR	Complete cytogenetic response
CML	Chronic myeloid phase
CNV	Copy number variation
CP	Chronic phase
COSMIC	Catalogue of Somatic Mutations in Cancer
ddPCR	Digital droplet PCR
DMR	Deep molecular response
EFS	Event-free survival
ELN	European LeukemiaNet
EMR	Early molecular response
ESMO	European Society of Medical Oncology
IS	International scale
ITD	Internal tandem duplications
Kb	Kilobase
KD	Kinase domain
LOD	Limit of detection
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MFC	Multi-parametric flow cytometry
MMR	Major molecular response
MRD	Minimal residual disease
NCCN	National Comprehensive Cancer Network
NGS	Next generation sequencing
OS	Overall survival
PCR	Polymerase chain reaction
PFS	Progression-free survival
Ph	Philadelphia chromosome
RNA-seq	RNA sequencing
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
SABER	Single-allele base extension reaction
smMIP	Single molecule molecular inversion probe
SNV	Single nucleotide variant
SV	Structural variant
TFR	Treatment free remission
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
UMI	Unique molecular identification
WES	Whole exome sequencing

Summary

Chronic myeloid leukaemia (CML) has been a prototype of a malignancy that is characterized by a single fusion, the Philadelphia chromosome. Introduction of the BCR::ABL1 targeting tyrosine kinase inhibitors (TKIs) revolutionized the landscape of CML therapy and overall survival over the past two decades has dramatically improved, approaching that of the general population but outcomes continue to remain heterogenous. A proportion of patients are able to permanently cease TKI treatment in a medically observed treatment-free remission (TFR) attempt whereas others progress to the terminal phase of CML, known as blast phase CML. To date, no TKI has been able to offer patients with blast phase CML a long-term curative option and so blast phase remains invariably fatal in the absence of an allogeneic haematopoietic stem cell transplant.

The overarching theme of this thesis is to investigate and identify potential biomarkers and novel investigative techniques that can be utilized to predict outcomes in CML. Chapter 2 details a retrospective analysis of a cohort of patients attempting TFR, highlighting the effect of *BCR::ABL1* halving time on the possibility of a successful TFR, appearing to be one of the strongest predictors of this outcome to date. The frequency of BCR::ABL1 monitoring following a TFR attempt with the potential of a novel monitoring strategy involving fewer assays while maximizing patient safety is addressed in Chapter 3. The development and validation of a customized mass spectrometry assay for detection of novel BCR::ABL1 kinase domain mutations that develop in response to TKI therapy, one of the best understood mechanisms of secondary resistance in CML, is addressed in chapter 4.

Additionally, somatic variants in genes linked to malignancy have been reported in CML in association with resistance and early disease progression to blast phase CML. These variants encompass single nucleotide variants (SNVs) and small insertions/deletions (indels), but also gene fusions and large focal gene deletions. Moreover, novel rearrangements associated with the Philadelphia chromosome has also been described and these events are frequently observed in TKI resistance and blast phase CML. The fifth chapter addresses the development and validation of highly sensitive techniques

that can be utilized to identify these somatic variants and Philadelphia-associated rearrangements (*BCR::ABL1*-dependent and *BCR::ABL1*-independent resistance pathways). Finally, the true incidence and effect of additional genetic abnormalities (AGAs) in newly diagnosed chronic phase (CP) CML patients has never been adequately explored. The sixth chapter of the thesis details the frequency of AGAs, including both somatic variants in cancer genes and Philadelphia-associated rearrangements, in a consecutively treated cohort of newly diagnosed CP-CML patients treated with first-line imatinib. The true effect of AGAs on long term survival and molecular outcomes was also reported in this patient cohort, highlighting that despite a highly proactive strategy of imatinib dose escalation or TKI switch in the event of sub-optimal responses, patients with AGAs had inferior outcomes.

This thesis demonstrates the importance of exploratory biomarkers on the spectrum of responses in CML in addition to the development and validation of novel methodologies utilized to identify them. The inclusion of halving time as a predictor for future TFR attempts will enhance TFR outcome prediction while demonstrating the importance of early TKI selection on future TFR potential. Likewise, the importance of AGAs on CP-CML outcome is highly novel and has the potential to guide optimal TKI selection. These findings illustrate the additive impact these biomarkers have on precision-based risk stratification and establishes the direction of future investigation.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge, it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of South Australia and where applicable, any partner institution responsible for the joint-award of this degree.

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Naranie Shanmuganathan



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Publications during candidature

Chapter 1

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2. Shanmuganathan N, Hughes TP. Molecular monitoring in CML: How deep? How often? How should it influence therapy? *Blood*. 2018;132(20):2125-2133.

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Chapter 5

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Chapter 6

1. Shanmuganathan N, Wadham C, Shahrin, N, et al. Impact of additional genetic abnormalities at diagnosis of chronic myeloid leukemia for first-line imatinib-treated patients receiving proactive treatment intervention. Haematologica. 2023; (early view)

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    a. Presented at the American Society of Hematology annual meeting in December 2022.
2. **Shanmuganathan N**, Wadham C, Shahrin NH, Thomson D, Feng J, Saunders VA et al: The presence of cancer-associated gene mutations and Ph-associated rearrangements at the time of diagnosis of chronic-phase CML predict for inferior outcomes
    a. Presented at the Baikie New Investigator session at Blood 2021
3. **Shanmuganathan N**, Wadham C, Shahrin NH, Thomson D, Feng J, Saunders VA et al: Mutated Cancer-Related Genes Detected at Diagnosis of CML and a Novel Class of Variant Associated with the Philadelphia Translocation Are Both Independent Predictors of Inferior Outcomes. *Blood* 2020, 136 (Supplement 1):46.
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Grants awarded during the candidature of the PhD

1. Cancer Council SA Beat Cancer Project Travel Grant (2018)
2. RACP Margorie Hooper Travel Scholarship (2019)
3. RCPA Grant-in-Aid (2019)……$20,000
4. Royal Adelaide Hospital Clinical Project Grant (2019)……..$50,000

5. Royal Adelaide Hospital Clinical Project Grant (2020)……..$50,000
6. Royal Adelaide Haematology Grant (2021)……..$20,000
7. European School of Haematology John Goldman Research Award (2022) €80,000

Introductory contextual statement

This thesis is submitted as an integrative body of work containing published research and substantive written components. Many concepts introduced within the literature review have been expanded, published and included in the Appendices.

While all patients diagnosed with CML have the diagnostic Ph chromosome, individual responses remain heterogenous despite BCR::ABL1 targeting TKI therapy. The vast majority of patients will have a reasonable response to TKI therapy, preventing disease progression, reverting CML into a 'chronic' illness. However, there are patients who are on the extremes of the disease response spectrum and the drivers of this is not clear, leading to the question of why do some patients progress to the terminal blast phase CML whereas others are able to eventually cease TKI therapy as part of a medically supervised treatment-free remission attempt. Exploration of the available evidence has not only highlighted substantial gaps in our understanding of these processes but also the inadequacy of current standard investigative techniques required to further current knowledge.

The body of work presented in this thesis has two primary aims.

1. An exploratory analysis into predictive biomarkers that can be utilized to discriminate CP- CML patient outcomes at key time points, including at diagnosis, treatment failure and/or prior to an attempt of TFR ***AND***
2. Develop and validate highly sensitive techniques that can be applied for the detection of predictive biomarkers in CP-CML

These aims explore the whole therapeutic spectrum of CP-CML, ranging from ideal optimal responses characterized by successful TFR attempts to therapy resistance and failure. Five specific projects were devised to investigate these aims.

<u>Project 1:</u> Exploration of biomarkers that can be utilized to predict outcomes in treatment free remission in chronic myeloid leukaemia

In recent years, there has been a shift in the focus of CP-CML management with TFR attempts increasingly becoming a key goal of therapy for many patients. An increasing proportion of patients on TKI therapy will achieve and maintain a deep molecular response making them eligible to stop their therapy in an attempt at achieving TFR. However ~50% of patients will require TKI recommencement due to molecular recurrence, the vast majority within the first 6 months of the TFR attempt. There are currently a number of clinical variables that have been able to demonstrate an association with TFR outcome but have poor predictive power regarding outcome prediction and have not been consistently demonstrated with outcome across clinical studies. The duration of TKI exposure and/or deep molecular response prior to the TFR attempt are two such key variables but other factors such as the *BCR::ABL1* transcript and patient age at TFR are others.

As indicated in the published manuscript titled "*Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia*"[1] (Chapter 2), a number of variables were analysed for correlation with TFR outcome. Duration of TKI exposure prior to the TFR attempt and the *BCR::ABL1* transcript type emerged on multivariate analysis as being predictive of a successful TFR attempt. More importantly however, this was the first report to demonstrate the strong relationship between TFR outcomes and the *BCR::ABL1* halving time. Short *BCR::ABL1* halving times indicate a more rapid decline in the leukaemic burden and was found to be associated with a higher potential for TFR success. The *BCR::ABL1* halving time is derived from the kinetics of molecular response within the first 3 months of TKI commencement. This data was also the first evidence that the initial management of CML can affect a patient's eventual TFR eligibility and the potential success of a TFR attempt, confirming that TFR should be a major consideration at the time of diagnosis and optimal therapy selection.

[1] Please note: Sections of the Thesis were published prior to the updated HUGO gene nomenclature committee (HGNC) recommendations in 2021 and so *BCR::ABL1* is also referred to *BCR-ABL1*.

<u>Project 2:</u> Optimization of a *BCR::ABL1* monitoring strategy for treatment free remission attempts that maximise cost-effectiveness and patient safety

A key requirement for a TFR attempt is monthly *BCR::ABL1* quantitative testing within the first year to monitor for molecular relapse which would warrant prompt TKI recommencement to minimize disease relapse/progression. However, this conservative strategy was derived from the preliminary clinical trials that explored the safety and viability of TFR attempts. In fact, this monitoring regime is currently prohibitive in many regions of the world, removing TFR as a viable option for patients due to financial, institutional or regulatory constraints. Modelling data enabled comparative testing of alternative monitoring strategies. Ultimately a strategy was devised which concurrently reduced the testing frequency while maximizing timely detection of molecular relapse, detailed in the published manuscript "*Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission*" (Chapter 3). Application of this novel strategy in resource-poor regions enables TFR to be offered to previously ineligible patients, expanding the health and financial benefits associated with TFR to a larger population.

<u>Project 3</u>: Development and validation of low-level *BCR::ABL1* kinase domain mutation detection by mass spectrometry testing including addition of asciminib-resistant mutations

BCR::ABL1 kinase domain mutations are the best understood mechanism of secondary resistance in CML. These point mutations in *ABL1* generally develop in response to TKI therapy. Conventional TKIs bind to the catalytic domain of *ABL1*, highlighting the historical location of kinase domain mutations development. However, more recently, kinase domain mutations in the myristoyl-binding site were reported in response to the novel 'Specifically Targeting the *ABL* Myristoyl Pocket' (STAMP) inhibitor, asciminib. Modification of the existing highly sensitive mass spectrometry assay was necessary to incorporate the identified asciminib-resistant mutations in addition to other kinase domain mutations of interest. The development, optimization and validation of the customized mass spectrometry is addressed in Chapter 4 (unpublished work).

<u>Project 4</u>: Development and validation of an RNA-based next generation sequencing strategy that can detect relevant genomic mutations and rearrangements in chronic myeloid leukaemia

Preliminary work has demonstrated that CML patients may have *BCR::ABL1* independent resistance pathways activated, with additional variants correlating with poorer outcomes and disease progression. This has emphasized the unmet need for a sensitive method to assist in identifying *BCR::ABL1* independent resistance pathways. Prior studies using RNA-seq and whole exome sequencing using DNA identified various variant subtypes in cancer-related genes associated with treatment failure in CML patients. Variants not only included single nucleotide variants (SNVs) and small insertions/deletions (indels) but also fusion transcripts and partial or whole gene deletions in addition to aberrant splicing. A single cost-effective hybridization capture next-generation sequencing method using total RNA was developed to detect all relevant mutation subtypes. Specific coverage of *BCR* and *ABL1* was essential to identify novel rearrangements associated with the *BCR::ABL1* fusion. The method can be applied to either DNA or RNA with excellent concordance. However, only RNA can interrogate the spliceosome and identify fusion transcripts. The development and validation of the customized next generation sequencing panel is covered in the manuscript titled "*RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia*" (Chapter 5).

<u>Project 5</u>: Correlation of additional genetic abnormalities identified at diagnosis with survival and molecular outcomes in chronic phase CML treated with frontline imatinib

While prior genomic studies of CML patients suggested a link between cancer gene variants and blast phase in highly selected patients, the true incidence and effect of potentially pathogenic variants at diagnosis of CP-CML is not known as their effect on treatment failure and molecular response has not been studied in a consecutive, uniformly treated cohort of patients. Therefore currently, evidence-based treatment decisions based on the genetic profile at diagnosis cannot be made. Genomic

investigation of diagnostic samples of a large consecutive cohort of newly diagnosed CP-CML patients who were uniformly treated with first-line imatinib 600mg daily was performed (Chapter 6). One third of patients with CP-CML had AGAs at diagnosis and those with AGAs had a higher incidence of treatment failure, *BCR::ABL1* kinase domain mutations, and inferior molecular responses. Importantly, switching to nilotinib in the event of suboptimal response failed to rescue deep molecular response in patients with AGAs. These findings establish the foundation for clinical trials where the genomic profile will determine the therapeutic approach. Of great interest will be whether more potent first-line TKI therapy will overcome the negative impact of AGAs or if a TKI plus drugs targeting other aberrant pathways will be more effective in this setting. This data provides critical evidence to support revised clinical guidelines to incorporate next generation sequencing for the diagnostic work up of CP-CML patients for genome-based risk stratification.

Together, the body of work presented in this thesis addresses some of the existing gaps in knowledge and technology in CML, demonstrating the importance and predictive capabilities of specific biomarkers in addition to the technical capabilities of the customized highly sensitive assays. These findings contribute to the evolving precision medicine approach to haematological malignancy, specifically in CML and presents a platform for ongoing research.

1 Background

This chapter comprises the literature review portion of the thesis. Concepts within this review were adapted into the five major studies within this thesis.

1.1 Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative neoplasm, characterized by a single diagnostic fusion oncogene (*BCR::ABL1*) termed the Philadelphia chromosome in an abnormal pluripotent hematopoietic stem cell[1]. The majority of patients are diagnosed in chronic phase (CP) but a small proportion (~5%) are identified in the more aggressive stages of accelerated phase (AP) or blast phase (BP). The incidence of CML in the United States of America is approximately 1.8 per 100,000 people with approximately 8,430 new cases diagnosed in 2017-2018 alone.[2] Australian data is similar with an incidence of 1.1 per 100,000 people in 2013. Survival in CML has historically been dismal with the 5-year relative survival in 1995 approximating 35%; allogeneic stem cell transplantation being the only option for long-term survival and prevention of progression to the more advanced stages of CML. However, many patients do not qualify for allogeneic transplantation due to the significant associated risks of morbidity and mortality. Consequently, the development of targeted agents with marked activity against the BCR::ABL1 fusion protein revolutionized CML therapy over 20 years ago. The introduction of these drugs, known as tyrosine kinase inhibitors (TKIs), has been associated with a dramatic survival benefit; from being virtually incurable in the absence of an allograft to a life approaching that of the general population;[3] reclassifying CML as a 'chronic disease'. However, despite these advances, patient responses remain heterogeneous.

Translocation of *ABL1* from chromosome 9 to chromosome 22 with the resultant fusion to *BCR* leads to the formation of the *BCR::ABL1* gene which is a key pre-requisite for the diagnosis of CML.[4] Physiological regulation of the *ABL1* gene is performed by the N-terminal cap which is lost through formation of the fusion gene, resulting in unrestrained kinase activity.[5] The TKIs target and bind to the fusion chimeric protein at varying intensities, inhibiting leukaemogenesis through reduction in clonogenicity, regulation of differentiation and restoration of the impaired apoptotic pathway.[6] The efficacy of imatinib, the first-generation TKI inhibitor, was established by the pivotal IRIS study which compared imatinib to the then current standard of care, interferon and cytarabine.[7] IRIS demonstrated that imatinib monotherapy was associated with dramatic improvements in tolerability, molecular, cytogenetic and survival outcomes;[7] fundamentally altering the landscape of CML therapy. However, primary treatment failure still occurs with an annual incidence of 1-7%[7] in CP-CML treated with frontline imatinib. Furthermore, progression to the more advanced stages of CML occurs in approximately 7% of imatinib treatment.[7, 8] To combat imatinib resistance, extremely potent second- and third-generation TKIs (nilotinib,[9] dasatinib,[10] bosutinib,[11] ponatinib)[12] were developed to be utilised upfront or sequentially. More recently, asciminib, originally known as ABL001, was developed and is a first-in-class allosteric inhibitor of BCR::ABL1 kinase activity through inhibition of the myristate site, mimicking the function of the N-terminal cap.[13] While the drug is not widely available, it has been licensed for use for the treatment of chronic phase CML in patients who have either failed at least 2 lines of therapy.[14] Although these more potent TKIs can rescue patients with resistance or provide more rapid molecular responses in the first line setting, this has not translated to a survival benefit as yet.[9-11]

The early investigation and management of CML[15] was expanded and published as a peer reviewed paper (Shanmuganathan N, Hughes TP. Early Management of CML. *Current hematologic malignancy reports.* 2019;14(6):480-91) that is included as Appendix A.

1.2 Molecular monitoring in CML

Measurement of *BCR::ABL1* mRNA expression through reverse-transcription quantitative polymerase chain reaction (RT-qPCR), reported according to the International Scale (IS), has become the primary tool for molecular monitoring and response assessment in CML. Patient results are reported according to the IS where results are expressed relative to a baseline of 100%, enabling standardization across individual laboratories.[16] The degree of leukaemic cell burden translates to critically important prognostic information and specific molecular milestones are incorporated into global treatment guidelines to assist with therapeutic decisions. Failure to achieve milestones or loss of previously attained response is concerning for the development of resistance and this should trigger further investigation.[17] TKI therapy may need to be altered to enable optimal outcomes through milestone achievement but to also prevent progression to BP.

This section has been expanded and published as a peer reviewed article,[18] (Shanmuganathan N, Hughes TP. Molecular monitoring in CML: How deep? How often? How should it influence therapy? *Blood*. 2018;132(20):2125-33) which has been included as Appendix B.

1.3 Landscape of responses in CML

The primary objective of TKI therapy is to prevent progression to BP-CML, characterized by the rapid expansion of either myeloid or lymphoid blasts.[1,19] Despite the improvement in patient outcomes with the introduction of TKI therapy, blast phase is refractory to TKI therapy and the only curative option is allogeneic stem cell transplantation.[20, 21] The *BCR::ABL1* oncogene is clearly sufficient to initiate chronic phase CML but transformation to blast phase requires acquisition of additional poorly defined driver mutations.[19, 22] Mutations implicated in other haematological malignancies (e.g. *RUNX1*, *ASXL1*, *IDH1*) have been identified in BP-CML with evidence of likely development at time of transformation.[19, 23] The significance of these mutations in the evolution to BP-CML is unknown but further definition of these previously unknown pathways may allow for more appropriate upfront TKI selection but also modification of therapy to prevent poor outcomes and blast phase transformation.

While achieving therapeutic milestones and optimal outcomes will minimize the risk of progression to BP, select patients with deep molecular responses may eventually discontinue therapy. Following a number of clinical trials demonstrating the success of medically supervised TKI cessation in carefully selected patients with optimal outcomes,[24, 25] treatment-free remission (TFR) is now entering mainstream practice and increasingly being adopted as an important goal of therapy. TFR success has a number of health and economic implications, including eliminating the morbidity associated with TKI toxicity and a reduction in therapy costs for either the patient, the drug funder, or both. Predictors of TFR success are predominantly clinical and include duration of TKI exposure and deep molecular response. Investigational biomarkers[26-28] have been identified as potential tools for predictors of TFR success but require validation in larger studies. To date, specific genes have not been associated with TFR eligibility nor optimal response but the development of more sophisticated tools to enable the early identification of optimal and poor responders (patients with multi-drug resistance and blast transformation) will be a useful prognostic tool at the time of diagnosis, enabling appropriate TKI selection to optimize patient outcomes.

1.4 Current risk stratification

The strongest predictor of patient outcomes remains initial molecular responses in the first year of TKI therapy, but at diagnosis, poor responders cannot be reliably differentiated from patients with eventual optimal outcomes. Clinical scoring systems, such as the Sokal and Hasford risk score, incorporate simple clinical and laboratory parameters to risk stratify CML patients.[29, 30] While these models were developed in the pre-TKI era, they remain widely utilized for prediction of molecular response.

The EUTOS score was subsequently developed and validated in an imatinib-treated cohort and was felt to be a simpler and more refined prognostic model, having removed factors in the prior risk calculators that fail to predict imatinib response.[31] This then led to the subsequent development of the EUTOS Long Term Survival score, best known as the ELTS score, which utilized the same variables as the Sokal model with different

weighting, to improve long-term survival prediction, specifically for CML-related death.[32] However, up until recently, only the Sokal and Hasford risk scores were incorporated into the NCCN[33] and the ELN guidelines.[34] However, the more recent iterations of the NCCN guidelines[35] include the ELTS score in the risk stratification tools while the 2020 ELN guidelines[17] preferentially recommends the ELTS model over alternative risk scores due to the expectation that the ELTS score is more reflective of current treatment practices and therefore more accurate in the TKI era.[36]

Although there is some evidence that patients deemed high risk by these traditional prognostic models have improved molecular responses with second-generation TKIs,[37, 38] the more potent agents do not necessarily abrogate the adverse effect of a high-risk score.[37] High-risk patients are less likely to achieve molecular targets but also have a higher risk of CML-related deaths. Poor disease control is associated with a higher risk of BP-CML development; indicating how our current risk stratification models are only indirectly predictive of disease progression.

1.5 Resistance in CML

TKI resistance mechanisms are not completely understood with both primary and secondary resistance being observed in CML patients. The mechanism of primary resistance, characterized by early refractoriness to TKI therapy, is poorly defined. Secondary resistance, described as initial achievement of therapeutic milestones with subsequent loss of response, is more frequently observed. The development of *BCR::ABL1* kinase domain (KD) mutations, primarily detected in secondary resistance,[39, 40] is the best understood mechanism of resistance.[41] KD mutations occur in the drug binding region and over 100 mutations have been described.[39] KD mutations confer varying degrees of drug resistance and direct the choice of appropriate sequential TKI therapy.[41] Despite appropriate TKI selection, directed by the sensitivity of KD mutations if present, patients do not always respond; indicating the presence of other resistance pathways.

1.5.1 Kinase domain mutations

Discovery of KD mutations in imatinib-treated patients is an adverse prognostic factor, conferring an increased risk of transformation to AP- or BP-CML while predicting for an inferior progression free survival (PFS) and overall survival (OS).[39] The risk of KD mutation development with upfront second-generation TKI therapy is lower compared with imatinib therapy although the prognostic implication is uncertain.[37, 42] The most common *BCR::ABL1* mutation, T315I, confers resistance against all first and second-generation TKIs, and results in inferior OS and PFS, even in comparison to the other KD mutations.[39] Harbouring multiple KD mutations is also associated with a poorer outcome.[43] IC_{50} (50% inhibitory concentration) values are published for the most frequently detected KD mutations, enabling appropriate sequential therapy selection.[40] Inadvertent selection of a TKI with reported resistance against the identified mutation is usually associated with therapy failure with expansion of the resistant clone.[44]

Direct (Sanger) sequencing is the currently accepted technique for investigation of KD mutations but has relatively low sensitivity.[40] Pre-screening samples of patients with suspected resistance with techniques such as denaturing-high performance liquid chromatography (D-HPLC) can minimize the number of samples required for sequencing, improving the detection limit to 1%. More sensitive tools such as mass spectrometry, having a detection limit of ~0.2%, have been utilised to identify low-level mutations in patients with imatinib resistance.[44] The improved detection limit capitalizes on the single-allele base extension reaction (SABER) protocol, which restricts primer extension to the mutant sequence.[45] The presence of low-level mutants (KD mutations detected on mass spectrometry but not by direct sequencing) has been shown to be predictive of therapy failure in the setting of inappropriate therapy selection.[44]

Advances in next generation sequencing (NGS) has enabled this technology to be applied to sensitive KD mutation detection in CML with recent data demonstrating the superiority of a targeted sequencing panel over the historical gold standard of direct sequencing. A recent Italian study investigated 236 CML patients who had experienced treatment failure with an amplicon based NGS panel.[46] Fifty-one patients with no

detectable KD mutation by direct sequencing had mutations identified by the NGS panel with a further 29 patients having additional low-level mutations; 53% of these events were deemed clinically relevant as they would have influenced treatment decisions.[46]

While KD mutations are the best understood mechanism of resistance in CML, they are only identified in 12-63% of imatinib failure and ~50% of patients with secondary resistance.[39] This suggests the presence of alternative resistance pathways which will be explored further.

Concepts presented in this section have been expanded and published as a peer reviewed article, (Shanmuganathan N, Branford S. Multiplex technologies for the assessment of minimal residual disease and low-level mutation detection in leukaemia: mass spectrometry versus next-generation sequencing. *British Journal of Haematology*. 2022;196(1):19-30.) which has been included as Appendix C.

The utility of NGS in CML for kinase domain mutation detection has also expanded as a commentary (Shanmuganathan N, Hughes T.P. What's NEXT for CML-NGS mutation screening. *Blood.* 2020;135(8):515–516.) written in response to one of the pivotal papers addressing mutation detection in CML was published in *Blood* and has been included as Appendix D.

1.5.2 Other Resistance mechanisms

1.5.2.1 Potential *BCR::ABL1* dependent resistance mechanisms

At diagnosis, additional chromosomal abnormalities are observed in ~5% of CP-patients but more frequently in AP and BP. Historically, major route abnormalities (trisomy 8, isochromosome 17q, a second Philadelphia chromosome, trisomy 19) at diagnosis predicted for poorer survival and molecular outcomes although a recent publication questions this tenet,[47] which may be explained by the small sample size or secondary to patients being treated upfront with more potent TKIs. Increased BCR::ABL1 expression (via a second Philadelphia chromosome, *BCR::ABL1* gene amplification or increased oncogene transcription), although uncommon, has also been associated with TKI

resistance. Efflux and influx transporters have been implicated in resistance mechanisms in many malignancies and *in vitro*, overexpression of ABCB1 and ABCG2 is associated with a reduction in intracellular TKI concentrations, potentially contributing to clinical TKI resistance. Influx transporters such as OCT-1 mediate imatinib entry into cells and patients with low OCT-1 activity have been shown to have inferior molecular and survival outcomes, increasing the risk of mutation development and disease progression.

1.5.2.2 Potential *BCR::ABL1* independent resistance mechanisms

While KD mutations are the major known cause of secondary resistance in CML, their role in primary resistance is probably minimal. Furthermore, despite appropriately selected therapy based on identified KD mutations, not all patients respond. Multiple signalling pathways (including STAT3, PI3K, SRC) have also been implicated as potential contributors to disease progression. More recently, mutations in other genes have been identified in BP-CML which are already implicated in a variety of myeloid malignancies. Mutations, such as *ASXL1*, confer a poorer prognosis in acute myeloid leukaemia[48] and its identification in CML patients may correlate with a similar adverse outcome.[49] The other mutations also associated with acute myeloid leukaemia, such as *IDH1*, have targeted therapeutic agents currently under investigation and approved in select scenarios,[50] raising the possibility of combination therapy using an IDH1 inhibitor with conventional TKIs if *IDH1* mutations are implicated in the pathogenesis of BP-CML.

Prior work investigated selected CML patients (n=65) on opposing ends of the therapeutic response spectrum (BP vs. good responders) with a combination of whole exome sequencing (WES), copy number variation and/or RNA-seq.[51] Mutations in cancer genes were identified in all patients (n=39) sequenced in BP-CML whereas these mutations were infrequent in patients with CP-CML with optimal responses. The most frequent mutations observed were *IKZF1, RUNX1 and ASXL1*, although multiple other mutations implicated in haematological malignancies were also identified.[51] Other somatic variants identified include gene fusions, including novel fusions associated with the Philadelphia translocation, and copy number variations.[51] Of the 33 patients who had RNA-Seq performed at BP-CML, 64% had gene fusions identified; a proportion

involving known leukaemia-associated fusions or having a known leukaemia-associated gene as a fusion partner.[51] WES analysis of 20 BP-CML patients revealed that 90% also had copy number variations.[51] Patterns of mutation acquisition in these extremes of responses were also evaluable in patients with matched samples at diagnosis and/or progression. All patients (n=5) diagnosed in either de novo AP- or BP-CML had 1-2 cancer gene mutations identified.[51] In the cohort tested at CP-CML, 18 patients had 1-4 cancer gene variants at diagnosis, including genes with known haematological significance (e.g. *RUNX1*, *TP53*, *IKZF1* and *ASXL1*).[51] Poor outcomes (evolution to BP-CML or primary TKI resistance) were observed in 15/18 patients.[51] A cohort of BP-CML patients who had absence of mutations at diagnosis of CP-CML demonstrated acquisition of mutations in known cancer genes at the time of BP[51]; potentially implicating these genes in the pathogenesis of BP-CML.

Further discussion and exploration of the data presented in this section is presented as a review article, (Shanmuganathan N, Branford S. The Hidden Pathogenesis of CML: Is *BCR-ABL1* the First Event? *Current hematologic malignancy reports*. 2019;14(6):501-6) which has been included as Appendix E.

1.5.3 Managing resistance in in CML

Despite the number of TKIs available for management of CML, not all patients reach therapeutic milestones while intolerance and toxicity remain legitimate concerns. Identification of the pan-TKI resistant KD mutation, T315I, triggered drug development to generate a TKI with activity against this mutation. Ponatinib, a third-generation TKI, is the only conventional agent with activity against this KD mutation. However ponatinib is associated with a high risk of arterial occlusive events (27% by 38 months),[12] significantly limiting its use. Utilizing fragment-based nuclear magnetic resonance, asciminib, the first in class 'Specifically Targeting the ABL Myristoyl Pocket' (STAMP) inhibitor, was developed. Unlike conventional TKIs which bind to the catalytic domain of *ABL1*, asciminib binds to the myristoyl pocket of *ABL1*, mimicking the physiological function of the N-terminal cap.[13] Preliminary phase I data demonstrated efficacy and tolerability in heavily pre-treated patients, including activity in patients with T315I

mutations.[13] *In vitro* studies demonstrated emergence of novel myristoyl-binding site mutations (e.g. A337V, V468F and I502L) conferring resistance to asciminib in the observed cell line.[13] Although these novel mutations were identified *in vitro*, the clinical relevance was not appreciable until the appearance of I502L and V468F correlated with progression to blast phase in the phase I study in a single patient.[13] Conventional mutational analysis with direct sequencing could fail to identify these novel mutations, especially if they are only present at low levels. More sensitive mutation analysis methods will need to be developed to ensure that these mutations are appropriately identified.

Further discussion and exploration of the data presented in this section is presented as a review article, (Shanmuganathan, N, Hughes, TP. Asciminib for chronic myeloid leukaemia: Next questions. *British Journal of Haematology*. 2022;00:1–10) which has been included as Appendix F and a review chapter (Hughes TP, Shanmuganathan N: Management of TKI-resistant chronic phase CML. *Hematology* 2022, 2022(1):129-137.), which has been included as Appendix G.

1.6 Research questions

1.6.1 Thesis rationale

While there have been substantial changes in the management of CML over the past 2 decades, a more personalized approach is required especially in the setting of resistance.

1.6.2 Overarching aim of the thesis

The overarching aim of this thesis is to develop novel techniques to study resistance mechanisms in CML in addition to the identification of potential biomarkers that can be utilized to predict specific outcomes such as achievement of treatment free remission and relevant molecular targets.

1.6.3 Research process

Several studies were conducted to address the aim of this thesis. The research projects presented in this body of work were undertaken in Adelaide, Australia. The projects were broadly divided into five related studies over the course of the candidature.

1.6.4 Summary of projects[1]

Project 1: Exploration of biomarkers that can be utilized to predict outcomes in treatment free remission in chronic myeloid leukaemia

Project 2: Optimization of a *BCR::ABL1* monitoring strategy for treatment free remission attempts that maximise cost-effectiveness and patient safety

Project 3: Development and validation of low-level BCR::ABL1 kinase domain mutation detection by mass spectrometry testing including addition of asciminib-resistant mutations

Project 4: Development and validation of an RNA-based next generation sequencing strategy that can detect relevant genomic mutations and rearrangements in chronic myeloid leukaemia

Project 5: Correlation of additional genetic abnormalities identified at diagnosis with survival and molecular outcomes in chronic phase chronic myeloid leukaemia treated with frontline imatinib

[1] Please note: Sections of the Thesis were published prior to the updated HUGO gene nomenclature committee (HGNC) recommendations in 2021[52] and so BCR::ABL1 is also referred to BCR-ABL1.

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2 Exploration of biomarkers that can be utilized to predict outcomes in treatment free remission in chronic myeloid leukaemia

Preface

TFR attempts are increasingly becoming a key goal of therapy for many patients at the time of diagnosis of CP-CML. The benefits of ceasing TKI in a medically supervised treatment-free remission (TFR) attempt include relief of financial pressures and to alleviate the burden of TKI induced toxicity. However, approximately 50% of patients will experience molecular relapse within the first year of TKI interruption, necessitating prompt TKI recommencement. While TFR remains an ambitious ideal for patients, there is a paucity of predictive biomarkers that correlate with TFR outcomes.

This chapter details the results of a study investigating the TFR cohort of South Australia, investigating 130 patients that underwent a medically supervised TFR attempt. The study's aim was to investigate multiple variables for an association with TFR outcome. The kinetics of *BCR::ABL1* decline within the first 3 months of TKI commencement was identified to be a powerful predictor of TFR outcome and was the first report of the effect of how the initial *BCR::ABL1* response influenced eventual TFR eligibility and outcome. The *BCR::ABL1* halving time was the preferential mode of measurement of the *BCR::ABL1* kinetics within the first 3 months of TKI therapy, with a short halving time correlating with a more rapid reduction in leukaemic burden. Patients with a short halving time (e.g. <9.35 days) had a higher probability of remaining in TFR at 12 months compared with those with longer halving times (e.g. >21.85 days). These results were validated using a cohort of 26 TFR patients from collaborators in Korea. Additionally, this study also confirmed that duration of TKI exposure and the *BCR::ABL1* transcript type correlated with TFR outcome.

The results of this study was published in Blood as "*Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia*", reference included. The supplemental files are included at the end of the chapter.

- Shanmuganathan N, Pagani IS, Ross DM, et al. Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia. *Blood*. 2021;137(9):1196-1207.

Early *BCR-ABL1* kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia

Shortened title: *BCR-ABL1* halving time and TFR

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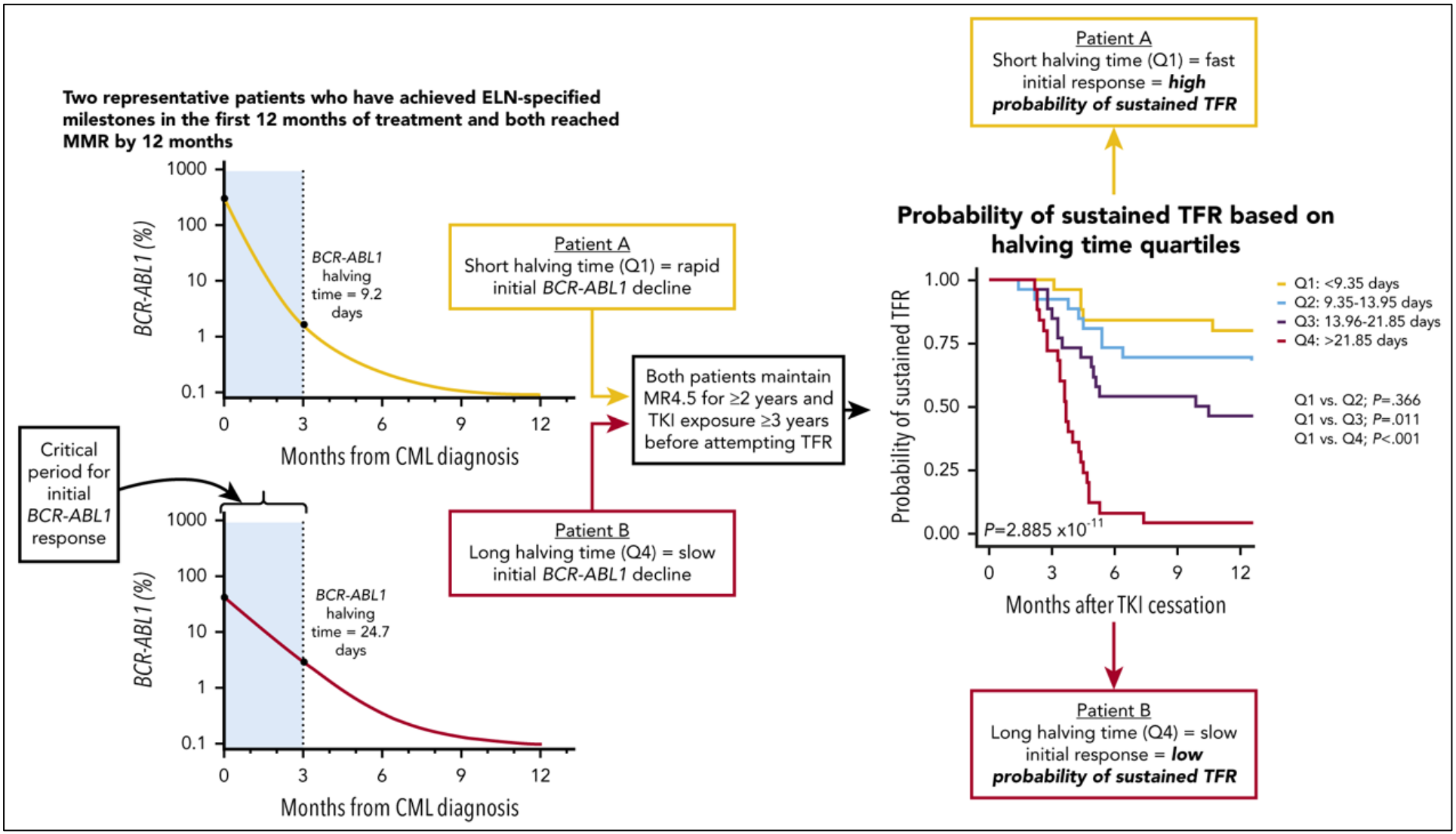
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Keywords: Chronic myeloid leukemia, *BCR-ABL1*, minimal residual disease, treatment-free remission, halving time

2.1 Key points

- Rate of reduction in *BCR-ABL1* transcripts over the first 3 months of therapy is a strong predictor of sustained treatment-free remission
- Slower rates of *BCR-ABL1* decline correlate with longer duration of drug exposure to become eligible for a treatment-free remission attempt

Visual abstract



2.2 Abstract

With treatment-free remission (TFR) rapidly becoming the ultimate goal of therapy in chronic myeloid leukemia (CML), there is a need to develop strategies to maximise sustained TFR by improving our understanding of its key determinants. Chronic phase CML patients attempting TFR were evaluated to identify the impact of multiple variables on the probability of sustained TFR. Early molecular response dynamics were included as a predictive variable, assessed by calculating the patient-specific halving time of *BCR-ABL1* after commencing tyrosine kinase inhibitor (TKI) therapy. Overall, 115 patients attempted TFR and had ≥12 months follow-up. The probability of sustained TFR, defined as remaining in major molecular response off TKI therapy for 12 months, was 55%. The time taken for the *BCR-ABL1* value to halve was the strongest independent predictor of sustained TFR: 80% in patients with a halving time of <9.35 days (first quartile) compared with only 4% if the halving time was >21.85 days (last quartile) (*P*<.001). The e14a2 *BCR-ABL1* transcript type and duration of TKI exposure before attempting TFR were also independent predictors of sustained TFR. However, the *BCR-ABL1* value measured at 3 months of TKI was not an independent predictor of sustained TFR. A more rapid initial *BCR-ABL1* decline after commencing TKI also correlated with an increased likelihood of achieving TFR eligibility. The association between sustained TFR and the time taken for *BCR-ABL1* to halve after commencing TKI was validated using an independent dataset. These data support the critical importance of the initial kinetics of *BCR-ABL1* decline for long-term outcomes.

2.3 Introduction

Sustained treatment-free remission (TFR), defined as remaining off tyrosine kinase inhibitor (TKI) therapy in a major molecular response (MMR, *BCR-ABL1* ≤0.1% on the International scale, IS), is increasingly accepted as the ultimate therapeutic goal for patients diagnosed with chronic phase chronic myeloid leukemia (CP-CML) in routine clinical practice.[1-3] To date, the published success rate of TFR attempts, that is TKI discontinuation in a stable deep molecular response (DMR; defined as either MR4.0, *BCR-ABL1*^{IS} ≤0.01% or MR4.5, *BCR-ABL1*^{IS} ≤0.0032%) leads to sustained TFR in 40-65% of patients.[4-8] Understanding TFR biology may assist in

determining why particular patients remain in a sustained TFR whereas others experience molecular relapse. TKI discontinuation trials[4,5,7,8] have investigated numerous parameters of patients attempting TFR, with the aim of identifying the key predictors of successful drug discontinuation to improve patient selection and maximize the chances of sustained TFR.

Currently few clinical variables demonstrate a consistent relationship with sustained TFR. The strongest to date are durations of TKI therapy and DMR prior to a TFR attempt. The French STop IMatinib (STIM) study[7] demonstrated a reduced risk of molecular relapse with imatinib exposure of >54 months. The interim analysis of the European Stop Kinase Inhibitor (Euro-SKI) study[4] validated the association and demonstrated that patients with >5.8 years of TKI exposure had a lower risk of molecular relapse. The Korean Imatinib Discontinuation (KID)[9] and De-Escalation and Stopping Treatment with Imatinib, Nilotinib, or sprYcel (DESTINY)[10] studies confirmed the link between TKI duration and sustained TFR. Likewise, a longer duration of nilotinib treatment prior to the TFR attempt, rather than total TKI exposure, was associated with a higher rate of sustained TFR in ENESTop.[11] However, duration of DMR was a stronger predictor of TFR outcomes in the Euro-SKI study where >3.1 years of MR4.0 was associated with a reduced molecular relapse rate at 6 months.[4] Similarly, duration of MR4.5 prior to the TFR attempt predicted molecular relapse in the ENESTop study.[11]

The majority of TFR studies have enrolled patients treated with the first-generation TKI, imatinib. First-line treatment with second-generation TKIs (2G-TKIs) lead to more rapid DMR,[12,13] which could increase the rate of TFR eligibility over the first 5 years of therapy.[14] However, the overall rate of sustained TFR is similar.[8,15] Older age was associated with increased sustained TFR in some clinical trials[16,17] but was not validated in other studies. Withdrawal syndrome secondary to TKI cessation had a weak association with sustained TFR in the KID study,[9] but has not been reported consistently. A weak association between high Sokal risk and molecular relapse was demonstrated in the Australian CML8 study,[5] supporting similar findings from the STIM trial.[7] The *BCR-ABL1* e14a2 transcript was associated with higher rates of sustained TFR in small clinical series.[18,19] Other biological variables were identified as predictors of TFR outcomes in individual studies but require further validation before being translated into clinical practice.[20-25]

Understanding the variables that predict TFR are critical for treatment decisions. The proportion of patients who achieve a sustained TFR depends on two key factors: the proportion of patients who achieve TFR eligibility, and the proportion of TFR-eligible patients who discontinue TKI and remain in MMR. An early molecular response (EMR, *BCR-ABL1*^{IS} <10% at 3 months of first-line TKI therapy) predicts subsequent DMR.[26] The initial rate of *BCR-ABL1* decline also influences outcomes. The German CML-IV study demonstrated that the relative change in *BCR-ABL1* value at 3 months of TKI therapy from the baseline value was associated with superior progression-free survival.[27] However, fold-reduction and absolute *BCR-ABL1* values measured at a defined time point fail to account for the influence of time, which can impact outcomes.[28] A 3-month *BCR-ABL1* value could theoretically be measured between 1.5 and 4.5 months,[29] whereas *BCR-ABL1* halving time measurements (Supplementary Figure 1) account for variability in the day of the 3-month sample collection and consistently predict MMR and DMR in multiple studies.[28,30-35] We explored the relationship between early response kinetics and sustained TFR.

2.4 Patients and methods

We performed a retrospective analysis of all adult patients receiving their primary CML management at two university hospitals in South Australia between January 2008 and October 2019, including patients diagnosed prior to 2008 who continued to be managed at these institutions. Ethics approval was obtained from the local Institutional Review Board and the study was performed in accordance with the Declaration of Helsinki. Clinical and laboratory records were reviewed and collated. Twenty patients were enrolled in the ALLG CML8, TWISTER study (ACTRN 12606000118505). The remaining patients discontinued TKI outside the setting of a clinical trial based on the physician's recommendation and patient preference. The criteria for TFR eligibility (Supplementary methods) were adapted from the CML8 study,[5] and included a minimum of three years of total TKI therapy and two years of sustained MR4.5. MR4.5 was confirmed by a minimum of four tests over two years. Non-quantifiable transcripts or a history of either blast crisis or accelerated phase CML were exclusion criteria for a TFR attempt. Validation of identified predictors of TFR outcome was performed on an independent dataset provided by The Catholic University of Korea, South Korea.

2.4.1 Molecular monitoring

Patients attempting TFR in South Australia were monitored with *BCR-ABL1* quantitative RT-PCR in a single laboratory.[36] The ratio of *BCR-ABL1* to the control gene, *BCR*, was converted to the *BCR-ABL1*^{IS} using the laboratory-specific conversion factor.[37] The consistency and reliability of this assay has been demonstrated, confirming its suitability for assessment of *BCR-ABL1* molecular dynamics.[26,28,37-40] Patients included in the validation cohort were monitored with an *ABL1* based assay.[41] All patients attempting TFR underwent monthly *BCR-ABL1* testing for a minimum of 12 months. Sustained TFR was defined as remaining off TKI with preservation of MMR at 12 months. TKI treatment was reinitiated for loss of MMR (molecular relapse) on a single test.[6] The TKI restart criteria for the 20 patients enrolled in the CML8 study[5] was more stringent and required TKI recommencement for positive *BCR-ABL1* values on two consecutive tests or loss of MMR. Patients recommencing TKI with *BCR-ABL1* values <0.1% were treated the same as patients recommencing TKI for MMR loss.

2.4.2 BCR-ABL1 rate of decline measurements

Halving time was calculated from available baseline (sample measured at or soon after diagnosis and prior to commencement of any TKI therapy) and 3-month *BCR-ABL1* values utilizing the formula below. Further detail is provided in the Supplementary methods and Supplementary File 2.[28] Shorter halving times indicate more rapid *BCR-ABL1* decline.

$$Halving\ time = ln(2)*x/ln(y/z)$$

x, number of days between the baseline and 3-month assessment; y, Baseline *BCR-ABL1* value; z, 3-month *BCR-ABL1* value

The *BCR-ABL1* fold-reduction was derived from the baseline *BCR-ABL1* value divided by the 3-month value. Unlike halving time, this calculation does not incorporate the time difference between the two measurements, which varied by almost 60 days (Table 1). This variability can have a major impact on the calculated rate of *BCR-ABL1* decline.[28]

2.4.3 Statistical analysis

Patient characteristics were described using frequencies and percentages for categorical variables, while continuous baseline measures were described using medians and interquartile ranges. The endpoint was the binary outcome of sustained TFR at 12 months. The secondary objective was molecular recurrence-free survival, defined as the time (in months) between cessation of TKI therapy and the first measurement of loss of MMR. Time-to-event modelling assessed the prognostic value of selected factors for molecular relapse. Time intervals (in either days or months) were entered into the analysis as continuous covariates. A series of univariate Cox proportional hazards regression models assessed and compared the prognostic value of each factor. Multivariate Cox proportional hazards regression modelling was performed on variables with a *P*-value of ≤0.1 on univariate analysis. Additional statistical tests to depict covariate significance include Analysis of Variance (ANOVA) and unpaired t-tests. A series of logistic regression analyses were utilized for predictive modelling of sustained TFR at 12 months using the final variables identified in the multivariate analysis. For the total CP-CML cohort, cumulative incidence graphs were applied to investigate the predictors of milestone achievement with the competing risks of allogeneic stem cell transplant, progression to blast crisis or death. Statistical analysis was performed using GraphPad Prism 8.0.0 and R version 3.6.1.

2.4.4 Data Sharing Statement

De-identified participant data collected for our study can be made available to researchers once appropriate ethical approval and a signed data access agreement is obtained.

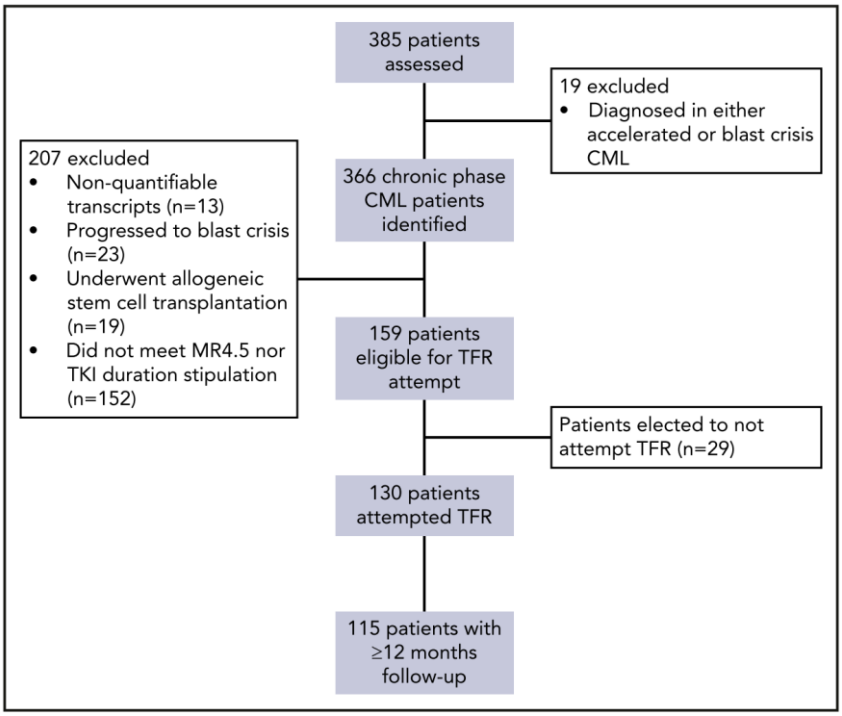
2.5 Results

2.5.1 Patient characteristics

Of the total cohort of 385 South Australian patients, 366 patients diagnosed in CP-CML were monitored during the defined observational period (Figure 1). In total, 130 patients (36%) attempted TFR and met our specified eligibility criteria, of whom 115 (88%) had ≥12 months of follow-up following TKI discontinuation. Table 1 summarizes the patient characteristics for

the 115 patients with ≥12 months follow-up and Supplementary Table 1 for the total 130 patients.

Figure 1: Flow diagram illustrating patient disposition.
TFR, treatment-free remission.



For the 115 TFR attempts with ≥12 months of follow-up, the median duration of follow-up after TKI cessation was 51.1 months (12.0-153.2 months). Males accounted for 53% of patients. All but one patient had a p210 transcript. The remaining patient had an e1a2 transcript, which was also monitored using quantitative PCR. The majority of patients (61%) ceased imatinib and 39% stopped a 2G-TKI. Of the 45 patients stopping 2G-TKIs, 17 (38%) were treated first-line, whereas 20 (44%) switched for imatinib intolerance and eight (18%) for resistance/sub-optimal response to imatinib. The median duration of TKI therapy prior to the TFR attempt was 7.0 years (3.1-17.6) and the median duration of MR4.5 was 3.3 years (2.2-12.5).

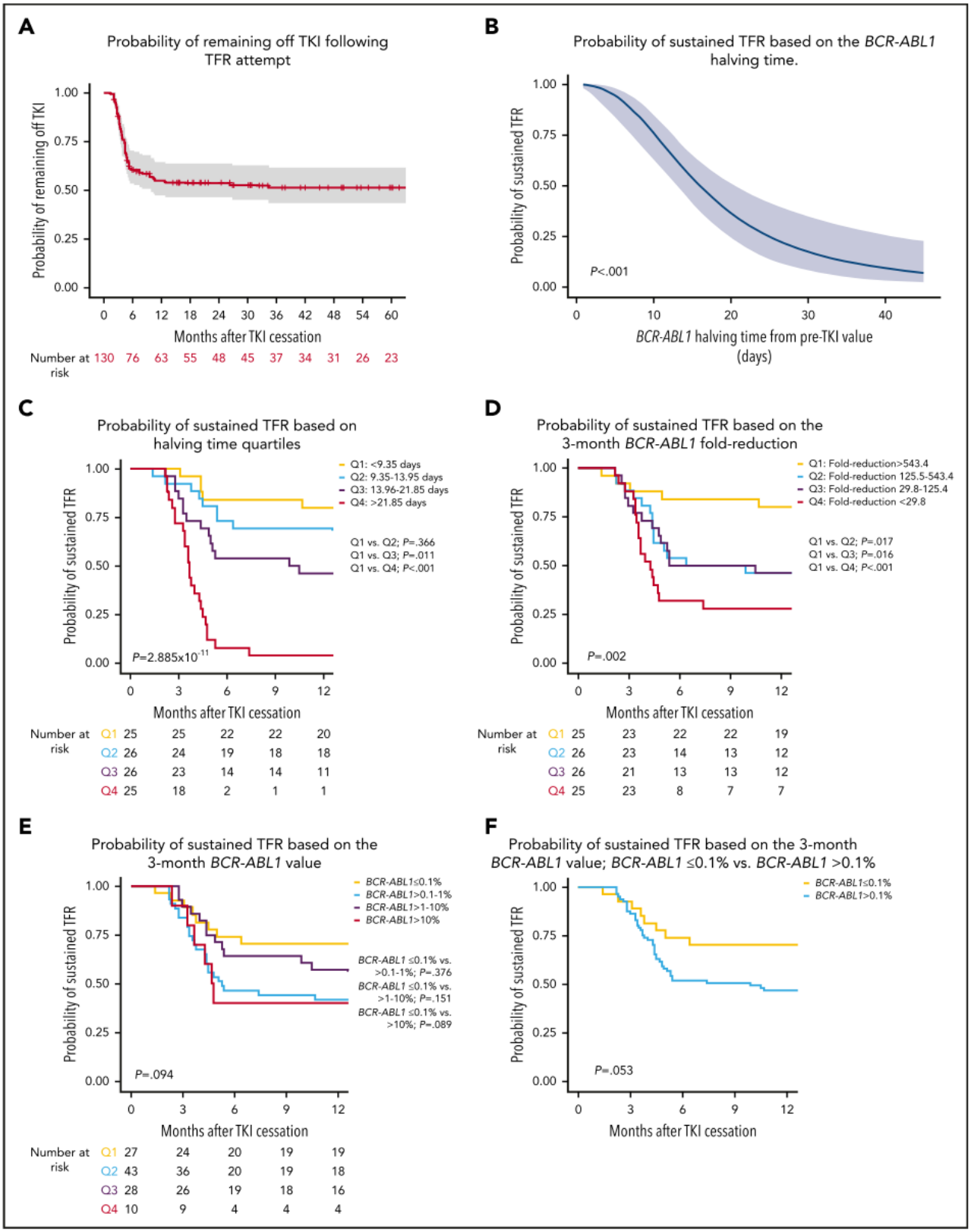
2.5.2 Outcomes of TFR attempts

Of the 130 patients who ceased TKI, 54% (n=70) remained off therapy at the last follow-up (Supplementary Figure 2). The restricted mean time to molecular recurrence was 5.4 months (1.4-33.4 months). Of the 115 patients with ≥12 months of follow-up after TKI cessation, 55%

(n=63) sustained TFR at 12 months (Figure 2A). Loss of MMR triggered TKI restart in 87% (n=45) of molecular relapses in the first 12 months. The remaining patients (n=7) restarted TKI due to progressive loss of DMR according to the more stringent recommencement strategy in the CML8 study.[5] All but 1 patient were predicted to lose MMR within one month based on the *BCR-ABL1* doubling time.[39] MMR was regained at a median of 2.0 months (0.2-9.3) in all patients and 96% regained MR4.5 after a median of 4.2 months (0.7-44.2). Six additional patients (5.2%) lost MR4.5 but remained in MMR at 12 months. Three of these patients eventually resumed TKI; one patient at 13 months for progressively rising *BCR-ABL1* levels (CML8 patient) while two patients lost MMR at 26.3 and 33.4 months, respectively. These two patients were the only late relapses in the follow-up of our cohort (2% of the >12-month cohort). Two of the six patients who lost MR4.5 spontaneously regained stable MR4.5 while the remaining patient had a fluctuating *BCR-ABL1* level below MMR at 36 months.

Figure 2: Probability of sustained TFR relative to halving time, 3-month BCR-ABL1 value and fold-reduction.

A. Kaplan-Meier analysis of probability of remaining off TKI in all TFR attempts with 95% confidence intervals shown. B. Logistic regression analysis demonstrating the probability of sustained TFR based on individual halving times demonstrating that the likelihood of TFR is reduced with longer halving times. C. Kaplan-Meier analysis of sustained TFR based on the BCR-ABL1 halving time quartiles. D. Kaplan-Meier analysis of sustained TFR based on the fold-reduction of BCR-ABL1 from baseline. Patients were divided into quartile values. E. Kaplan-Meier analysis of sustained TFR based on the 3-month BCR-ABL1 value. Comparison of BCR-ABL1^{IS} ≤0.1% vs. other groups shown. F. Kaplan-Meier analysis of sustained TFR based on whether patients achieved a BCR-ABL1^{IS} ≤0.1% at 3 months. Q, quartile; TFR, treatment-free remission; TKI, tyrosine kinase inhibitor.


A
Probability of remaining off TKI following TFR attempt
Probability of remaining off TKI
1.00
0.75
0.50
0.25
0.00
0 6 12 18 24 30 36 42 48 54 60
Months after TKI cessation
Number at risk 130 76 63 55 48 45 37 34 31 26 23
B
Probability of sustained TFR based on the BCR-ABL1 halving time.
Probability of sustained TFR
P<.001
0 10 20 30 40
BCR-ABL1 halving time from pre-TKI value (days)
C
Probability of sustained TFR based on halving time quartiles
Probability of sustained TFR
Q1: <9.35 days
Q2: 9.35-13.95 days
Q3: 13.96-21.85 days
Q4: >21.85 days
Q1 vs. Q2; P=.366
Q1 vs. Q3; P=.011
Q1 vs. Q4; P<.001
P=2.885x10-11
0 3 6 9 12
Months after TKI cessation
Number at risk
Q1 25 25 22 22 20
Q2 26 24 19 18 18
Q3 26 23 14 14 11
Q4 25 18 2 1 1
D
Probability of sustained TFR based on the 3-month BCR-ABL1 fold-reduction
Probability of sustained TFR
Q1: Fold-reduction>543.4
Q2: Fold-reduction 125.5-543.4
Q3: Fold-reduction 29.8-125.4
Q4: Fold-reduction <29.8
Q1 vs. Q2; P=.017
Q1 vs. Q3; P=.016
Q1 vs. Q4; P<.001
P=.002
0 3 6 9 12
Months after TKI cessation
Number at risk
Q1 25 23 22 22 19
Q2 26 23 14 13 12
Q3 26 21 13 13 12
Q4 25 23 8 7 7
E
Probability of sustained TFR based on the 3-month BCR-ABL1 value
Probability of sustained TFR
BCR-ABL1≤0.1%
BCR-ABL1>0.1-1%
BCR-ABL1>1-10%
BCR-ABL1>10%
BCR-ABL1 ≤0.1% vs. >0.1-1%; P=.376
BCR-ABL1 ≤0.1% vs. >1-10%; P=.151
BCR-ABL1 ≤0.1% vs. >10%; P=.089
P=.094
0 3 6 9 12
Months after TKI cessation
Number at risk
Q1 27 24 20 19 19
Q2 43 36 20 19 18
Q3 28 26 19 18 16
Q4 10 9 4 4 4
F
Probability of sustained TFR based on the 3-month BCR-ABL1 value; BCR-ABL1 ≤0.1% vs. BCR-ABL1 >0.1%
Probability of sustained TFR
BCR-ABL1≤0.1%
BCR-ABL1>0.1%
P=.053
0 3 6 9 12
Months after TKI cessation

2.5.3 Predictors of TFR outcome

The candidate prognostic variables shown in Table 1 were investigated for association with sustained TFR: sex, age at TFR attempt, Sokal and ELTS[42] scores, transcript type, time to MR4.5, duration of MR4.5, total duration of TKI therapy, TKI at TFR attempt, first-line TKI, first-line versus second-line TKI, EMR, baseline and 3-month *BCR-ABL1* value, 3-month *BCR-ABL1* fold-reduction, 3-month *BCR-ABL1* halving time, and MMR at 12 months of TKI therapy.

Table 1: Characteristics of all CML patients attempting TFR with ≥12 months of follow-up (n=115)

Patient characteristic	**Patients attempting TFR with ≥12 months follow-up** Number (%) or Median [range]
Male	61 (53.0)
Age at TFR attempt (years)	60.9 [33.4-89.8]
Sokal score - Low-risk (<0.8) - Intermediate-risk (0.8-1.2) - High-risk (>1.2) - Not available	 49 (42.6) 41 (35.7) 17 (14.8) 8 (7.0)
ELTS score - Low-risk (≤1.5680) - Intermediate-risk (>1.5680 – ≤2.2185) - High-risk (>2.2185) - Not available	 72 (63.2) 26 (22.4) 8 (6.4) 9 (8.0)
Transcript type - e14a2 - e13a2 - Both e14a2 and e13a2 - e1a2	 51 (44.3) 43 (37.4) 20 (17.4) 1 (0.9)
Baseline *BCR-ABL1*^{IS} value available	111 (96.5)
Time to MR4.5 (years)	2.2 [0.2-13.2]
Duration of MR4.5 (years)	3.3 [2.0-12.5]
Duration of TKI therapy (years)	7.0 [3.0-17.6]
Therapy ceased - Imatinib - First-line 2G-TKI (nilotinib/dasatinib) or 2G-TKI for imatinib-intolerance - 2G-TKI following imatinib failure	 70 (60.9) 37 (32.2) 8 (7.0)
First-line treatment - Imatinib	 89 (77.4)

- 2G-TKI - Other (interferon/autograft)	17 (14.8) 9 (7.8)
EMR achieved	98 (90.7)
3-month *BCR-ABL1* value available - *BCR-ABL1*^{IS} >10% - *BCR-ABL1*^{IS} >1-10% - *BCR-ABL1*^{IS} >0.1-1% - *BCR-ABL1*^{IS} ≤0.1%	108 (93.9) 10 (9.3) 28 (25.9) 43 (39.8) 27 (25.0)
3-month halving time available - Median halving time	102 (88.7) 13.95 [3.6-267.8]
3-month *BCR-ABL1* fold-reduction	125.5 [3.1-8700]
Months between baseline and 3-month *BCR-ABL1* value	3.1 [2.0-3.9]
12-month *BCR-ABL1* value available - MMR achieved	112 (97) 86 (76.8)

EMR, Early molecular response; IS, International Scale; MMR, Major molecular response; 2G-TKI, Second-generation TKI; TFR, Treatment-free remission; TKI, Tyrosine kinase inhibitor

Univariate Cox regression analysis for prediction of sustained TFR at 12 months is shown in Table 2. Variables demonstrating a significant association on univariate analysis were halving time (Figure 2B-C), transcript type, *BCR-ABL1* fold-reduction (Figure 2D) and 3-month *BCR-ABL1*^{IS} value (Figure 2E-F). Variables with a *P*-value of ≤0.1 were included in the multivariate Cox regression analysis. Halving time and transcript type remained significant predictors of sustained TFR. Duration of TKI exposure was also an independent predictor (Table 2). A second multivariate analysis was performed using the fold-reduction value from baseline to 3-months, excluding halving time to avoid collinearity. Fold-reduction, transcript type, and duration of TKI exposure were independent predictors of sustained TFR (Supplementary Table 2). However, when comparing the concordance-index of the two models, the analysis including halving time had a higher value, implying that this model has slightly better discriminatory power for predicting sustained TFR.

***Table 2: Univariate and multivariate Cox regression analysis of variables investigated as* potential predictors of sustained TFR at 12 months**

Variable	Number of patients (%)	Patients in sustained TFR (%)	Univariate analysis			Multivariate analysis			
			β-coefficient	Hazard ratio (95% CI)	*P* value	β-coefficient	Hazard ratio (95% CI)	*P* value	Concordance index§
Halving time (days)*	102 (88.7)		0.02	1.02 (0.01-1.01)	2.26×10^{-8}	0.03	1.03 (1.02-1.05)	1.91×10^{-6}	0.745
Transcript									
- e14a2	51 (44.3)	34 (66.7)	Ref	-	-	Ref	-	-	
- e13a2	43 (37.4)	17 (39.5)	0.88	2.41 (1.30-4.44)	0.005	0.91	2.49 (1.39-4.46)	0.002	
- Both transcripts	20 (17.4)	11 (55.0)	0.33	1.39 (0.62-3.11)	0.43	-	-	-	
Duration of TKI therapy (years)*	115 (100)		0.56	1.75 (0.92-3.33)	0.09	0.95	2.61 (1.05-6.09)	0.03	
Time to MR4.5 (years)*	115 (100)		-0.08	1.08 (0.99-1.18)	0.08	-0.07	0.93 (0.86-1.06)	0.26	
3-month *BCR-ABL1*^{IS} value*	108 (94)		0.03	1.03 (1.00-1.05)	0.03	-0.004	1.00 (0.96-1.03)	0.80	
3-month *BCR-ABL1* fold-reduction†	102 (88.7)		2.8×10^{-6}	1.00 (1.00-1.01)	0.008				
Baseline *BCR-ABL1*^{IS} value*	111 (96.5)		-0.002	0.99 (0.99-1.00)	0.35				
Duration of MR4.5 (years)*	115 (100)		0.29	1.33 (0.75-2.38)	0.33				
Sex									
- Male	61 (53.0)	32 (52.5)	Ref	-	-				
- Female	54 (47.0)	31(57.4)	-0.16	0.85 (0.49-1.47)	0.57				
Age at TFR attempt*	115 (100)		0.26	1.30 (0.76-2.25)	0.34				
Sokal score									
- Low	49 (45.8)	25 (51.0)	Ref	-	-				
- Intermediate	41 (38.3)	22 (53.7)	-0.10	0.91 (0.50-1.65)	0.74				
- High	17 (15.9)	9 (52.9)	<0.01	1.01 (0.45-2.24)	0.99				
ELTS									
- Low	72 (67.9)	38 (52.8)	Ref	-	-				

- Intermediate - High	26 (24.5) 8 (7.5)	11 (42.3) 7 (87.5)	0.28 -1.57	1.33 (0.72-2.44) 0.20 (0.03-1.52)	0.36 0.12				
TKI ceased - First-line imatinib - First-line 2G-TKI - 2G-TKI for imatinib resistance	60 (52.2) 17 (14.8) 8 (7.0)	40 (66.7) 11 (64.7) 6 (75.0)	Ref 0.26 -0.09	- 1.29 (0.73-2.30) 0.92 (0.28-3.02)	- 0.37 0.89				
First-line TKI - Imatinib - 2G-TKI	89 (77.4) 17 (14.8)	51 (57.3) 12 (70.6)	Ref -0.68	- 0.47 (0.20-1.27)	- 0.14				
EMR achieved	98 (85.2)	53 (54.1)	-0.38	0.68 (0.29-1.61)	0.38				
MMR by 12 months	86 (76.8)	46 (53.5)	0.09	1.10 (0.57-2.10)	0.78				

CI, Confidence interval; EMR, Early molecular response; MMR, Major molecular response; Ref, Reference variable; 2G-TKI, Second-generation TKI; TFR, Treatment-free remission

* Continuous variables

§ Harrell's Concordance index is a measure of the goodness of fit of the prediction model.[58]

† 3-month fold-reduction is assessed in a separate multivariate analysis model to avoid collinearity with the 3-month halving time (Supplementary Table 2)

Of the 115 patients who attempted TFR with ≥12 months of follow-up after TKI discontinuation, *BCR-ABL1* halving times[28] could be calculated in 102 (89%) patients. The remaining 11% did not commence TKI as first-line treatment (n=9) or either the baseline or the 3-month value, or both, were unavailable. Patients with a sustained TFR had a shorter median halving time (10.1 days, range 3.6-26.4) compared with patients with molecular relapse (21.7 days, range 7.1-267.8), *P*<.001 (Supplementary Figure 3). Logistic regression also confirmed that the likelihood of sustained TFR at 12 months was lower in patients with longer halving times (Figure 2B). For example, patients with a halving time of 10 days who attempted TFR once eligible had almost 75% probability of sustained TFR, falling to ~12% for patients with a halving time of 40 days. Patients were divided into quartiles based on halving time: the probability of sustained TFR was 80% in patients in the first quartile (Q1, halving time of <9.35 days) compared with 4% in patients in the fourth quartile (Q4, halving time of >21.85 days), *P*<.001 (Figure 2C). The late relapses had halving times in the third and fourth quartiles with fluctuating *BCR-ABL1* values until eventual molecular relapse (Supplementary Figure 2). All patients with a halving time in Q4 (>21.85 days) eventually experienced molecular relapse during follow-up. Interestingly, there was an association between the *BCR-ABL1* doubling time at molecular relapse and the initial halving time, such that patients with the shortest halving time (Q1) had longer median doubling times compared with patients in Q2-4 (Supplementary Figure 4). Division of patients based on the *BCR-ABL1* fold-reduction confirmed that a more rapid *BCR-ABL1* decline (i.e. Q1: >543.4 fold-reduction) was associated with an 80% probability of sustained TFR (Figure 2D). Patients with a fold-reduction of <29.8 (Q4) still had a 28% probability of sustained TFR, *P*=.002. Follow up of patients also demonstrated that unlike the patients with a Q4: halving time, patients with a Q4: fold-reduction were able to remain in long-term TFR (Supplementary Figure 5).

As the *BCR-ABL1* halving time predicted sustained TFR, the 3-month *BCR-ABL1*^{IS} value was assessed for similar predictive power using Kaplan-Meier analysis (Figure 2D). Patients with a 3-month *BCR-ABL1*^{IS} of ≤0.1% had a 70% probability of sustained TFR compared with 40% for patients with a *BCR-ABL1*^{IS} of >10%, *P*=.089. Splitting patients further into those with and without a *BCR-ABL1*^{IS} value of ≤0.1% at 3 months (Figure 2E)

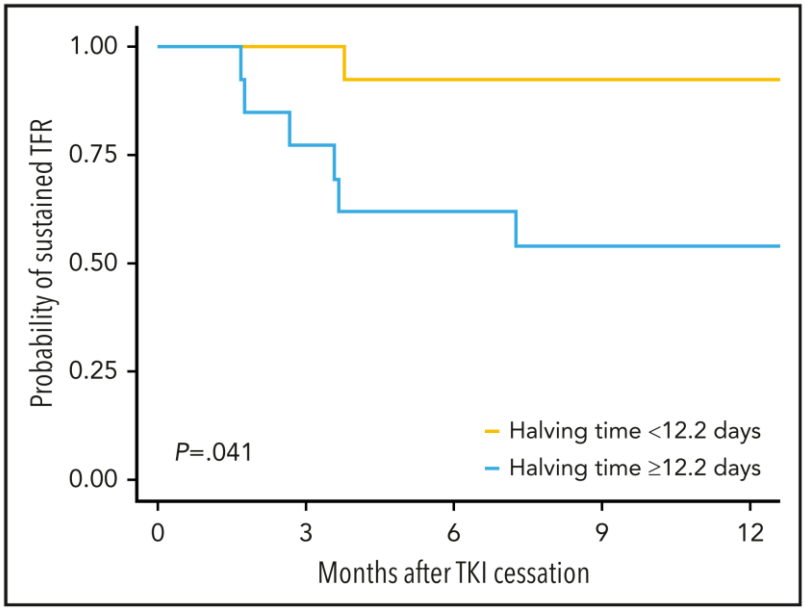
approached statistical significance (*P*=.053). However, the 3-month *BCR-ABL1* value was not a significant predictor of sustained TFR on multivariate analysis (Table 2). This confirmed that the 3-month *BCR-ABL1* value is not interchangeable with the halving time as a predictor of sustained TFR.

The estimated proportion of e14a2 transcript patients in sustained TFR was 67% compared with 40% for e13a2 patients (*P*=.005). The simultaneous expression of both transcripts did not predict sustained TFR (Table 2). Additionally, there was no association between the halving time and transcript type (Supplementary Figure 6A). However, for patients with either e14a2 or e13a2 transcript types, the halving times of patients in sustained TFR (Supplementary Figure 6B-C) were shorter than those with molecular relapse. Patients with the e14a2 transcript and sustained TFR had a median halving time of 10.2 versus 22.3 days for those with molecular relapse, *P*=.01. Similarly, patients with the e13a2 transcript and sustained TFR had a median halving time of 10.7 versus 21.5 days for those with molecular relapse, *P*=.012.

2.5.4 Validation of halving time as a predictor of TFR outcome

Independent validation of the initial *BCR-ABL1* halving time as a predictor of sustained TFR was performed using an external dataset of 26 first-line TKI treated patients attempting TFR in Korea. The characteristics are detailed in Supplementary Table 3. This dataset involved a combination of patients enrolled in the KID study[9] (n=19) and local registries (n=7). The rate of sustained TFR at 12 months was 73%. The median *BCR-ABL1* halving time of all 26 patients was 12.2 days (range 6.0-35.0). Halving time was validated as a predictor of sustained TFR (Figure 3). Patients with halving time below the median of 12.2 days had a 92% probability of sustained TFR, whereas patients with longer halving times had a 54% probability of sustained TFR (*P*=.041).

Figure 3: Probability of sustained TFR in validation cohort based upon the median halving time.

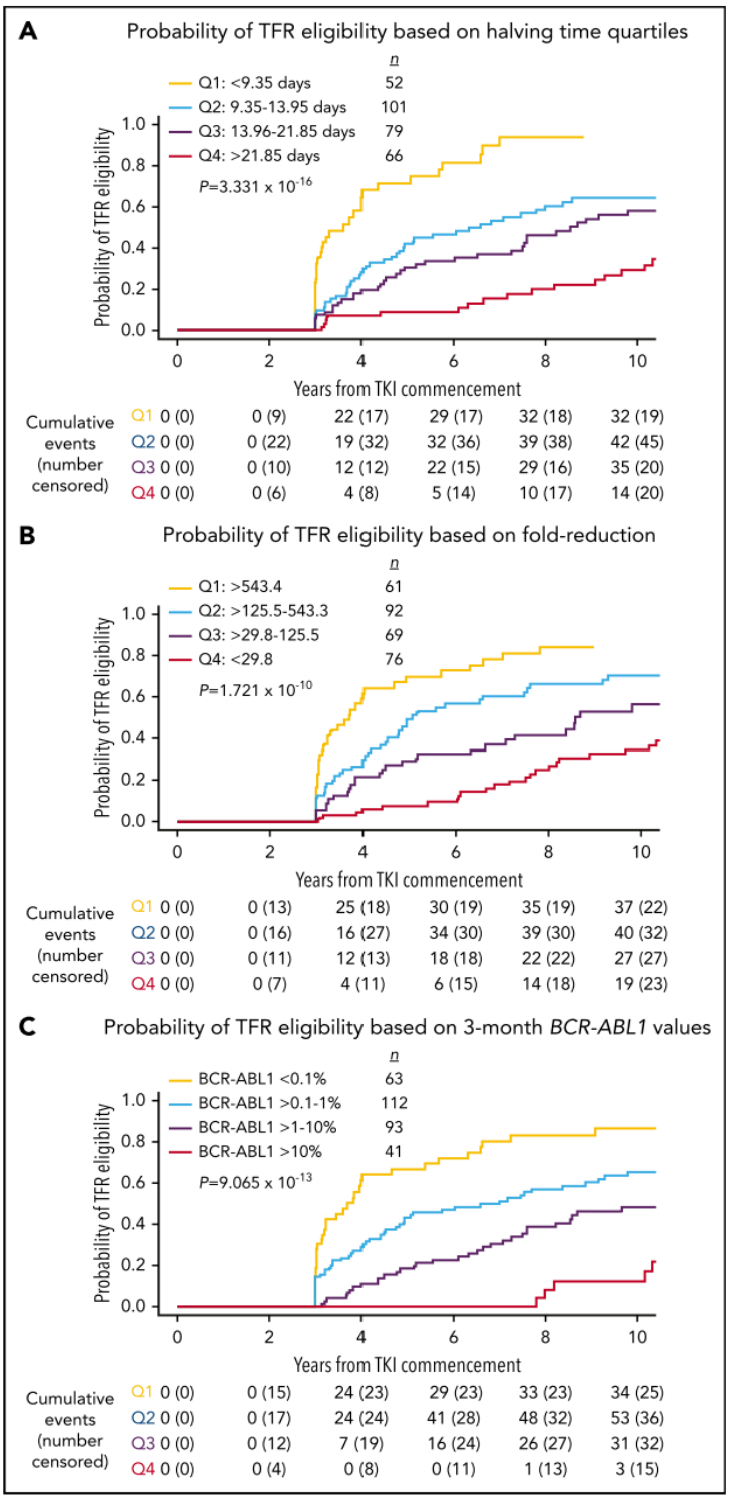


2.5.5 Prediction of TFR eligibility

We assessed the initial *BCR-ABL1* halving time, the fold reduction of *BCR-ABL1* from baseline to 3 months and the 3-month *BCR-ABL1* value for prediction of eligibility for a TFR attempt. All CP-CML patients with available halving times (n=298) were divided into the same quartile groups that were derived from the cohort attempting TFR with 12 months of follow-up (Figure 4A). Cumulative incidence analysis stratified by halving time demonstrated that by 5 years of TKI therapy, 71% of patients in Q1 (halving time of <9.35 days) achieved TFR eligibility, versus only 8.9% of Q4 (halving time of >21.85 days), *P*<.001. Logistic regression analysis of halving time at specific durations (5 and 8 years) of TKI therapy confirmed that shorter halving times remained predictive of TFR eligibility (Supplementary Figures 7-8). For example, a halving time of 10 days predicted for ~50% probability of TFR eligibility by 5 years, increasing to ~56% by 8 years. Patients with a halving time of 40 days had a 25% probability of TFR eligibility by 5 years, increasing to only ~35% by 8 years of TKI therapy. Similarly, cumulative incidence analysis demonstrated that the *BCR-ABL1* fold-reduction predicted for TFR eligibility (Figure 4B). Confirming our previous published work (14.8% patient overlap),[26] the 3-month *BCR-ABL1* value predicted TFR eligibility in 309 patients with available 3-month *BCR-ABL1* values, *P*<.001 (Figure 4C). This analysis demonstrates that the *BCR-ABL1* response at 3 months of first-line TKI is a powerful predictor of TFR eligibility, whether measured as

the rate of *BCR-ABL1* decline from baseline or by assessing the actual *BCR-ABL1* value. However, the 3-month value on its own did not predict sustained TFR.

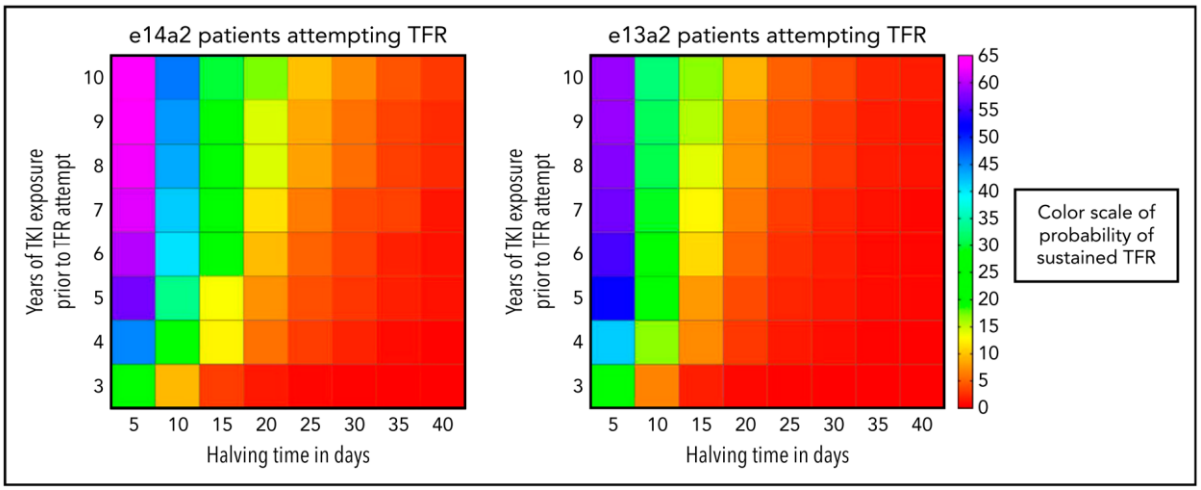
Figure 4: Prediction of TFR eligibility in the total CP-CML cohort of 366 patients.
A. Cumulative incidence of TFR eligibility based upon halving time quartiles. B. Cumulative incidence of TFR eligibility based upon the BCR-ABL1 fold-reduction. C. Cumulative incidence of TFR eligibility based upon the 3-month BCR-ABL1 value.



2.5.6 Modelling sustained TFR in all chronic phase CML patients

We modelled prediction of sustained TFR in all CP-CML patients based on the strongest combination of variables that emerged from the multivariate analysis: halving time, transcript type and duration of TKI exposure. A series of logistic regression analyses were performed to calculate the probability of TFR eligibility and sustained TFR based on these variables. These calculations were combined to develop a composite prediction model (Figure 5). Using the model, which assumes that only eligible patients attempt TFR, clinicians can predict the probability of sustained TFR based on halving time, years of TKI exposure and transcript type. For example, a patient with an e14a2 transcript and an initial *BCR-ABL1* halving time of 5 days has a 30% probability of sustained TFR, if attempted after 3 years of TKI exposure. This increases to ~65% if the TFR attempt is deferred to 8 years of TKI exposure. In contrast, patients with a slower initial *BCR-ABL1* decline (halving time ≥35 days) have <5% chance of attaining a sustained TFR irrespective of the transcript type and length of TKI exposure. This model could be useful for patient counselling prior to TFR attempts.

Figure 5: Personalized prediction model for sustained TFR based on halving time (days), transcript type and duration of TKI exposure (years).
The color scale to predict the probability of sustained TFR is depicted on the right side of the figure and represents the percent probability. Patients were divided into the major transcript groups e14a2 and e13a2. The vertical axis is the years of TKI exposure prior to TFR attempt and the horizontal axis is the halving time.



2.6 Discussion

We have shown that a rapid initial decline in *BCR-ABL1* transcripts for first-line TKI-treated patients is an independent predictor of TFR eligibility and subsequent sustained TFR. *BCR-ABL1* transcript type and duration of TKI therapy before the TFR attempt were also independent predictors of sustained TFR. Duration of MR4.5 was not a predictor of sustained TFR in our data compared with the Euro-SKI study.[4] This likely reflects our proactive approach to TFR attempts whereby many patients promptly ceased TKI shortly after attaining TFR eligibility. A model including *BCR-ABL1* halving time measured at 3 months had greater discriminatory power to predict sustained TFR than the fold-reduction of *BCR-ABL1* at 3 months. We also demonstrate that no patients with long halving times (Q4) remained in long-term TFR (Supplementary Figure 2) while fold-reduction, outside of Q1, is less able to differentiate patients for long-term TFR (Supplementary Figure 5). This strengthens our argument that that while fold-reduction can predict TFR outcomes, halving time is a far better discriminator, presumably because it more accurately reflects the true rate of leukemic decline by incorporating the number of days between the start of TKI therapy and the day of *BCR-ABL1* measurement,[28] which varied by almost 60 days in our cohort for the 3-month time point (Table 1).

Importantly, we were able to validate the association between the initial rate of *BCR-ABL1* decline and sustained TFR in an independent dataset. Such datasets with available *BCR-ABL1* values measured at diagnosis and at 3 months of first-line TKI using internationally standardized quantitative methods are not readily available. This relates to the long duration of therapy before attempting TFR in most studies. Many patients enrolled in cessation studies were from regional centres where molecular monitoring was not performed or the patient was diagnosed before methods were standardized and in routine use. Furthermore, quantification of *BCR-ABL1* transcripts at diagnosis is not routinely performed for all patients and is not mandated by the European LeukemiaNet.[3] We suggest that quantification of *BCR-ABL1* transcripts at diagnosis is essential for outcome prediction and should be incorporated into the monitoring strategy.

Shorter halving times or a greater *BCR-ABL1* fold-reduction indicate a more rapid decline in measurable leukemia after commencing TKI, likely reflecting both the intensity of kinase inhibition and the individual sensitivity of the leukemic cells. Our data are consistent with the concept that the initial rate of *BCR-ABL1* decline has a stronger correlation with the degree of suppression of the leukemic clone than an absolute *BCR-ABL1* value at a pre-determined time point. Whether strategies targeting a rapid initial *BCR-ABL1* decline using more potent TKIs will translate into a higher overall probability of sustained TFR is yet to be determined. Decisions about first-line therapy must always be made in the context of the patient's comorbidities, since 2G-TKIs are associated with significantly higher rates of serious toxicity.[12,13,43] Dedicated 2G-TKI TFR studies addressing the rapidity of the initial *BCR-ABL1* response will be required to investigate this hypothesis further prior to incorporating considerations of halving times into treatment recommendations.

We developed a prediction model for sustained TFR comprising the strongest combination of variables (halving time, transcript type and duration of TKI exposure, Figure 5). This could potentially be used when counselling patients about the potential for sustained TFR. However, this model was developed from a population that was predominantly treated with first-line imatinib and refinement will likely occur with data from more first-line 2G-TKI-treated patients.

The impact of transcript type on CML outcomes has long been debated.[44-46] The e14a2 transcript and the shorter e13a2 transcript encode protein products that differ by 25 amino acids.[44,47] Superior molecular responses have been reported in patients with e14a2 transcripts, with more rapid achievement of molecular milestones such as MMR and MR4.5 on imatinib treatment,[48] although the differences were less pronounced with 2G-TKI therapy.[44] Overall, survival outcomes are largely similar between the transcript groups.[44,46,48,49] Claudiani *et al.*[18] provided the first report that the e14a2 transcript was associated with a higher rate of sustained TFR in a retrospective analysis of 64 patients attempting TFR. In our larger cohort, we observed that the e14a2 transcript type is an independent predictor of sustained TFR. Additionally, the effect of transcript type on sustained TFR is independent of halving time (Supplementary Figure 6). While the exact

mechanism for superior responses in e14a2 patients is not understood, the transcript is predicted to be more immunogenic[50,51] leading to increased immune-mediated clearance, perhaps contributing to the higher probability of sustained TFR.[52]

The control gene in our assay is *BCR* and the decline of *BCR-ABL1* relative to *BCR* has been shown to be exponential, ratifying the halving time formula used in this work.[28] However, the use of an *ABL1*-based assay is more widespread and our validation data were derived from an *ABL1*-based method. A number of groups have now established the reliability of *ABL1*-obtained *BCR-ABL1* values in halving time calculations.[30-34,53,54] Use of the halving time formula based on values derived from *BCR-ABL1* assays with alternative control genes such as *GUSB*[54] and *GAPDH*[35] has also been published. We have demonstrated a significant association between halving time, TFR eligibility and sustained TFR, but it is essential that the initial *BCR-ABL1* decline demonstrates an exponential response for the halving time formula to be valid, regardless of the control gene utilized.[28]

TFR attempts remain extremely safe, although blast crisis is a rare event that has been reported in <1% of patients.[6,55-57] In our analysis, no patient who attempted TFR has progressed to blast crisis with up to 153.2 months of follow-up. The timing of blast crisis in the few reported cases was variable and in a single instance, occurred months after TKI recommencement despite re-achievement of MMR.[6] Hence, while there is no clear predictor as yet for the development of blast crisis in patients attempting TFR, clinicians should remain vigilant for this rare complication by continued regular monitoring.

In conclusion, we have demonstrated that the initial rate of *BCR-ABL1* decline, specifically measured as halving time, is a powerful predictor of sustained TFR. Understanding the extrinsic determinants and the intrinsic biological factors that influence the rate of leukemic decline will be imperative to maximise the number of patients that can successfully attempt TFR. A model incorporating the initial rate of *BCR-ABL1* decline and other independent predictors of sustained TFR will likely enhance long-term clinical management decisions for clinicians and patients.

2.7 Acknowledgements

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2.8 Authorship Contributions

NS collected and analysed the data and wrote the paper. IP provided key insights into the paper and reviewed the manuscript. DY, DH, AY, and DMR reviewed the manuscript. JB and HA performed the molecular analysis and reviewed the manuscript. SP and D-WK contributed data and reviewed the paper. SB contributed key concepts and methodology and reviewed the manuscript. TPH conceptualised the paper and reviewed the manuscript.

2.9 Disclosure of Conflicts of Interest

NS received honoraria from Novartis and travel and accommodation expenses from Novartis, Gilead, Amgen, and Janssen. SB is a member of the advisory boards of Qiagen, Novartis and Cepheid, received honoraria from Qiagen, Novartis, Bristol-Myers Squibb, Incyte and Cepheid, and research funding from Novartis. AY received research funding from Novartis, BMS and Celgene and honoraria from Novartis and BMS. DMR received research funding and honoraria from Novartis and BMS. TPH is a member of advisory boards and has received research funding and honoraria from Novartis and Bristol-Myers Squibb. D-WK received research funding and honoraria from Novartis, Bristol-Myers Squibb, Otsuka, Takeda, Sun Pharma and ILYANG. The other authors declare no conflict of interest.

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Supplement

Supplemental Methods, Results, Tables and Figures to Shanmuganathan N, Pagani IS, Ross DM, et al. Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia. *Blood*. 2021;137(9):1196-1207 (Chapter 2)

Supplementary methods

TFR eligibility criteria

The TFR eligibility criteria for this study were adapted from the CML8 ("Twister") study published by Ross *et al*.[1]

Inclusion criteria

- Adult patients diagnosed with chronic phase CML receiving primary CML management at either of the treatment institutions
- Receiving current TKI therapy irrespective of agent or line of therapy
- Minimum 3 years of TKI therapy
- Minimum 2 years of deep molecular response (DMR)
    - Defined as MR4.5 (*BCR-ABL1* ≤0.0032% IS) with a minimum of 4 tests over the 2 year period

Exclusion criteria

- Non-quantifiable *BCR-ABL1* transcripts
- History of blast crisis or accelerated phase
- History of an allogeneic stem cell transplant

Halving time calculation

The rate of *BCR-ABL1* decline following TKI commencement from the patient's baseline can be calculated at the 3 month sample timepoint using the halving time formula shown below, adapted from Branford *et al*.[2]

$$Halving\ time = ln(2)*x/ln(y/z)$$

This formula estimates the number of days taken to achieve a 50% reduction in the baseline *BCR-ABL1* value. The number of days (x) between the baseline and 3-month *BCR-ABL1* value is calculated prior to being multiplied by the natural log of 2. We then divide this by the natural log of baseline (y) *BCR-ABL1* value divided by the 3-month (z) *BCR-ABL1* value. We have also included an excel spreadsheet which will assist in the calculation of the halving time (Supplementary file 2).

Supplementary Tables

Supplementary Table 1: Characteristics of all CML patients attempting TFR (n=130)

Patient characteristic	**Patients attempting TFR** Number (%) or Median [range]
Male	69 (53.1)
Age at TFR attempt (years)	61.0 [33.4-89.8]
Sokal score - Low-risk (<0.8) - Intermediate-risk (0.8-1.2) - High-risk (>1.2) - Not available	 53 (40.8) 48 (36.9) 20 (15.4) 9 (6.9)
ELTS score - Low-risk (≤1.5680) - Intermediate-risk (>1.5680 – ≤2.2185) - High-risk (>2.2185) - Not available	 82 (63.1) 30 (23.1) 8 (6.2) 10 (7.7)
Transcript type - e14a2 - e13a2 - Both e14a2 and e13a2 - e1a2	 56 (43.1) 50 (38.5) 23 (17.7) 1 (0.8)
Time to MR4.5 (years)	2.1 [0.1-13.2]
Duration of MR4.5 (years)	3.3 [2.0-12.5]
Duration of TKI therapy (years)	6.6 [3.0-17.6]
Therapy ceased - Imatinib - First-line 2G-TKI (nilotinib/dasatinib) or 2G-TKI for imatinib-intolerance - 2G-TKI following imatinib failure	 74 (56.9) 46 (35.4) 10 (7.7)
First-line treatment - Imatinib - 2G-TKI - Other (interferon/autograft)	 94 (72.3) 26 (20.0) 10 (7.7)
EMR achieved	113 (86.9)
3-month *BCR-ABL1* value available - *BCR-ABL1*^{IS} >10% - *BCR-ABL1*^{IS} >1-10% - *BCR-ABL1*^{IS} >0.1-1% - *BCR-ABL1*^{IS} ≤0.1%	123 (94.6) 10 (8.1) 32 (26.0) 49 (39.8) 32 (26.0)
3-month halving time available - Median halving time	115 (88.5) 13.6 [3.6-267.8]
3-month *BCR-ABL1* fold-reduction	148 [3.1-8700]
Months between baseline and 3-month *BCR-ABL1* value	3.1 [2.0-4.3]
12-month *BCR-ABL1* value available - MMR achieved	125 (96.2) 97 (77.6)

EMR, Early molecular response; IS, International Scale; MMR, Major molecular response; 2G-TKI, Second-generation TKI; TFR, Treatment-free remission; TKI, Tyrosine kinase inhibitor

Supplementary Table 2: Multivariate Cox regression analysis assessing the 3-month BCR-ABL1 fold-reduction independent from the 3-month halving time as a potential predictor of sustained TFR at 12 months

Variable	Number of patients (%)	Patients in sustained TFR (%)	Multivariate analysis			
			β-coefficient	Hazard ratio (95% CI)	*P* value	Concordance index§
Transcript						0.644
- e14a2	51 (44.3)	34 (66.7)	Ref	-	-	
- e13a2	43 (37.4)	17 (39.5)	0.76	2.13 (1.15-3.96)	0.02	
- Both transcripts	20 (17.4)	11 (55.0)	-	-	-	
Duration of TKI therapy (years)*	115 (100)		1.07	2.93 (1.13-7.60)	0.03	
Time to MR4.5 (years)*	115 (100)		-0.05	0.95 (0.82-1.08)	0.45	
3-month *BCR-ABL1*^{IS} value*	108 (94)		-0.02	0.98 (0.98-1.05)	0.31	
3-month *BCR-ABL1* fold-reduction	102 (88.7)		2.5×10^{-6}	1.00 (1.00-1.01)	0.02	

CI, Confidence interval; Ref, Reference variable; TFR, Treatment-free remission

* Continuous variables

§ Concordance index is a measure of the goodness of fit of the prediction model

Supplementary Table 3: Characteristics of the validation cohort (n=26)

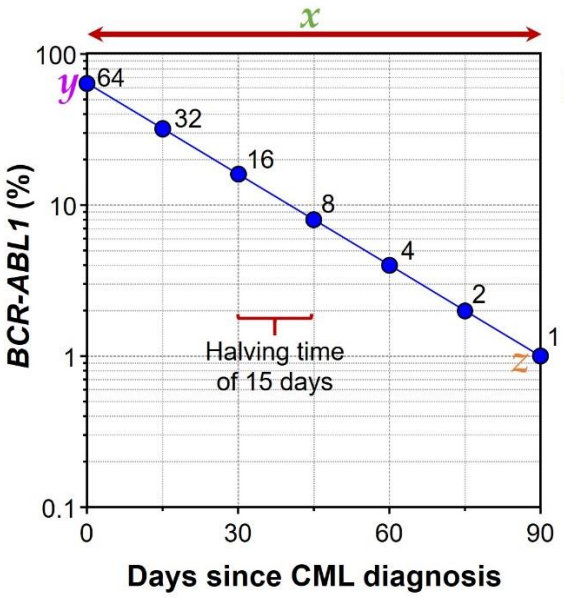
Patient characteristic	**Patients attempting TFR** Number (%) or Median [range]
Male	10 (47.6)
Age at TFR attempt (years)	39 [25-68]
Sokal score - Low-risk (<0.8) - Intermediate-risk (0.8-1.2) - High-risk (>1.2) - Not available	 13 (50) 7 (26.9) 5 (19.2) 1 (3.8)
Transcript type - e14a2 - e13a2	 22 (84.6) 4 (15.4)
Duration of TKI therapy (years)	5.75 [2.6-13.1]
First-line TKI - Imatinib - 2G-TKI	 21 (80.8) 5 (19.2)
Median halving time	12.2 [6.0-35.0]
3-month *BCR-ABL1* value available - *BCR-ABL1*^{IS} >10% - *BCR-ABL1*^{IS} >1-10% - *BCR-ABL1*^{IS} >0.1-1% - *BCR-ABL1*^{IS} ≤0.1%	 1 (3.8) 9 (34.6) 6 (23.1) 10 (38.5)
Median fold-reduction	148.7 [0.3-16000]

IS, International Scale; 2G-TKI, Second-generation TKI; TFR, Treatment-free remission; TKI, Tyrosine kinase inhibitor

Supplementary Figures

Supplementary Figure 1: Graphical representation of halving time calculation

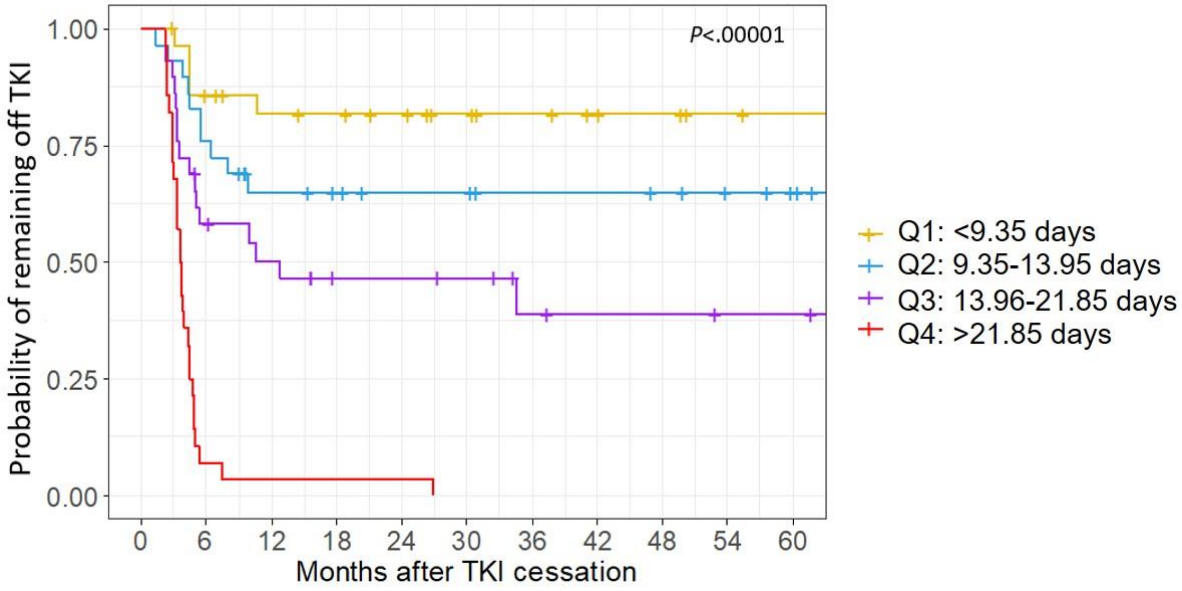
This graph illustrates a patient from our institution who had a baseline BCR-ABL1 value of 64% (y) and a 3-month BCR-ABL1 value of 1% (z). Assuming a constant rate of decline,[2] the expected interval values are plotted. The difference in days (x) between the baseline and 3-month BCR-ABL1 time points is 90 days. Based on the halving time formula, the halving time is 15 days, which means that the patient's BCR-ABL1 value halves every 15 days.



Halving time =ln(2)*x/ln(y/z)
=ln(2)*90/ln(64/1)
=15

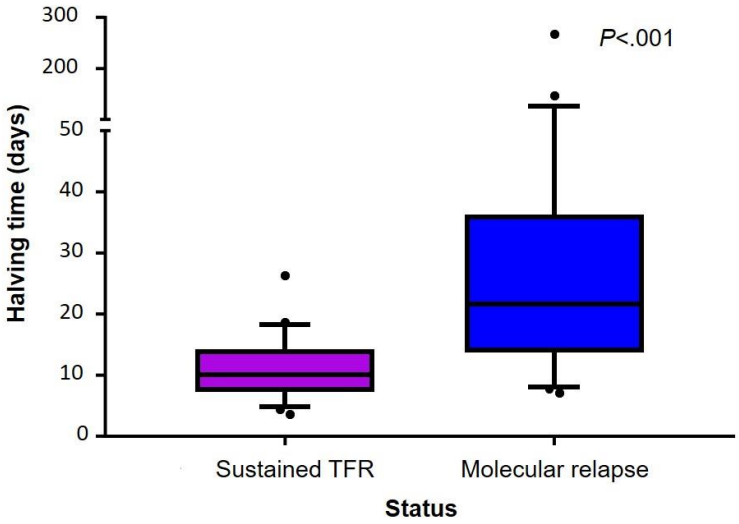
Supplementary Figure 2: Probability of remaining off TKI based on halving time quartiles in all 130 patients attempting TFR.

The halving time was available for 115 of 130 patients assessed. All late relapses (molecular relapses >12 months after TKI cessation) had halving times in either Q3 or Q4. All patients with a halving time in Q4 (halving time >21.85 days) experienced molecular relapse with no patients remaining in long-term TFR.

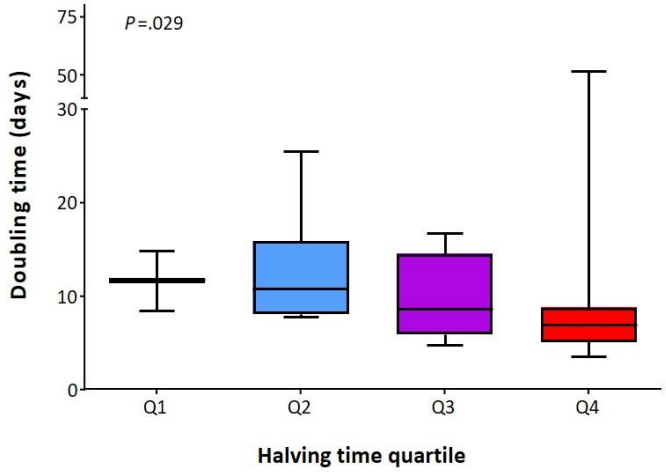


Supplementary Figure 3: Box and whisker plot of halving time between patients in sustained TFR (n=63) compared with patients with molecular relapse (n=52).

Median values, 5-95% percentile range and outliers are indicated

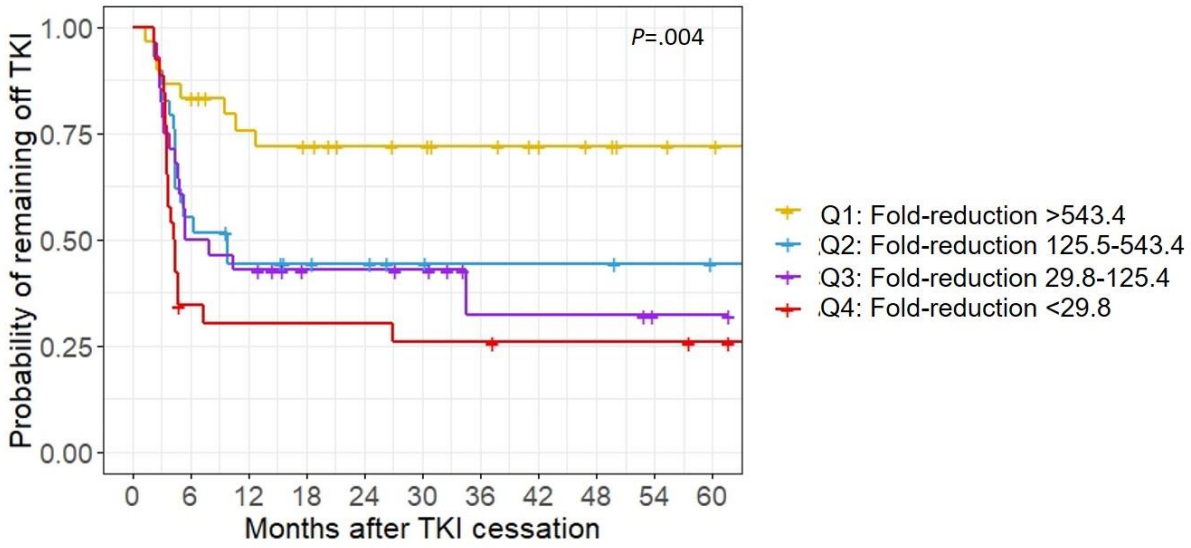


Supplementary Figure 4: Box plot illustrating the inverse relationship between halving time and rate of molecular relapse (doubling time) in patients who required TKI recommencement.
There was an association between the BCR-ABL1 doubling time at molecular relapse and the initial halving time, such that patients with the shortest halving time (Q1) had longer median doubling times compared with patients in Q2-4.



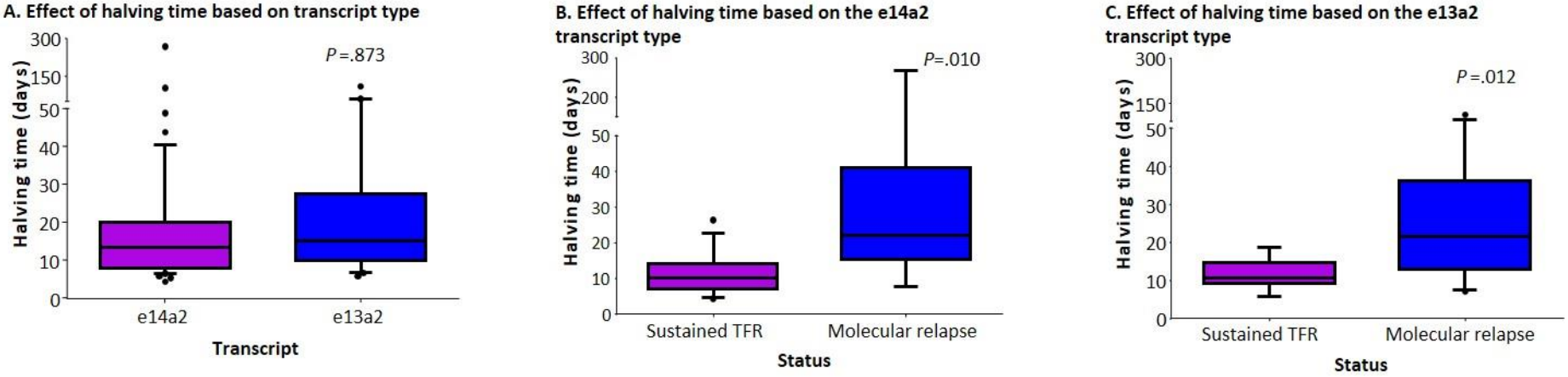
Supplementary Figure 5: Probability of remaining off TKI based on fold-reduction quartiles in all 130 patients attempting TFR.

The fold-reduction was available for 115 of 130 patients assessed. When comparing to the halving time quartiles, the fold-reduction was less discriminatory in differentiating patients who eventually experienced molecular relapse in Q2-4. Furthermore, while all patients with a Q4: halving time eventually relapsed, almost 25% with a Q4: fold-reduction still were able to remain in long-term TFR.

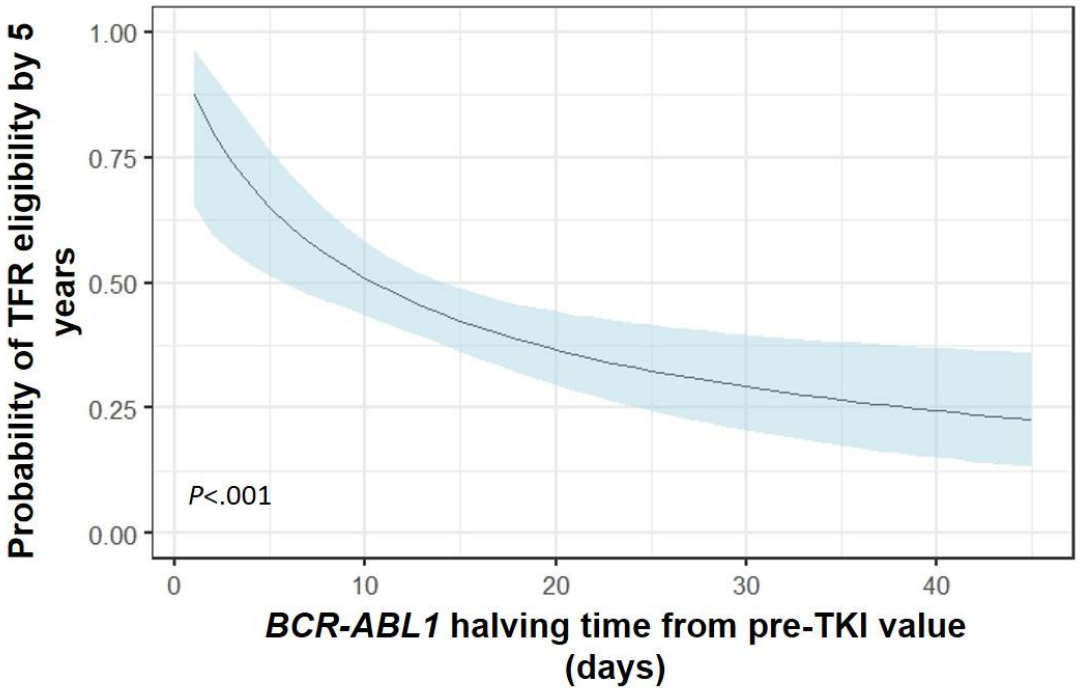


Supplementary Figure 6: Association between halving time and transcript type.

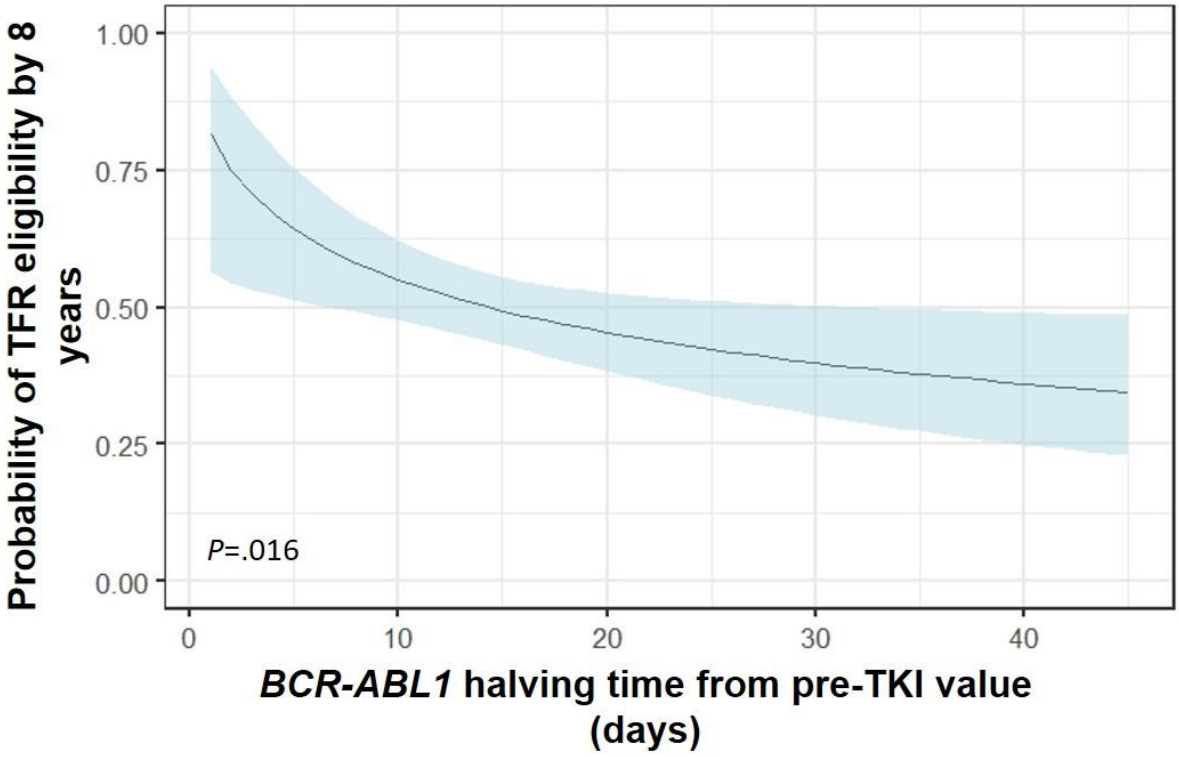
A. There was no association between the halving time based upon transcript type, e14a2 (n=45) vs. e13a2 (n=40). B. Among patients with the e14a2 transcript, there was an association between the halving time of those with sustained TFR (n=28) compared with those with molecular relapse (n=17) by 12 months. C. Among patients with the e13a2 transcript, there was an association between the halving time of those remaining in sustained TFR (n=14) compared with those with molecular relapse (n=26) by 12 months.



Supplementary Figure 7: Probability of TFR eligibility by 5 years based on halving time with 95% confidence intervals



Supplementary Figure 8: Probability of TFR eligibility by 8 years based on halving time with 95% confidence intervals



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3 Optimization of a *BCR::ABL1* monitoring strategy for treatment free remission attempts that maximise cost-effectiveness and patient safety

Preface

Monthly *BCR::ABL1* quantitative testing within the first year of a TFR attempt is a mandatory requisite to ensure the safety of the endeavour. However, this monitoring regime is prohibitive for patients in some regions of the world due to financial, institutional or regulatory constraints. In some first world nations, BCR::ABL1 testing may not be funded beyond quarterly assessments outside of clinical trials whereas in developing countries, access to and the cost of sensitive BCR::ABL1 molecular testing can be challenging for patients. These conditions limit the option of TFR for a large proportion of the CML global community due to inability to promptly detect molecular relapse in the event of a TFR attempt.

This chapter presents the modelling data of a study investigating potential monitoring strategies that would be able to reduce the frequency of molecular monitoring while maximizing timely detection of molecular relapse. Application of this novel strategy in resource-poor regions enables TFR to be offered to previously ineligible patients, expanding the health and financial benefits associated with TFR to a wider population.

The results of this study were published in Blood as "*Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission*", reference included.

- Shanmuganathan N, Braley JA, Yong ASM, et al. Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission. *Blood*. 2019;134(1):85-89.

Modelling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission

Shortened title: Monitoring Frequency after TKI cessation

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Keywords: Chronic myeloid leukemia, *BCR-ABL1*, minimal residual disease, molecular monitoring, treatment-free remission

3.1 Letter to *Blood*

With the increasing adoption of treatment-free remission (TFR) as a goal for patients with chronic myeloid leukemia (CML), rigorous molecular monitoring has been recommended to ensure timely tyrosine kinase inhibitor (TKI) recommencement in the event of molecular relapse. The NCCN has now incorporated TFR into its most recent guidelines, recommending monthly quantitative PCR monitoring of *BCR-ABL1* for the first 12 months following TKI discontinuation.[1] Molecular relapse, the trigger to re-start TKI, is currently defined as loss of major molecular response[2] (MMR; *BCR-ABL1* ≤0.1% IS), and predominantly occurs in the first 6 months following TKI cessation.[3,4] Delay in detection of molecular relapse may place patients at unnecessary risk of adverse outcomes, such as loss of complete hematological response (CHR). At a minimum, it delays the re-achievement of MMR and deep molecular response (DMR: MR4 *BCR-ABL1* ≤0.01% or MR4.5 *BCR-ABL1* ≤0.0032%). Conversely, an overly rigorous monitoring schedule imposes unnecessary costs associated with a TFR attempt, adding to logistical difficulties experienced by some patients in accessing highly sensitive, standardized *BCR-ABL1* testing[5] and may prevent some patients from stopping therapy. This is especially important in countries where funding of molecular testing is limited, and patients may be required to pay for additional tests.

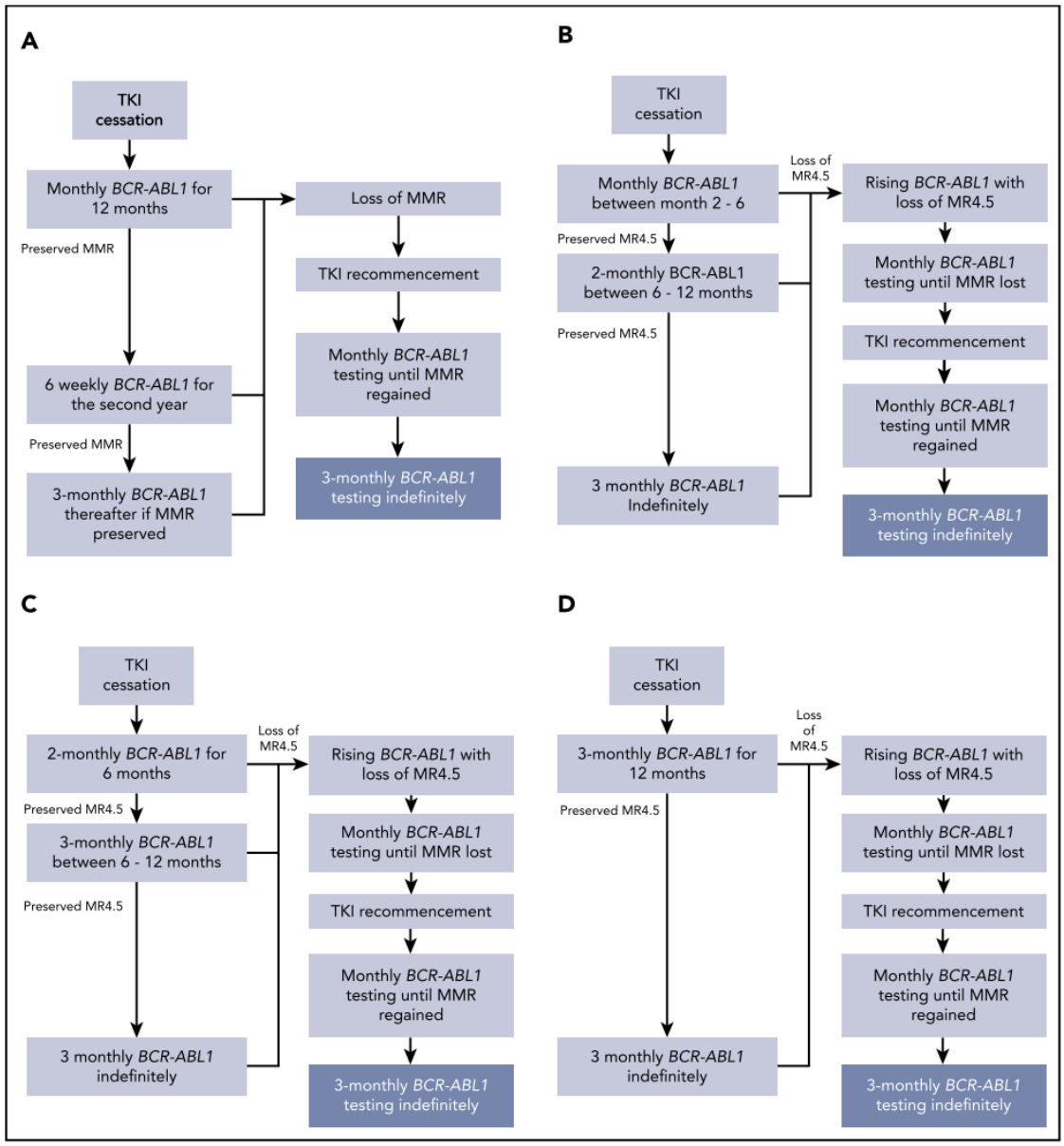
Recommendations for qualification for a TFR attempt include a minimum of 3 years of prior TKI therapy and ≥2 years of DMR.[1,6] Studies have shown that the median *BCR-ABL1* doubling time for patients with relapse is 9 days (range 6.9-26.5 days),[7] or approximately 1-log per month.[3,8] Assuming patients attempt TFR with stable MR4.5 and that the rise of *BCR-ABL1* follows this linear model,[7] patients who fail a TFR attempt will take at least

2 months to lose MMR. Thus, a reduction in monitoring to 2-monthly should still capture the majority of patients losing MMR while still in a complete cytogenetic response (CCyR; equating to a *BCR-ABL1* of <1%).[9] Furthermore, as molecular relapse is most likely to occur in the first 6 months following TKI cessation,[3,4] concentrating testing in this time-frame will be more cost-effective. Less frequent monitoring can potentially follow.

We assessed the impact of 4 monitoring algorithms, shown in Figure 1. Figure 1A delineates the current NCCN recommendations of monthly molecular monitoring after cessation (Algorithm A, 12 tests for all patients) and alternative algorithms with less frequent monitoring (Algorithms B-D). All of these algorithms include an absolute requirement for monthly monitoring if there is loss of MR4.5. Algorithm B commences monthly testing at month 2 for the first six months, followed by 2-monthly monitoring. Algorithm C introduces 2-monthly testing for 6 months followed by 3-monthly testing. Algorithm D uses 3-monthly monitoring.

Figure 1: Treatment-free remission (TFR) monitoring based on the NCCN guidelines and potential monitoring strategies.

A: Monitoring regime as recommended by the NCCN, monthly testing for the first 12 months of a TFR attempt (12 tests for all patients) B: Algorithm B commences monthly testing at month 2 for the first six months post TKI cessation followed by 2-monthly monitoring between 6-12 months, C: Algorithm C commences 2-monthly testing for the first 6 months post TKI cessation followed by 3-monthly monitoring, D: Algorithm D maintains 3-monthly testing throughout the TFR attempt. All algorithms incorporate monthly testing following loss of MR4.5.

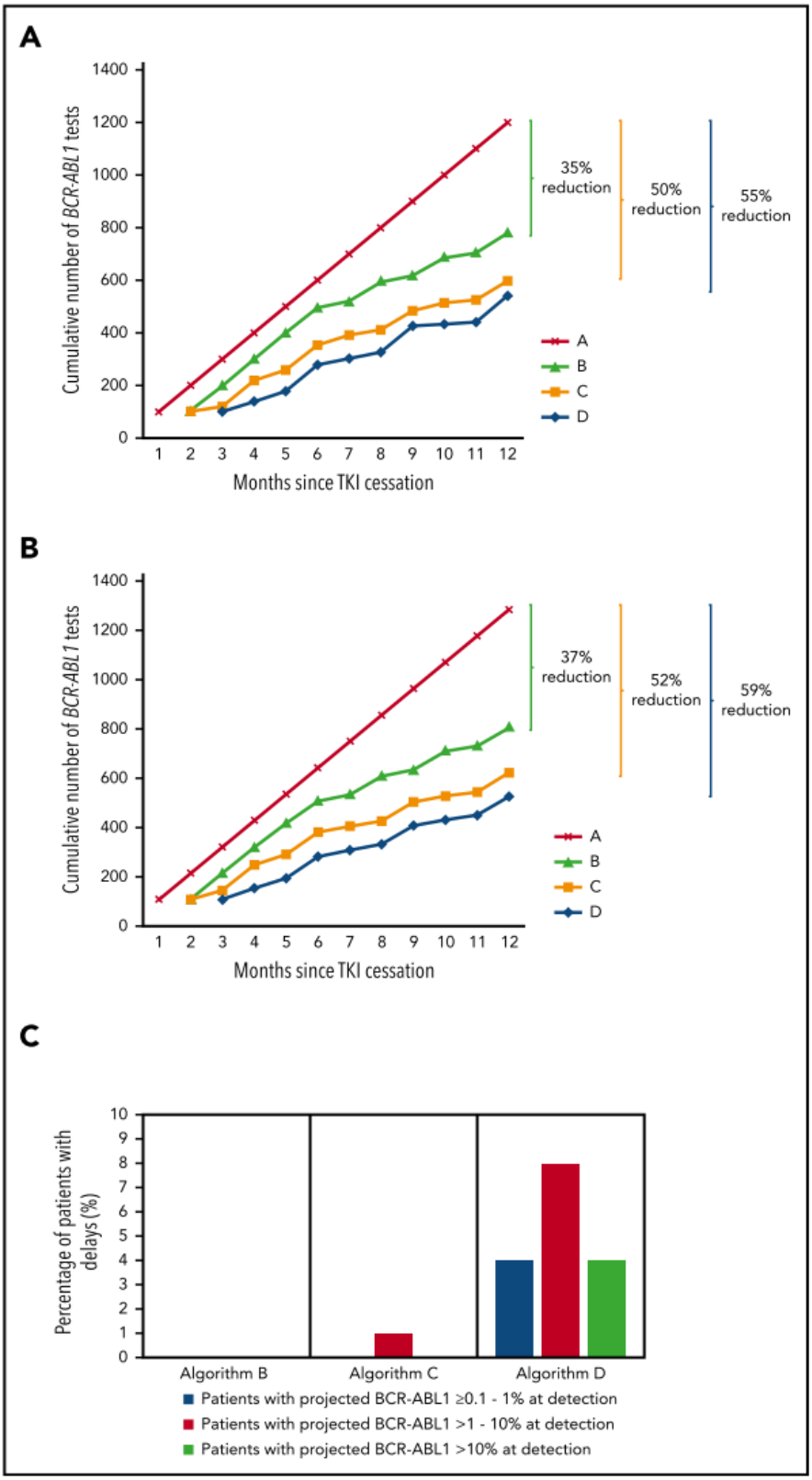


A theoretical cohort of 100 patients was used to predict the impact of reduced monitoring frequencies for patients attempting TFR, recommencing TKI in the event of MMR loss. Based on EURO-SKI data, 39% of patients will relapse and recommence TKI by 6 months with a further ~7% predicted to lose MMR by 12 months.[6] Following TKI recommencement, the NCCN recommend monthly *BCR-ABL1* monitoring until MMR is regained followed by resumption of 3-monthly testing.[1] We estimated the number of tests over the first 12 months based on the EURO-SKI molecular relapse rate,[6] the kinetics of molecular relapse[3,7,8] (Supplemental Methods) and the increased monitoring

requirement following TKI recommencement according to the 4 algorithms outlined. Compared to Algorithm A, each alternative algorithm decreased the total number of *BCR-ABL1* tests in the theoretical cohort (Figure 2A); Algorithms B, C and D would reduce testing by 35%, 50%, and 55%, respectively.[7]

The different monitoring algorithms were then retrospectively applied to the actual data of 107 patients who attempted TFR with monitoring at our institution (Supplemental Methods). The TKI at the time of cessation were imatinib (68%, n=73), nilotinib (21%, n=22), dasatinib (10%, n=11) or bosutinib (1%, n=1). The actual number of tests per patient was 12 (total 1284). Molecular relapse occurred in 49 patients (46%) by 12 months and 54% remained off TKI, consistent with previous studies.[2-4,10-12] TKI was recommenced in 5 patients with rising *BCR-ABL1* values on consecutive tests prior to MMR loss, all of whom were predicted to lose MMR within 1-2 months based on the calculated doubling times.[7] Loss of CCyR occurred in 7 patients (7%) at the time of molecular relapse and TKI was recommenced within 1 month of molecular relapse in 38/49 patients. Seven patients recommenced TKI between 1-1.5 months and 4 recommenced at 1.7-2.3 months. One of these 4 had >10% *BCR-ABL1* and CHR loss, which was related to delayed TKI recommencement secondary to patient concerns regarding previous TKI toxicity. All patients rapidly regained MMR (median 2.0 months) with no significant difference in the *BCR-ABL1* halving times[13] between patients who restarted TKI with *BCR-ABL1* values of 0.1-1% vs. >1%, confirming TKI sensitivity on recommencement regardless of *BCR-ABL1* value.

Figure 2: Cumulative frequency of BCR-ABL1 testing over the course of the first 12 months following TKI cessation utilizing the various testing algorithms demonstrated in Figure 1.
A: Theoretical cohort of 100 patients, B: 107 real patients who underwent a TFR attempt and were monitored at our institution. C: Predicted effect of detection delays based upon algorithms B-D for the 107 real patients.



The estimated cumulative number of *BCR-ABL1* tests over 12 months after TKI cessation using actual patient data in each of the four monitoring algorithms is shown in Figure 2B. Three patients lost MR4.5 early, with fluctuating *BCR-ABL1* levels, but remained in MMR at 12 months; such patients require monthly testing regardless of which monitoring algorithm is applied. Application of algorithm B would have resulted in a 37% reduction in testing (807 tests, average 7.5 tests/patient). Importantly, no delay to molecular relapse detection or re-starting TKI was predicted (Figure 2C). A reduction of 52% (621 tests, average 5.8 tests/patient) would occur if algorithm C were employed. One patient was predicted to have a delay of one month to detection of molecular relapse and re-starting TKI. This would likely result in loss of CCyR at the time of relapse detection as calculated from the *BCR-ABL1* doubling time.[7] Monitoring patients according to algorithm D would account for the largest reduction in testing at 59% (525 tests, average 4.9 tests/patient). However, this algorithm would delay the detection of molecular relapse by 1-2 months in 17 patients (16%) with associated TKI recommencement delays. Furthermore, 13 of these 17 patients were projected to lose CCyR, and 4 were projected to have *BCR-ABL1* >10% at the time of relapse detection.

The monthly monitoring undertaken in the first TKI studies was a cautious measure instituted at a time when the potential risks of discontinuing TKI therapy were unknown. We have demonstrated that a reduced frequency of monitoring in the first 12 months of a TFR attempt is likely to be safe. A planned monitoring frequency of 2-monthly in the first 6 months and 3-monthly between 6 and 12 months (Algorithm C) may provide the best balance between reduced testing and minimization of delays in relapse detection

and TKI recommencement. Patients and clinicians in settings without constraints on molecular monitoring may still prefer more frequent testing for the reassurance that it provides. We recommend re-initiation of monthly *BCR-ABL1* testing at loss of MR4.5, including in the few patients with fluctuating *BCR-ABL1* levels without MMR loss, which was 3% of our cohort. We have not examined the recommendation for stringent monthly monitoring in patients requiring TKI recommencement due to molecular relapse. Resistance to TKI re-treatment is exceedingly rare. Nevertheless, we agree with the current cautious recommendation of monthly monitoring until regaining MMR and subsequent 3-monthly monitoring thereafter.[1] Our data show that *BCR-ABL1* can increase substantially in the time between detection of molecular relapse and re-starting TKI therapy. This highlights the importance of a rapid turnaround time for *BCR-ABL1* results (and clinical action to the result), especially with a reduced monitoring frequency. Less frequent monitoring would make TFR attempts more cost-effective. More importantly, in some settings reduced monitoring may enable clinicians to offer TFR to patients for whom the availability of molecular monitoring is a barrier. Further prospective studies are needed to validate this proposal prior to incorporation into the current standard of care.

3.2 Acknowledgements

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4 Validation of low-level *BCR::ABL1* kinase domain mutation detection by mass spectrometry testing: addition of asciminib-resistant mutations

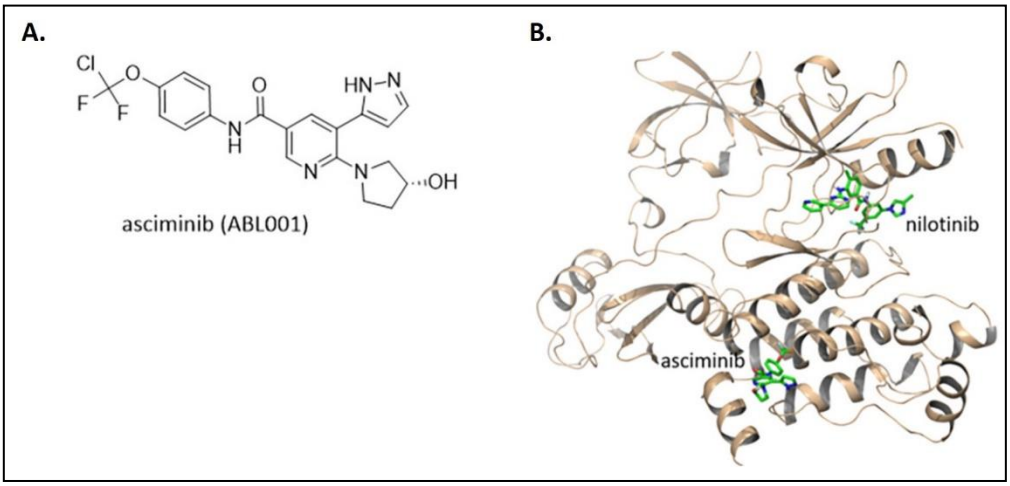
4.1 Introduction

Although the therapeutic landscape in CML was revolutionized with the introduction of TKIs, TKI failure still occurs with a small proportion of patients progressing to accelerated (AP) or blast phase (BP).[1-4] Intrinsically, *BCR::ABL1* promotes genetic instability, providing the perfect environment for the further gain of additional mutations.[5-7] *BCR::ABL1* kinase domain mutations are currently the best understood resistance mechanism in CML. They are also an adverse prognostic factor, conferring an increased risk of transformation to AP/BP CML while predicting for an inferior progression free and overall survival.[3,4] However the current gold standard of detection and monitoring of these mutations is Sanger sequencing which, although fast and relatively cheap, has a mutation detection limit of 10-20%,[8,9] negating its applicability for low-level mutation testing. The prognostic importance of low-level mutation detection has been repeatedly demonstrated[9-11] as it provides crucial information that can guide subsequent therapy selection in patients with TKI resistance. Inappropriate TKI selection will lead to expansion of the resistant clone resulting in a high risk of treatment failure and disease progression. A highly sensitive, multiplexed mass spectrometry assay was previously developed by SA Pathology, which has the capability of identifying low-level kinase domain mutations and has demonstrated clinical utility.[9-11] The assay is based on a multiplex single-allele base extension reaction (SABER) protocol which excludes amplification of wild type (unmutated) sequences, maximizing amplification of any mutant present. Thirty-one mutations in the *BCR::ABL1* kinase domain were able to be detected by this assay to a detection limit of 0.05-0.5%. These mutations represent approximately 92% of all mutations detected in patients resistant to first- and second-generation TKIs. However, a number of

additional BCR::ABL1 inhibitors have since been developed for the treatment of CML patients, including the selective allosteric ABL1 inhibitor, asciminib. The emergence of specific asciminib-resistant kinase domain mutations was predicted from pre-clinical studies[12] due to its novel mechanism of action of binding to the myristoyl pocket of ABL1. This is different from other BCR::ABL1 inhibitors, which bind to the catalytic domain (Figure 1).[13] Our laboratory was the first to report asciminib resistant mutations in a patient treated in a Phase 1 trial.[12]

Figure 1: The structure and binding site of asciminib[14]

A. The chemical structure of asciminib, B. The binding site of nilotinib compared with asciminib which is shown binding to the novel myristate pocket of BCR::ABL1



The Phase III international multi-centre Asciminib study (www.clinicaltrials.gov identifier NCT03106779), sponsored by Novartis Pharmaceuticals, was planned for investigation of the efficacy of asciminib in heavily pre-treated CP-CML patients that were either resistant or intolerant to ≥2 TKIs.[15] Patients were randomised to either 40mg twice daily of asciminib or 500mg daily of bosutinib. As part of the study, The Leukaemia Unit of SA Pathology was asked to perform low-level kinase domain mutation testing by Novartis Pharmaceuticals in conjunction with NGS for cancer gene mutations at key time points. Correlative studies, including both mass spectrometry and NGS, were planned at study entry and either at the end of the clinical trial or study withdrawal, assuming BCR::ABL1 transcripts ≥0.1%^{IS} for the mass spectrometry assay. Mass spectrometry was specifically included to detect the presence of low-level kinase domain mutations that could potentially predict TKI resistance. It has been demonstrated that the use of a TKI (such as bosutinib) with demonstrable resistance to a low-

level mutant (e.g. T315I) will permit the expansion of that specific TKI-resistant clone, resulting in therapy failure.[16]

As a large proportion of patients enrolled in the study had a history of TKI resistance to imatinib, dasatinib, nilotinib and/or ponatinib, non-myristoyl kinase domain mutations were expected to be present at study enrolment, including low-level mutations. However, asciminib resistance-specific mutations, which would potentially emerge during the clinical trial due to asciminib resistance, would not be detectable by the original mass spectrometry design. Therefore, redesign of the assay was required, including comprehensive validation of the analytical performance of the new assay in order to reliably and sensitively detect asciminib-resistant mutations. Additional mutations were also included in the redesigned assay that had been found to play a role in resistance to the third-generation TKI, ponatinib.

The re-development and the comprehensive validation study of the mass spectrometry assay is included in this chapter. The mass spectrometry results of the clinical trial are not available since the study follow up is ongoing.

For the purposes of clarity, the original mass spectrometry assay will be referred to as MSA v1 while the new modified method will be referred to as MSA v2.

4.2 Mutation list

An additional 11 mutations were incorporated into the current mass spectrometry assay, as listed in Table 1. This list was finalised after significant discussion between the Novartis team and the Leukaemia Unit, SA Pathology, to ensure that all relevant mutations observed in the asciminib clinical trials were encompassed. The included asciminib-specific mutations were reported either in cell lines or patients in publication format or conference presentation by March 2019. Other rare mutations observed in CML or Philadelphia positive acute lymphoblastic leukaemia (Ph+ ALL) that have developed in response to other TKIs were also included. While both the T315M and I315M were included in the initial plan, mutation naming

convention dictates that they are the same mutation. Consequently, only the T315M will be referred to in this document. In June 2019, the mutation list was finalised.

Subsequent to the mutation list being finalised, the Phase I asciminib study was published in December 2019,17 revealing additional asciminib-resistant mutations reported in patients (G463D, A337T, G109D, Y115N). These were identified via NGS with 3 mutations only identified at <10%. By this stage, the development phase of the mass spectrometry assay had commenced preventing incorporation of the mutations into the modified design. None of these mutations will be identified by MSA v2.

Table 1: BCR::ABL1 kinase domain mutations detectable by the modified mass spectrometry assay. *ABL1 reference sequence NM_005157.*

Kinase domain mutation	Nucleotide position change	Nucleotide change within the codon[+]	Reason for inclusion
M244V	730A>G	ATG>GTG	
L248V	742C>G	CTG>GTG	
D276G	827A>G	GAC>GGC	
G250E	749G>A	GGG>GAG	
Q252H	756G>C	CAG>CAC	
Q252H	756G>T	CAG>CAT	
Y253H	757T>C	TAC>CAC	
Y253F	758A>T	TAC>TTC	
E255K	763G>A	GAG>AAG	
E255V	764A>T	GAG>GTG	
E279K	835G>A	GAG>AAG	
V299L	895G>C	GTG>CTG	
V299L	895G>T	GTG>TTG	
F311I[α]	931T>A	TTC>ATC	Rare imatinib resistant mutation
T315A	943A>G	ACT>GCT	
T315I	944C>T	ACT>ATT	

T315L[α]	943A>C 944C>T 945T>A	ACT>CTA	[P,CL]Rare ponatinib resistant mutation[18,19]
T315M[α]	944 C>T 945 T>G	ACT>ATG	[P,CL]Rare ponatinib resistant mutation[18,19]
F317I	949T>A	TTC>ATC	
F317L	949T>C	TTC>CTC	
F317V	949T>G	TTC>GTC	
F317C	950T>G	TTC>TGC	
F317S	950T>C	TTC>TCC	
F317L	951C>G	TTC>TTG	
F317L	951C>A	TTC>TTA	
A337V[α]	1010C>T	GCC>GTC	[P,CL]Asciminib resistant mutation[12,20]
M351T	1052T>C	ATG>ACG	
E355G	1064A>G	GAG>GGG	
F359I	1075T>A	TTC>ATC	
F359V	1075T>G	TTC>GTC	
F359C	1076T>G	TTC>TGC	
H396P	1187A>C	CAT>CCT	
H396R	1187A>G	CAT>CGT	
E450G[α]	1349A>G	GAG>GGG	Rare imatinib resistant mutation
E459K	1375G>A	GAG>AAG	
G463S[α]	1387G>A	GGC>AGC	[P]Asciminib resistant mutation[17]
C464W[α]	1392C>G	TGC>TGG	[CL]Asciminib resistant mutation[21]
P465S[α]	1393C>T	CCA>TCA	[P,CL]Asciminib resistant mutation[12]
V468F[α]	1402G>T	GTC>TTC	[P,CL]Asciminib resistant mutation[12]
F486S	1457T>C	TTT>TCT	
F497L[α]	1491C>G	TTC>TTG	[CL]Asciminib resistant mutation[22]
I502L[α]	1504A>C	ATC>CTC	[P,CL]Asciminib resistant mutation[12]

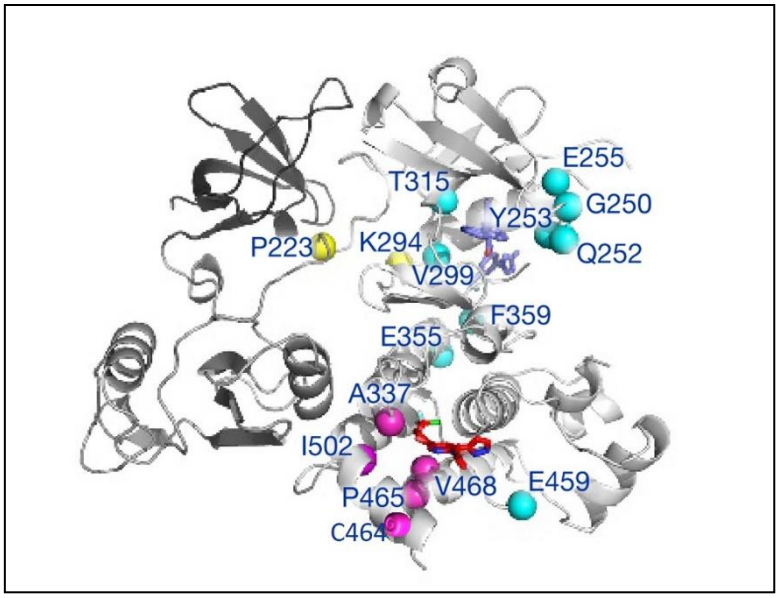
[+]Nucleotide change indicated in red

[α]Additional mutations incorporated into the mass spectrometry assay

[CL]Mutation reported in cell line data

[P]Reported in patients

Figure 2: Location of resistance mutations overlaid on the ABL1 structure[12,21]
Asciminib-resistant mutations (A337, C464, P465, V468 and I502) located at the myristoyl site are highlighted in magenta.



4.3 Study design

4.3.1 Principle of the mass spectrometry assay

The low-level mass spectrometry assay is based on a SABER protocol which analyses amplified cDNA products by chip-based MALDI-TOF mass spectrometry.[23] The SABER protocol ensures increased sensitivity as extension products are not generated for the wild type (unmutated) sequence, which may be present in abundance, maximizing the availability of the extension primer to extend onto the mutant sequence in later cycles.[11] While this approach will preclude estimating the mutant percentage, the high sensitivity of the assay over alternative methods of mutation detection outweighs this loss. The Agena MassARRAY system, using iPLEX reagents, is the platform used to sensitively detect the mutations. This assay is not quantitative but generates an estimation of the mutant percentages based on dilutions of standards of known mutant quantity. All values greater than our highest standard dilution

(2%) are reported as >2%, irrespective of the actual mutant percentage quantified by other methods, such as 100% by Sanger sequencing.

The original mass spectrometry assay[11] (MSA v1) design involved four separate primer mixes to detect the 31 *ABL1* kinase domain mutations. The mutant base nucleotide determined which mix the primer pair was assigned. Incorporation of additional mutations would either require inclusion of additional primers into the existing four mixes or the preparation of four other primer mixes specifically for the novel mutants. The first option would require retesting and revalidation of all existing mutations as well as the new additional mutations. Additional samples would be required for the validation study, whereas the second option would only require validation of the new primer mixes. However, this would require almost double the labour and consumables given the expansion of the new assay to eight mixes. Ultimately, the eight primer mix assay option was selected.

Development of the new assay required careful primer design. The primers also necessitated careful consideration of mix allocation of the designed primers to ensure that the required nucleotide only extended onto the mutant base. New assay controls and standards were required. Unlike the plasmid standards utilised in MSA v1, unique double stranded DNA fragments, known as gBlocks, were designed and validated prior to inclusion as assay standards.

Prior to the validation study being conducted, the MSA v2 design required method optimization, confirmation that the gBlock fragments functioned as appropriate assay standards and verification that all investigated mutants could be detected at a very low level. The gBlock assay standards enabled estimation of detected mutants as the SABER technique does not permit mutant quantification. Appropriate retrospective patient samples with relevant mutations required identification and preparation for the validation study. Samples selected for the optimization and validation studies required PCR isolation of the *BCR::ABL1* kinase domain to exclude the normal *ABL1* allele in anticipation for mass spectrometry.[24] Following optimization, the method required comprehensive validation, which included

determination of intra- and inter-assay reproducibility, limit of detection (LOD), and retrospective patient testing.

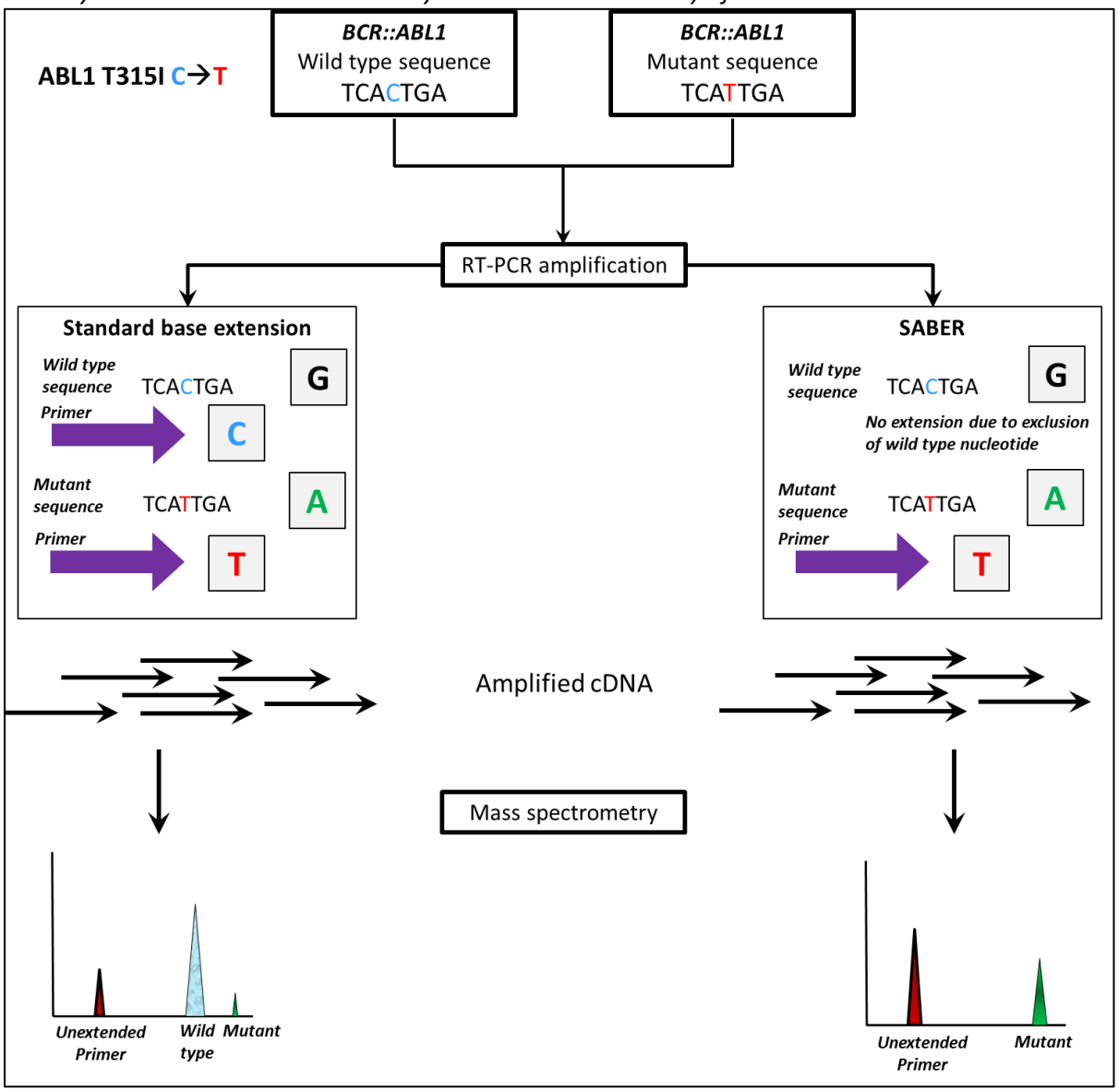
4.3.2 Assay design

4.3.2.1 Primer design

Universal and extension primers were designed using the Agena MassARRAY® Assay Design Suite v2.0. Universal primers amplify the region surrounding individual mutations within the *BCR::ABL1* kinase domain. Extension primers hybridize directly adjacent to the target mutated base on the amplified cDNA fragment. The mutation is identified by extension of a single mass-modified terminator nucleotide that is complimentary to the mutated base. Extension primers were designed in the forward or reverse direction to minimise overlap of the last 5 bases of the extension primer with other mutations, as their presence would reduce binding efficiency of the primer. Mutations were then grouped according to the iPLEX terminator nucleotide required for extension onto the mutated base. Four multiplex genotyping reactions, in addition to the existing four reactions in MSA v1, were designed using the SABER protocol. Increased sensitivity of the reaction was ensured through exclusion of extension of the wild type sequence through omission of the corresponding iPLEX terminator. Complex mutations involving multiple nucleotide exchanges were included in MSA v2 as detailed in Table 1 for T315L and T315M. Detection of these mutations is reliant on the combined detection of nucleotide exchange using the existing T315I or T315A assays in MSA v1 and nucleotide exchange in the new assay MSA v2. The SABER protocol and primer design ensured that only the targeted mutations would be identified (Figure 3).

Figure 3: Method of mutant detection by mass spectrometry: standard base extension compared with the SABER protocol

Comparison of mutant detection according to the mass spectrometry base extension method. The ABL1 kinase domain mutation T315I is used as an example. The BCR::ABL1 kinase domain containing both mutant and non-mutated molecules are isolated and amplified by RT-PCR. The standard base extension method will detect both mutated and non-mutated molecules, whereas the SABER technique will only detect the mutant and thereby enhances the sensitivity of mutant detection.



4.3.2.2 Standards and control samples

For the MSA v1, plasmids containing mutant and wild type *BCR::ABL1* were generated for each of the genotyping assays.[11] Double-stranded DNA fragments of ~1Kb of the *ABL1* kinase domain containing mutated and wild type *BCR::ABL1* were generated to use as standards for the MSA v2 assay, known as gBlock Gene Fragments (manufactured by Integrated DNA Technologies). The gBlock gene fragments were composed of kinase domain sequences that

were modified to incorporate specific mutant bases. Coverage of all additional mutations required multiple gBlocks to be designed to minimize interference by overlapping of primer and mutant sequences. Ultimately, four gBlocks were designed. Three gBlocks covered all the required mutations while the final fragment had the wild type sequence. There was overlap in mutation coverage across the gBlocks to ensure that each gBlock could serve as standards for multiple multiplex reactions. The gBlock sequences are included in Appendix I. To generate the various mutant dilutions for the standards, the mutant gBlocks were diluted in the wild type gBlock to form mutant mixes of 100%, 50%, 20%, 2%, 1%, 1.5%, 0.5%, 0.2%, and 0.05%. Due to the multiple nucleotide change for T315L and T315M, two mutations on two gBlocks were confirmed on MSA v1 using the T315A or the T315I assays.

Each multiplex reaction also had extension primers designed to extend onto either nucleotide 969C or 961G within exon 6 (ABL1 mRNA NCBI Reference Sequence: NM_005157). These nucleotides have no known sequence variations and serve as loading and reaction positive controls for all samples, including wild type controls and patients with no evidence of mutations.

BCR::ABL1 positive cell lines KU812 (containing F497L)[22] and BV173 (containing E255K and E459K) were both diluted to 50% mutant to serve as positive controls. The negative control was the wild type *BCR::ABL1* plasmid while the remaining negative cluster standards were composed of *BCR::ABL1* negative patient samples and the cell line HeLa. The standards, positive and negative controls occupy 10 positions in each primer mix irrespective of the number of samples analysed.

4.3.2.3 Criteria for mass spectrometry mutation calling

The new assay design required that each sample be tested using the 8 primer mixes to determine the mutation status. Typer software (Version 4.1, Agena) was used to analyse each mass spectrometry run. The software generates automated mutation calls using the default computational algorithms for SNP genotyping. However, as the *BCR::ABL1* assay is designed for somatic mutation detection, manual calling is necessary for some low-level mutations,[11] just as it is for Sanger sequencing. Manual calling is performed by visual inspection of the details plot and Typer Yield Call Cluster Plot, which compares the area under the curve of the

extension product peak with that of the unextended primer peak (yield). Patient samples are called positive if the following criteria are met:

1. Extension primer controls are positive
2. Negative control samples are clustered close to the origin
3. Plasmid/gBlock standards are positioned away from the negative cluster
4. The positive control sample is positive

Additionally, each sample is run in duplicate. Mutations are only called positive if detected in both runs.

The detailed method is documented in PRC-GMO-464 (Appendix I).

4.4 Assay specificity

4.4.1 gBlock analytical specificity

Once reconstituted, the gBlocks underwent PCR amplification using specially designed primers (see Appendix 2 from PRC-GMO-464/Appendix I) to amplify the desired region followed by confirmation of the expected mutations using Sanger sequencing. The wild type gBlock matched the reference sequence.

4.4.2 Confirmation of gBlock specificity

Mass spectrometry confirmed that only the targeted mutations were identified in each of the undiluted gBlocks and no additional mutations were detected. The gBlocks were also analysed using the MSA v1 assay in conjunction with the relevant plasmid to confirm the serial dilutions performed in the same manner as the plasmids (Figure 4). As some of the novel mutations were reliant on MSA v1 primers for detection, some of the newly designed gBlocks could be detected by MSA v1 primer mixes. Demonstrated below are the three gBlocks with confirmation of the anticipated mutations using Sanger sequencing (Figures 5-7).

No additional unexpected mutations were identified on any of the four gBlocks.

Figure 4: Confirmation of matching dilution series of gBlocks and plasmids

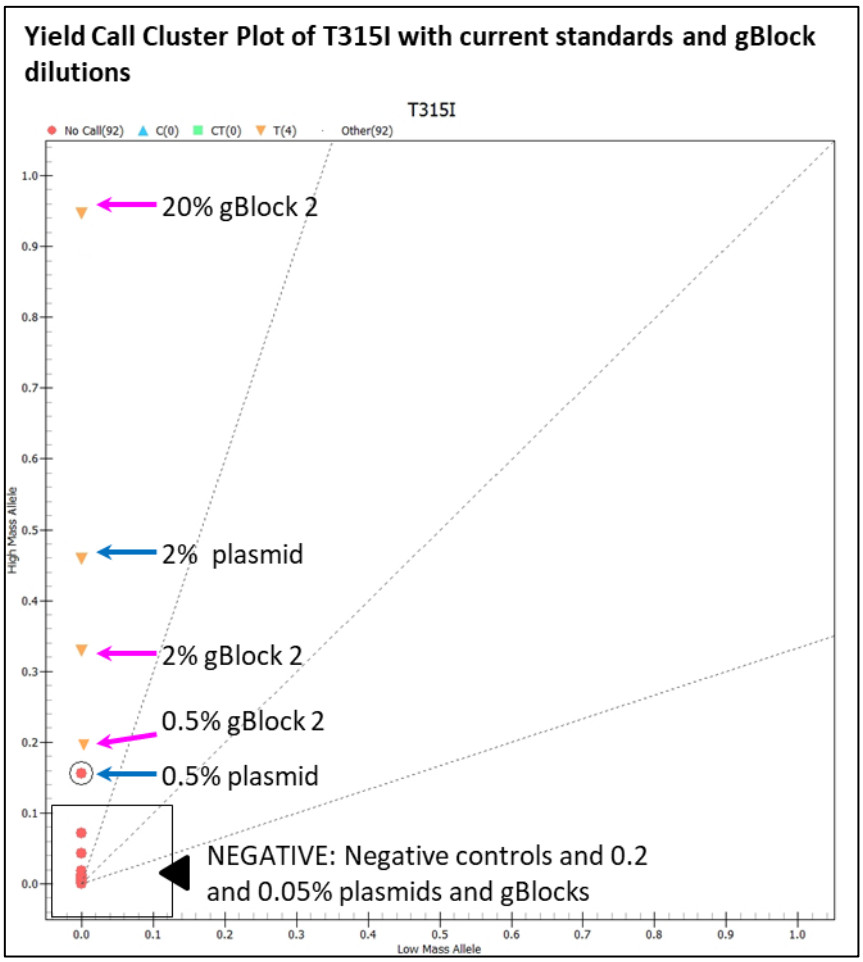


Figure 5: Sanger sequencing of gBlock-1 confirming the presence of control, T315L, E450G and I502L mutations.

A. Nucleotide sequence; row 1= reference sequence, row 2 = wild type gBlock, row 3= gBlock-1. B. Amino acid sequence of reference sequence (row 1) and gBlock-1 (second row). C. Chromatogram of reference sequence. D. Chromatogram of gBlock-1 with nucleotide substitution (red arrow) and control (green star).

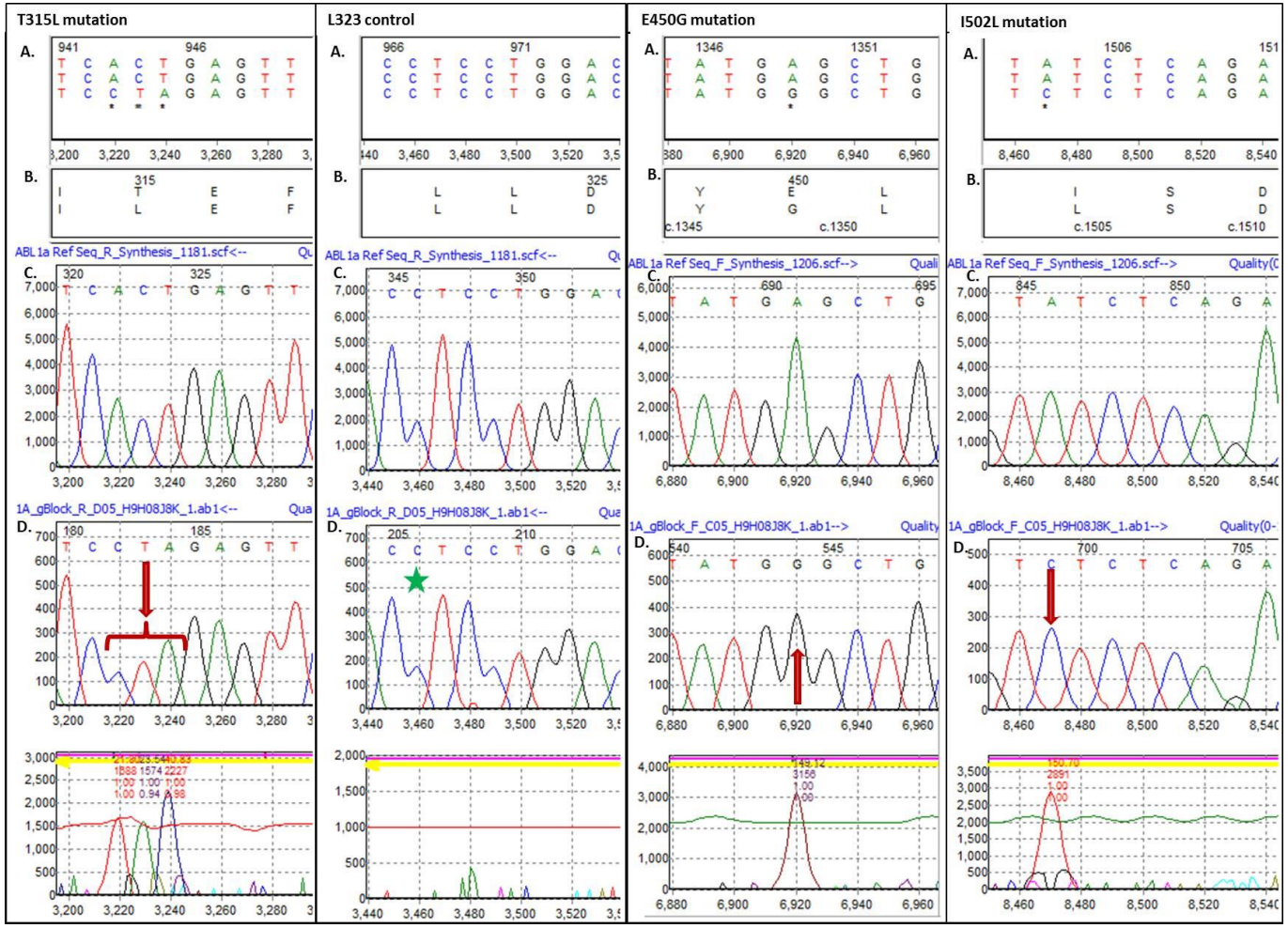


Figure 6: Sanger sequencing of gBlock-2 confirming the presence of control, T315M, A337V, C464W, V468F and F497L mutations.

A. Nucleotide sequence: row 1 = reference sequence, row 2 = wild type gBlock and row 3= gBlock -2. B. Amino acid sequence of reference sequence (row 1) and gBlock-2 (row 2). C. Chromatogram of reference sequence. D. Chromatogram of gBlock-2 with nucleotide substitution (red arrow) and control (green star).

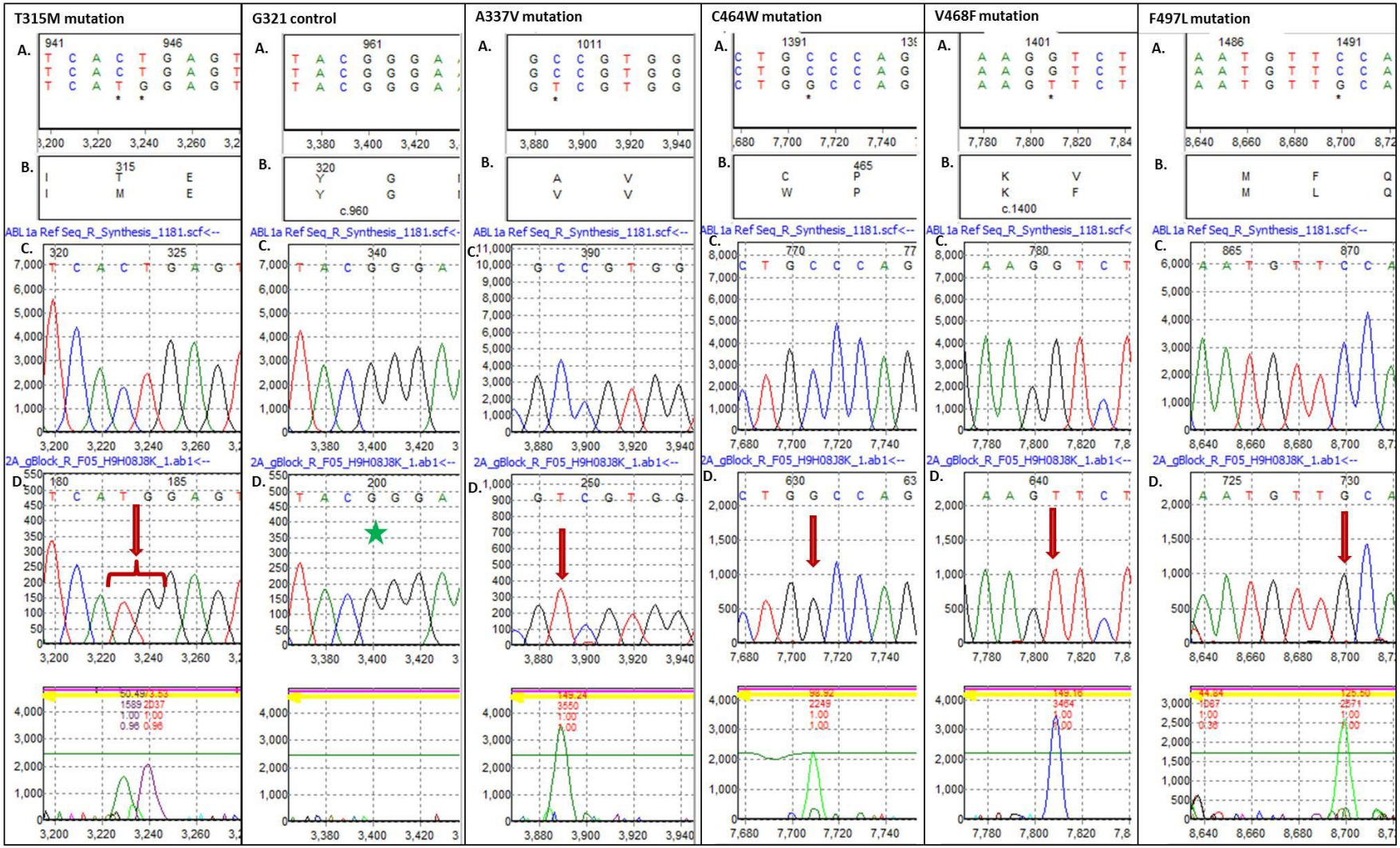
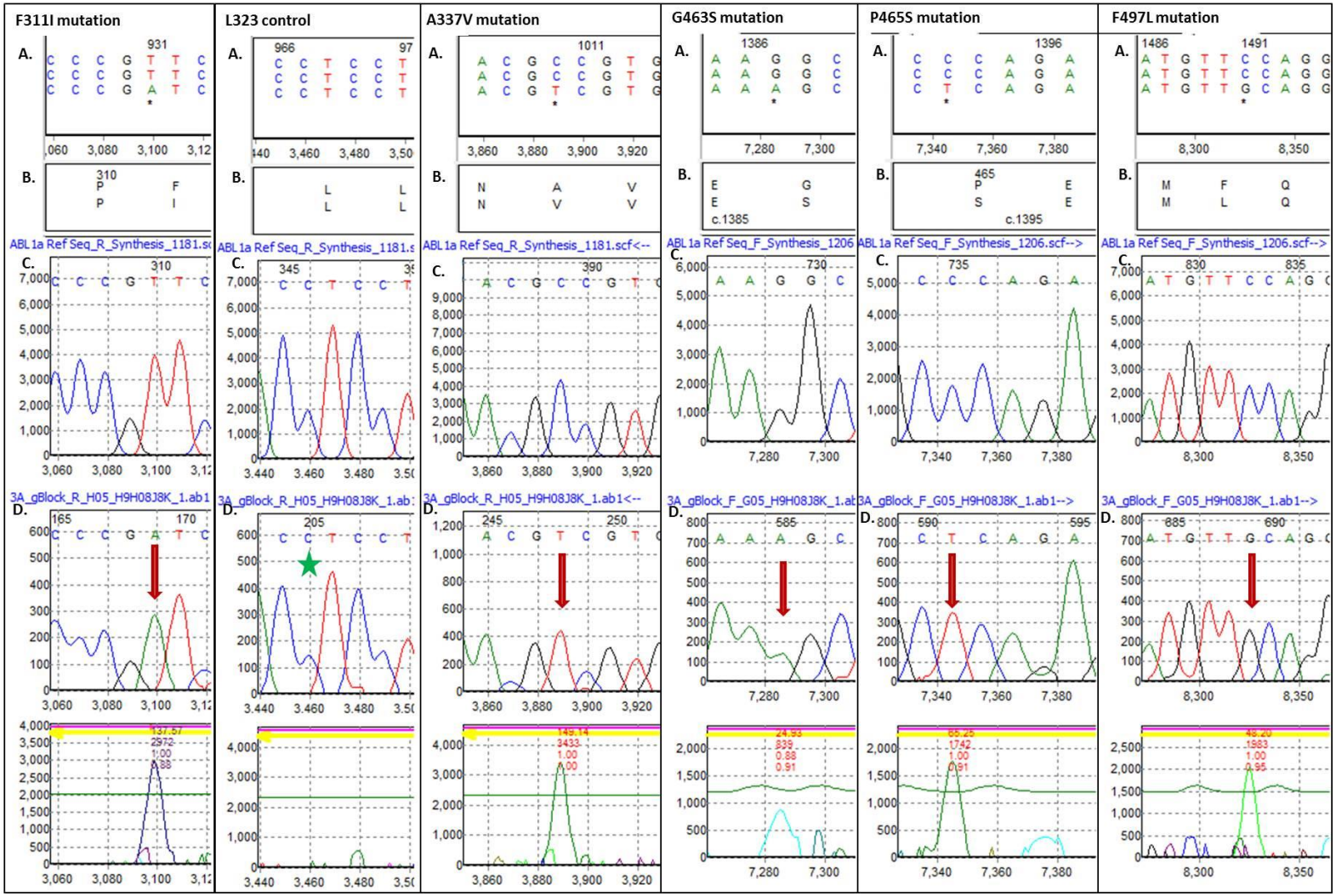


Figure 7: Sanger sequencing of gBlock-3 confirming the presence of control, F311I, A337V, G463S, P465S and F497L mutations.

A. Nucleotide sequence: row 1= reference sequence, row 2 = wild type gBlock and row 3 = gBlock-3. B. Amino acid sequence of reference sequence (row 1) and gBlock-3 (row 2). C. Chromatogram of reference sequence. D. Chromatogram of gBlock-3 with nucleotide substitution (red arrow) and control (green star).

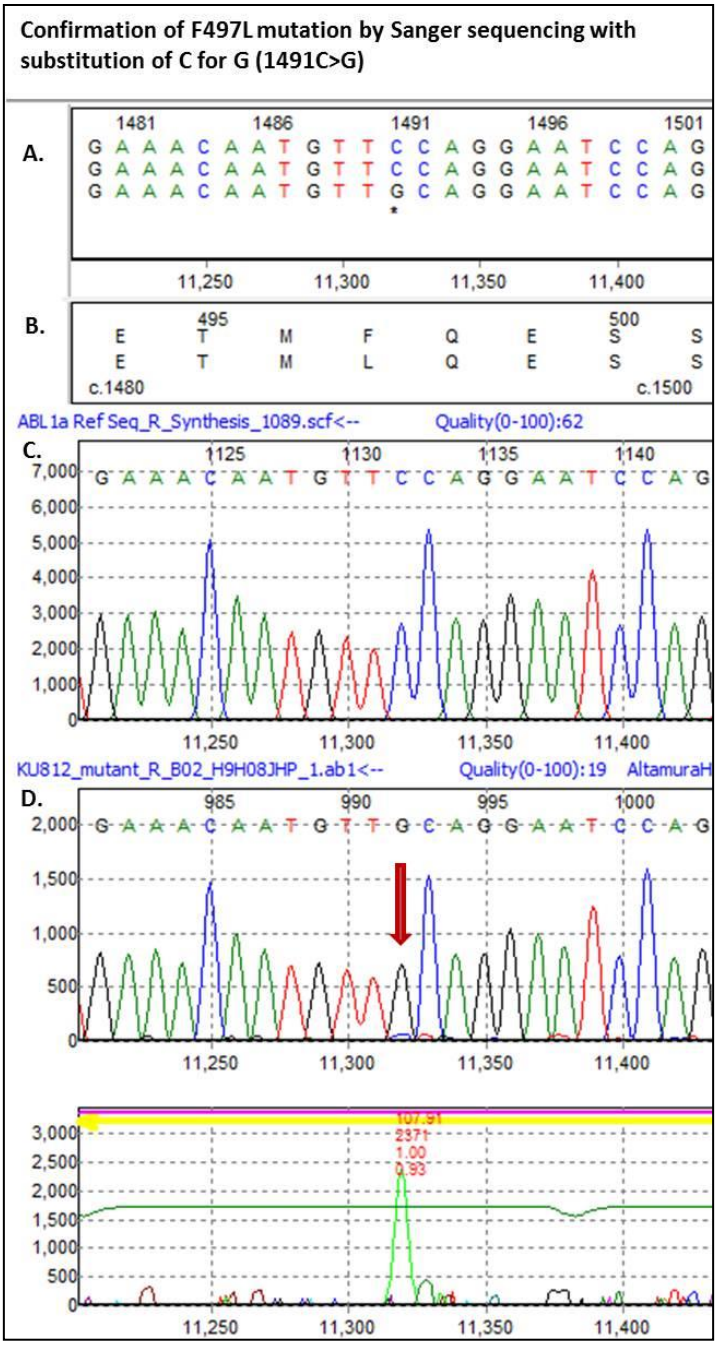


4.4.3 Mutant KU812 cell line analytical specificity

Sanger sequencing (Figure 8) confirmed the F497L mutation.

Figure 8: Sanger sequencing confirmation of F497L

A. Nucleotide sequence: row 1 = reference sequence, row 2 = KU812 cell line and row 3 = wild type KU812 cell line. B. Amino acid sequence of reference sequence (row 1) and mutated KU812 cell-line (row 2). C. Chromatogram of reference sequence. D. Chromatogram of KU812 F497L cell line with nucleotide substitution (red arrow).



4.4.5 Retrospective samples

Patients with known kinase domain mutations confirmed by Sanger sequencing were included to demonstrate the analytical performance of MSA v2. All 8 multiplex reactions were performed on all patient samples in addition to all positive and negative controls to confirm the robustness of the assay in addition to identifying any low-level mutations that were not previously identified by Sanger sequencing. Patients with low-level *BCR::ABL1* values were also included to confirm the ability of the assay to detect kinase domain mutations in patients with minimal disease. Extra samples, in addition to the samples included for inter- and intra-assay precision, were analysed for confirmation of the testing method. Ten different patients together with the KU812 mutant cell line were utilised for the complete validation of MSA v2 totalling 16 different samples. Twenty-four kinase domain mutations were identified in these samples through historical testing by Sanger sequencing.

Table 2: Clinical details of the patient samples used in the validation study
Samples 1-10 were used in the intra- and inter-assay validation

Sample no.	Patient no.	Date of sample	Transcript type	*BCR::ABL1*^{IS} value (%)	Mutation identified by SS	Mutation percentage by SS (%)
1	KU812	Cultured 02-Dec-19	e14a2		F497L$^{\alpha}$	50
2	Patient 1	20-Nov-19	e14a2	110	No mutation	
3	Patient 1	16-Jan-20	e14a2	240	P465S$^{\alpha}$	65
4	Patient 2	22-Dec-14	e14a2	36	V468F$^{\alpha}$	75
					I502L$^{\alpha}$	15
5	Patient 2	05-Jan-15	e14a2	44	V468F$^{\alpha}$	20
					I502L$^{\alpha}$	80
6	Patient 3	14-Sep-07	e13a2, e14a2	77	E450G$^{\alpha}$	100
7	Patient 4	10-May-11	e1a2	20	T315L$^{\alpha}$	100

8	Patient 4	24-May-11	e1a2	1.4	T315L$^{\alpha}$	100
9	Patient 4	02-Jun-11	e1a2	0.47	T315L$^{\alpha}$	100
10	Patient 5	16-May-18	e1a2	6.0	T315I	50
					T315M$^{\alpha}$	50
11	Patient 6	29-Sep-10	e13a2, e14a2	86	E255K	70
12	Patient 7	20-Mar-13	e14a2	200	V299L (895G>T)	65
					V299L (895G>C)	25
					F486S	100
13	Patient 7	17-Jun-13	e14a2	65	Q252H	35
					E255V	55
					V299L (895G>C)	30
					F486S	100
14	Patient 8	05-Aug-18	e1a2	3.9	F311I$^{\alpha}$	40
					E459K	60
15	Patient 9	21-Aug-19	e13a2, e14a2	75	F311I$^{\alpha}$	80
16	Patient 10	18-May-18	e13a2	15	F311I$^{\alpha}$	90

SS: Sanger sequencing; $^{\alpha}$New mutations MSA v2

4.5 Validation study design

Table 3: Mass spectrometry runs and samples used in the validation study

Run ID	Date of run	Instrument	Samples	Reason
G1252393	28-Apr-20	MassARRAY® Analyser 4 MALDI-TOF Mass Spectrometer	Samples 1-16	Inter-assay validation
G1227502	14-May-20	MassARRAY® Analyser 4 MALDI-TOF Mass Spectrometer	Samples 1-16	Inter-assay validation
G1228001	26-May-20	MassARRAY® Analyser 4 MALDI-TOF Mass Spectrometer	Samples 1-10 in triplicate	Intra-assay validation and Inter-assay validation

4.5.1 Intra- and inter-assay variation

Ten samples from one cell line and five patient samples were used for both intra- and inter-assay precision analysis (Table 3). Three patients had >1 time point tested to confirm mutations at differing *BCR::ABL1* values. The KU812 cell line containing the F497L mutation at a 50% variant allele frequency was selected as it was the only cell line available with an asciminib-resistant mutation.[22]

4.5.2 Limit of detection (LOD)

The MassARRAY assay is not a quantitative assay; however, the gBlock standards can provide an indication of the relative abundance of the detected mutation. The LOD experiment for the MSA v2 assay involved serially titrated dilutions of the gBlocks and KU812 F497L mutant CML cell line. Dilutions of 50%, 20%, 2%, 1.5%, 1%, 0.5%, 0.2% and 0.05% of each gBlock into the wild type gBlock were used to estimate the LOD. However, in the development phase, the 0.2% and 0.05% mutant concentrations were inconsistently detected and were not included in the validation phase. The LOD was determined from 4 runs. The lowest dilution consistently detected was considered the LOD for individual mutations. For confirmation of the F497L gBlock LOD, serial dilutions of the KU812 F497L CML cell line into the wild-type KU812 cell line were performed in triplicate. The dilutions tested were 2%, 1.5%, 1%, 0.5%, 0.2%, and 0.05%.

4.5.3 Recall analytics

The recall rate of kinase domain mutations was calculated according to the joint consensus recommendations of the Association for Molecular Pathology and College of American Pathologists.[19]

Positive Percentage Agreement: Number of kinase domain mutations detected by Sanger sequencing divided by the (number of kinase domain mutations detected by mass spectrometry that were previously characterized + number of kinase domain mutations previously characterised that were not detected by mass spectrometry).

Positive Predictive Value: Number of kinase domain mutations detected by Sanger sequencing that were previously characterised divided by the (number of kinase domain mutations detected by mass spectrometry that were previously characterised + additional kinase domain mutations detected by mass spectrometry that were not previously detected).

Negative Percentage agreement: Number of kinase domain mutations not detected by both methods divided by the (number of kinase domain mutations negative by both methods + number of kinase domain mutations detectable by Sanger sequencing but not by mass spectrometry).

Negative Predictive Value: Number of kinase domain mutations negative by both methods divided by the (number of kinase domain mutations negative by both methods + number of additional kinase domain mutations only detected by mass spectrometry).

4.6 Qualitative limit of detection

4.6.1 Results of LOD analysis

Serial gBlock dilutions were evaluated to confirm the LOD for each mutation assay performed in four separate runs. Assessments were performed using 2%, 1.5%, 1%, and 0.5% dilutions.

Concordance in all runs was required to confirm the lowest mutant percentage detected. The LOD for all mutation assays are demonstrated in Table 4 and Figure 9.

Table 4: Summary of LOD analysis for each mass spectrometry run

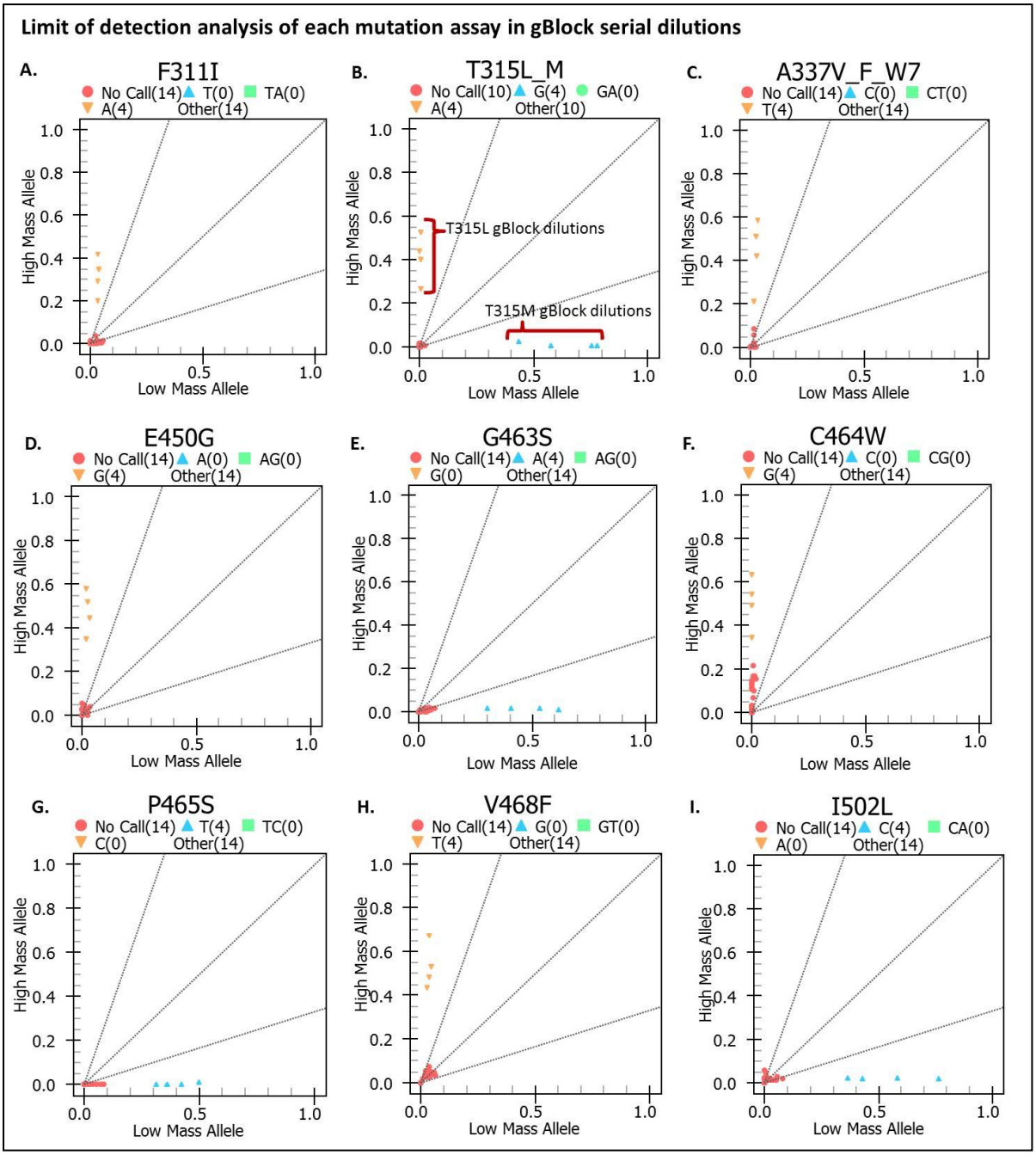
		17-Mar-20	27-Mar-20	09-Apr-20	16-Apr-20	
		G1227509	G1249750	G1249417	G1249417	
Mutation	gBlock dilution	Run 1	Run 2	Run 3	Run 4	LOD assigned for individual mutations
F311I	2%	D	D	D	D	1.5%
	1.5%	D	D	D	D	
	1%	D	D	D	ND	
	0.5%	D	D	ND	ND	
T315M	2%	D	D	D	D	1%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	ND	D	D	
T315L	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
A337V	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
E450G	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	

	1%	D	D	D	D	
	0.5%	D	D	D	D	
G463S	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
C464W	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
P465S	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
V468F	2%	ND	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
F497L	2%	D	D	D	D	0.5%
	1.5%	D	ND	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	
I502L	2%	D	D	D	D	0.5%
	1.5%	D	D	D	D	
	1%	D	D	D	D	
	0.5%	D	D	D	D	

D: Mutation detected, ND: Mutation not detected

Figure 9: Graphs of LOD analysis of 10 of 11 mutations using gBlock serial dilutions of 2%, 1.5%, 1% and 0.5%.

Orange/blue triangles indicate specific mutation detection, red circles depict no mutation detection. The 2% mutant mix is detected at the highest intensity with the lower mixes observed in a descending order. A. F311I, B. T315L and T315M using 2 gBlocks, C. A337V, D. E450G, E. G463S, F. C464W, G. P465S, H. V468F and I. I502L.



Irrespective of whether the extension primer extends in the forward or the reverse direction, the added nucleotide is demonstrated in the forward direction.

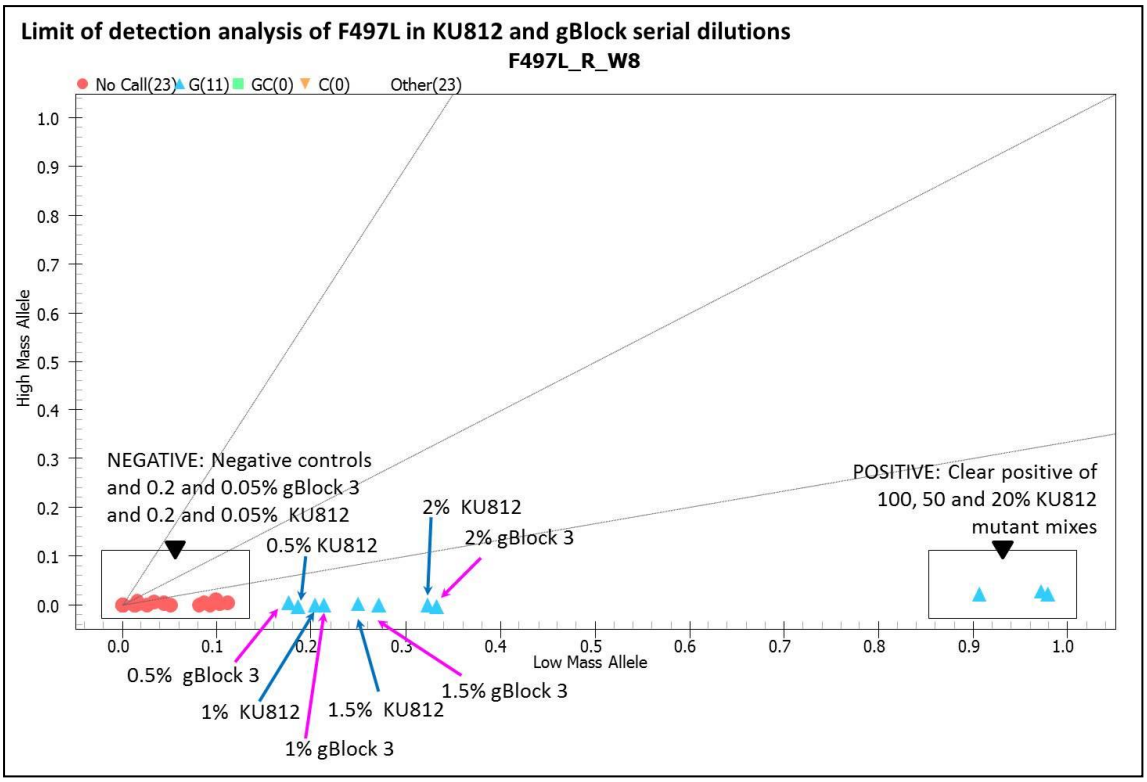
4.6.2 Confirmation of LOD of the F497L mutation using the KU812 cell line

To confirm the F497L gBlock assay LOD, the KU812 F497L dilutions were analysed in three runs, including the lower two cell line dilutions (0.05% and 0.2%) to further corroborate the LOD of the F497L mutant. Table 5 confirms that the lowest detectable percentage of the F497L mutation in the KU812 cell line was 0.5%, which matched the gBlock estimation. The detectable mutant percentages also corresponded to matching gBlock-3 dilutions, providing further confidence in the results (Figure 10).

Table 5: Triplicate results of serial dilutions of F497L mutated KU812 cell line

	17-Mar-20	27-Mar-20	09-Apr-20
	G1227509	G1249750	G1249417
KU812 mutant concentrations	Run 1	Run 2	Run 3
100%	Detected	Detected	Detected
50%	Detected	Detected	Detected
20%	Detected	Detected	Detected
2%	Detected	Detected	Detected
1.5%	Detected	Detected	Detected
1%	Detected	Detected	Detected
0.5%	Detected	Detected	Detected
0.2%	Not Detected	Not Detected	Not Detected
0.05%	Not Detected	Not Detected	Not Detected

Figure 10: LOD for the F497L mutation at 0.5% using gBlock-3 was confirmed using KU812 F497L cell line dilutions.



4.7 Precision: Intra- and inter-assay analysis

4.7.1 Repeatability: Intra-assay assessment

Ten samples from 5 patients and the undiluted KU812 cell line were analysed in triplicate in one run with the data demonstrated in Table 6.

4.7.1.1 Intra-assay analysis results

Fifty-one mutations in total were detected by mass spectrometry in the samples used for the intra-assay analysis. Twelve different mutations were represented, including 7 of the 12 mutations in the new assay: T315L, T315M, E450G, P465S, V468F, F497L, and I502L. Each mutation was detected in each of the triplicate samples and the estimated mutation allele frequencies were the same in each triplicate, Table 6.

- 100% concordance of mutation detection across the samples run in triplicate
- 100% concordance of percentage mutation allele frequency estimation

Summary: The data demonstrate a high level of repeatability for the tested mutations and estimated mutant percentage.

4.7.2 Reproducibility: Inter-assay assessment

Ten samples from five patients and the KU812 F497L cell line were analysed in three separate runs (Table 6).

4.7.2.1 Inter-assay analysis results

Similar to the intra-assay analysis, all mutations detected in each sample were detected in each run for 100% concordance. Similarly, the estimated mutation allele frequencies were the same in each run, Table 6.

- 100% concordance of mutation detection across the three runs
- 100% concordance of estimated mutation allele frequency percentage

Summary: The data demonstrate a high level of reproducibility for the tested mutations and mutant percentage.

Table 6: Intra- and inter-assay results

Sample no.	Patient no.	*BCR::ABL1*^{IS} value (%)	Mutation	Intra-assay precision					Inter-assay precision				
				Mutant estimate (%)			Concordance of mutation detection (%)	Concordance of estimated allele frequency (%)	Mutant estimate (%)			Concordance of mutation detection (%)	Concordance of estimated allele frequency (%)
				1	2	3			1†	2	3		
1	KU812		F497L$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100
2	Patient 1	110	M244V	0.5-2^{+}	0.5-2	0.5-2	100	100	0.5-2	0.5-2	0.5-2	100	100
3	Patient 1	240	P465S$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100
4	Patient 2	36	V468F$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100
			I502L$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100
			E355G	0.5-2^{+}	0.5-2	0.5-2	100	100	0.5-2	0.5-2	0.5-2	100	100
5	Patient 2	44	V468F$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100
			I502L$^{\alpha}$	>2	>2	>2	100	100	>2	>2	>2	100	100

			E355G	0.5-2[+]	0.5-2	0.5-2	100	100	0.5-2	0.5-2	0.5-2	100	100
6	Patient 3	77	E450G[α]	>2	>2	>2	100	100	>2	>2	>2	100	100
			E459K	>2	>2	>2	100	100	>2	>2	>2	100	100
7	Patient 4	20	T315L[α]	>2	>2	>2	100	100	>2	>2	>2	100	100
8	Patient 4	1.4	T315L[α]	>2	>2	>2	100	100	>2	>2	>2	100	100
9	Patient 4	0.47	T315L[α]	>2	>2	>2	100	100	>2	>2	>2	100	100
10	Patient 5	6	E255K	>2	>2	>2	100	100	>2	>2	>2	100	100
			E255V	>2	>2	>2	100	100	>2	>2	>2	100	100
			T315M[α]	>2	>2	>2	100	100	>2	>2	>2	100	100

[α]New mutations MSA v2

†The result of the first triplicate of each sample in the intra-assay run was used for the inter-assay comparison.

[+] Mutations detected using the original assay where a 1% and 1.5% plasmid standard was not included.

4.8 Recall rate in retrospective samples

Samples of ten different patients together with the KU812 F497L mutant cell line were utilised for the complete validation of MSA v2, totalling 16 different samples. Twenty-four kinase domain mutations were identified through historical testing using Sanger sequencing. A total of 29 mutations were identified using mass spectrometry, including 6 low-level mutations that were previously undetected by Sanger sequencing. A single mutation (T315I) detected by Sanger sequencing was not able to be distinguished by mass spectrometry as this patient had a T315M mutation, which concealed the presence of a co-existing T315I. The results are documented in Table 7.

4.8.1 Additional mutations identified

Summary of the additional kinase domain mutations identified:

- Patient 1 had a known P465S mutation (65%) at 16-Jan-20 with a history of treatment with asciminib. The earlier time point (20-Nov-19) had no detectable mutation using Sanger sequencing but a low-level M244V (0.5-2%) was identified using mass spectrometry, which is a known imatinib-resistant kinase domain mutation. This patient had been treated with multiple TKIs, including imatinib, prior to commencement of asciminib. Our data suggest that the M244V mutation was a high confidence, low-level mutation, since it was detected when tested 5 times for the validation study. However, the mutation never became a predominant mutation.
- Patient 2, with known V468F and I502L asciminib resistant mutations, was tested at two time points by MSA v2. The mass spectrometry assay confirmed the V468F and I502L mutations (both >2%), plus an E355G mutation at low-level (0.5-2%). The E355G mutation was detected by Sanger sequencing at a prior time point. The detection of the low-level E355G by mass spectrometry demonstrates the deselection of the E355G with the dominant emergence of the two asciminib-resistant mutations. This is a high confidence result due to consistency with the previous mutation profile.
- Patient 3 had an E450G mutation detected by Sanger at the time point selected for MSA v2 with no other historical results available. In addition to the E450G, an E459K was identified by MSA v2, estimated at >2%. When reviewing the original Sanger

mutation data, the E459K was detectable on a single Sanger sequencing run at 15% mutant. Since it was not detected in replicates, this result was not reported but provides supporting evidence of reliable low-level mutation detection by mass spectrometry.

- Patient 5 had T315I and T315M mutations detected by Sanger sequencing at the time point selected for mass spectrometry analysis. The T315M was confirmed by mass spectrometry. However, E255V and E255K were also detected at >2%. These two mutations subsequently emerged a month later and were detected by Sanger sequencing at 25% and 20%, respectively. This demonstrates high confidence that the mass spectrometry assay detected low-level mutations prior to emergence at a level detectable by conventional Sanger mutation testing.

Table 7: Validation run summary

Sample No.	Patient number	Date of sample	Transcript	*BCR::ABL1*^{IS} value (%)	Mutation identified by SS	Mutation percentage by SS	Mutation identified by MSA	Estimated mutation percentage by MSA
1	KU812	02-Dec-19			F497L	50	F497L$^{\alpha}$	>2%
2	Patient 1	20-Nov-19	e14a2	110			M244V	0.5-2%
3	Patient 1	16-Jan-20	e14a2	240	P465S	65	P465S$^{\alpha}$	>2%
4	Patient 2	22-Dec-14	e14a2	36	V468F	75	V468F$^{\alpha}$	>2%
					I502L	15	I502L$^{\alpha}$	>2%
							E355G	0.5-2%
5	Patient 2	05-Jan-15	e14a2	44	V468F	20	V468F$^{\alpha}$	>2%
					I502L	80	I502L$^{\alpha}$	>2%
							E355G	0.5-2%
6	Patient 3	14-Sep-07	e13a2, e14a2	77	E450G	100	E450G$^{\alpha}$	>2%
							E459K	>2%
7	Patient 4	10-May-11	e1a2	20	T315L	100	T315L$^{\alpha}$	>2%
8	Patient 4	24-May-11	e1a2	1.4	T315L	100	T315L$^{\alpha}$	>2%

9	Patient 4	02-Jun-11	e1a2	0.47	T315L	100	T315L[α]	>2%
10	Patient 5	16-May-18	e1a2	6.0			E255K	>2%
							E255V	>2%
					T315M	50	T315M[α]	>2%
					T315I	50		
11	Patient 6	29-Sep-10	e13a2, e14a2	86	E255K	70	E255K	>2%
12	Patient 7	20-Mar-13	e14a2	200	V299L (895G>T)	65	V299L (895G>T)	>2%
					V299L (895G>C)	25	V299L (895G>C)	>2%
					F486S	100	F486S	>2%
13	Patient 7	17-Jun-13	e14a2	65	Q252H	35	Q252H	>2%
					E255V	55	E255V	>2%
					V299L (895G>C)	30	V299L (895G>C)	>2%
					F486S	100	F486S	>2%
14	Patient 8	05-Aug-18	e1a2	3.9	F311I	40	F311I[α]	>2%
					E459K	60	E459K	>2%
15	Patient 9	21-Aug-19	e13a2, e14a2	75	F311I	80	F311I[α]	>2%

16	Patient 10	18-May-18	e13a2	15	F311I	90	F311I[α]	>2%

[α]New mutations MSA v2

MSA: Mass spectrometry assay; SS: Sanger seq

Samples 11-12 were run in 2 runs each and the concordance of data for each sample was 100%

4.8.2 BCR::ABL1 values

The *BCR::ABL1*^{IS} levels at the time of mass spectrometry testing ranged from 0.47-240%. Multiple samples from patient 4 collected at different timepoints were specifically included to confirm the sensitivity of mass spectrometry to detect mutations in samples with *BCR::ABL1* values below 5%. Of the three time points tested, two had *BCR::ABL1* values of 0.47% and 1.4% and all samples had the T315L detectable by mass spectrometry.

4.8.3 Recall rate summary

Table 8: Method comparison[25,26] of kinase domain mutation detection between Sanger sequencing and mass spectrometry

		Sanger sequencing		
	Kinase domain mutations	Positive	Negative	Total
Mass spectrometry	Positive	True positive: A 23	False negative: B 6	A+B 29
	Negative	False positive: C 1	True negative: D 658	C+D 659
	Total	A+C 24	B+D 664	

4.8.3.1 Positive percentage agreement (A/A+C)

Twenty-four mutations were detected in the patient samples and cell line when originally tested using Sanger sequencing (Table 2). Twenty-three of 24 kinase domain mutations were identified by mass spectrometry. The T315I mutation in patient 5 was not detected due to a co-existing T315M.

Positive percentage agreement: 96%

4.8.3.2 Positive predictive value (A/A+B)

Six kinase domain mutations in addition to those identified by Sanger sequencing were detectable by mass spectrometry.

Positive predictive value: 79%

4.8.3.3 Negative percentage agreement (D/C+D)

658 mutation assays were negative for kinase domain mutations by both Sanger sequencing and mass spectrometry. All but one mutation detected by Sanger sequencing were identified on mass spectrometry analysis.

Negative percentage agreement: 99.8%

4.8.3.4 Negative predictive value (D/B+D)

In all samples, there were 664 mutation assays that were negative for kinase domain mutations by Sanger sequencing. Mass spectrometry identified an additional six kinase domain mutations in these samples.

Negative predictive value: 99%

4.9 Limitations

There are four limitations with the mass spectrometry assay and two encompass the complex nucleotide rearrangement comprising the T315L and T315M mutations.

Table 9: Nucleotide sequences for the various mutations located at amino acid 315 in the forward direction

Primer mix number	Available terminators	Mutation	Wild type nucleotide sequence	Mutation nucleotide sequence
2	CGT	T315A	ACT	GCT
2/5	CGT/CGT	T315L	ACT	CTA
4	TG	T315I	ACT	ATT

4/5	TG/CGT	T315M	ACT	ATG

N.B: Nucleotide change indicated in red

4.9.1 Limitations in the T315L assay

The rare T315L mutation requires a three nucleotide change from ACT to CTA, which is not easily detectable using a single base extension assay such as the SABER mass spectrometry assay. Through a literature review, we determined that the T315L mutation is detectable with high confidence using mass spectrometry by detection of the first and third nucleotide of the T315L mutation. The second nucleotide will not be detected.

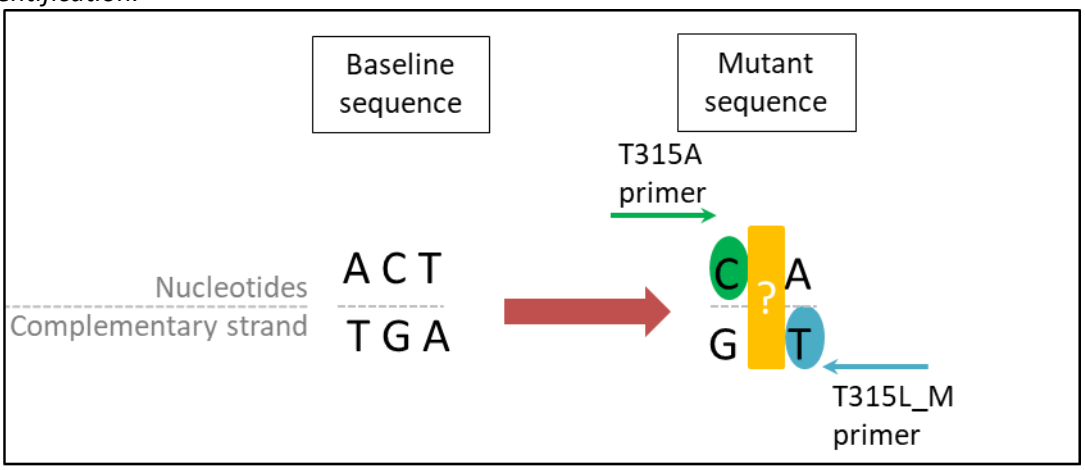
The possible alternative nucleotide combinations are listed below but none of these kinase domain mutations have been reported in patient samples in the literature. Consequently, we make the assumption that if the first base of T315 is changed to a C and the third base to an A, the mutation is T315L (Figure 11).

- T315R (ACT>CGA)
- T315Q (ACT>CAA)
- T315P (ACT>CCA)

Note that T315P has been reported in patients but the exchange is ACT >CCT

Figure 11: T315L mutation detection

With the T315L mutation, the baseline ACT has a 3 nucleotide change to CTA. As the primers will only detect a single base, 2 separate assays (the T315A and the T315L_M) will be required for identification.



Conclusion: Detection of the second nucleotide would be very difficult using the SABER protocol for mass spectrometry. It is possible using Sanger sequencing and next generation sequencing (NGS). Due to the low likelihood of detecting an alternative second nucleotide in the already rare event of multiple nucleotide alterations, we assume the mutation is T315L. Therefore, there is a low probability of misidentification of the T315L mutation.

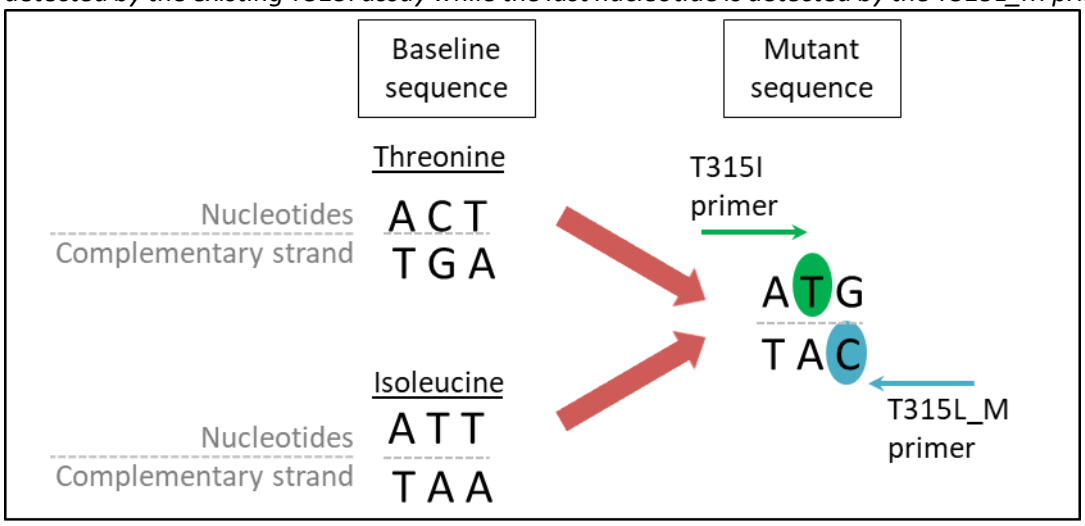
4.9.2 Limitations with the T315M assay

Detection of the rare T315M mutation is dependent on identifying a two nucleotide exchange that can occur in two ways (Figure 12):

- The unmutated threonine can gain a two nucleotide exchange (ACT>ATG) resulting in a methionine = T315M
- A second nucleotide exchange can occur in a patient with an existing T315I mutation resulting in the T315M (ATT>ATG)

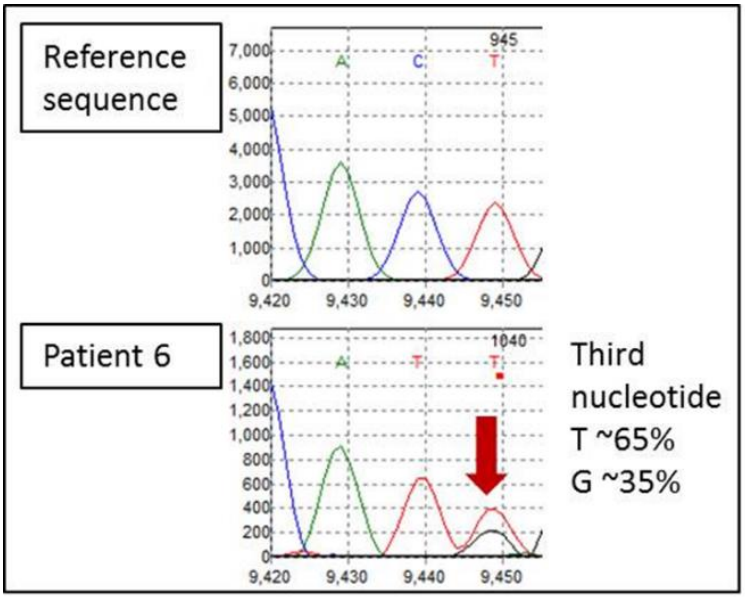
Figure 12: T315M detection

Detection of the rare T315M mutation is dependent on identifying a two nucleotide exchange that can occur in two ways: The unmutated threonine can gain a two nucleotide exchange resulting in a methionine or a second nucleotide exchange can occur in a patient with an existing T315I mutation resulting in the methionine. The first base change of the T315M mutation is detected by the existing T315I assay while the last nucleotide is detected by the T315L_M primer.



The first base change of the T315M mutation is detected by the T315I assay. Therefore, a co-existing T315I mutation cannot be distinguished in the presence of T315M by mass spectrometry (Figure 13).

Figure 13: Concurrent T315I and T315M cannot be distinguished by mass spectrometry
The T315I primer identifies the second nucleotide as a T and this is common to both the T315I mutation and the T315M. However, the T315M primer identifies the third nucleotide and will identify if there is a mutant at this location. As the T315I has a wild type nucleotide at this position, it cannot be identified since the premise of SABER is to exclude the wild type nucleotide. Therefore, co-existing T315I and T315M mutations can only be identified using Sanger sequencing or NGS.



4.9.3 Mutation in an adjacent nucleotide

The presence of mutations in adjacent nucleotides may disrupt binding of extension primers. While this occurrence was not observed in the current validation study, it was previously reported in one patient with the D276G mutation and the extremely rare T277S mutation.[11] The presence of T277S interfered with primer binding resulting in lack of detection of D276G by the mass spectrometry assay.

4.9.4 Limitations in detection of additional mutations

Alternative mutations, including subsequently identified asciminib-resistant mutations that are not listed in Table 1, will not be detected by this method.

4.10 Validation findings

In this validation study, we modified the existing design of the low-level *BCR::ABL1* kinase domain mutation mass spectrometry assay to include additional kinase domain mutations. These mutations include asciminib-resistant mutations.

The validation study involved inter- and intra-assay precision, limit of detection and mutation recall rate of 10 patients and the KU812 F497L cell line over 16 different samples:

- Twenty-four kinase domain mutations were identified by Sanger sequencing
- A total of 29 mutations were identified by mass spectrometry, including low-level mutations that were previously undetected
- A T315I mutation was not detected by mass spectrometry due to a co-existing T315M

Key analytical components for kinase domain mutation detection for MSA v2

- Positive percentage agreement: 96%
- Positive predictive value: 79%
- Negative percentage agreement: 99.8%
- Negative predictive value: 99%
- Intra-assay repeatability: 100%
- Inter-assay reproducibility: 100%

Limit of detection for kinase domain mutations

- F311I 1.5%
- T315M 1%
- T315L 0.5%
- A337V 0.5%
- E450G 0.5%
- G463S 0.5%
- C464W 0.5%
- P465S 0.5%
- V468F 0.5%
- F497L 0.5%
- I502L 0.5%

4.11 Conclusions

The mass spectrometry assay based on the SABER protocol is a reliable and sensitive method for the detection of specific kinase domain mutations compared with the gold standard of Sanger sequencing. The LOD for the F311I mutation is 1.5% while the LOD for the T315M mutation is 1%. All other mutations can be detected to a level of 0.5%. NGS has now been recommended by the European LeukemiaNet for kinase domain mutation testing but the limit of detection is 3%.[16,27] An LOD of 0.5% mutant using NGS would require a high depth of coverage, which could increase costs. High sensitivity results can be achieved with error correction and duplex sequencing.[28] While the mass spectrometer can achieve superior sensitivity, it is limited to detection of known specific mutations whereas NGS can be utilized for a full investigation into resistance, evaluating for *BCR::ABL1*-dependent and independent mechanisms. Another advantage of mass spectrometry is turn around time, but a dedicated instrument is required. Some NGS techniques require substantial data processing and infrastructure, but many laboratories now have various NGS instruments available, facilitating the development of the sensitive techniques required for MRD detection. With additional kinase domain mutations secondary to asciminib being constantly reported and with additional novel TKIs on the horizon, NGS has the advantage for resistance testing in CML over mass spectrometry.

4.12 References

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5 Development and validation of an RNA-based next generation sequencing strategy that can detect relevant genomic mutations and rearrangements in chronic myeloid leukaemia

Preface

Historically, single nucleotide variants within the BCR::ABL1 kinase domain have been the only illustrated mechanism of TKI resistance and treatment failure. However, these mutations are only implicated in about half of the cases of resistance. Preliminary work has demonstrated that CML patients have *BCR::ABL1* independent resistance pathways activated, with additional variants correlating with poorer outcomes and disease progression. This has emphasized the unmet need for a sensitive method to assist in identifying *BCR::ABL1* independent resistance pathways.

Prior studies using RNA-seq and whole exome sequencing (WES) using DNA identified various variant subtypes in cancer-related genes that drive other haematological malignancies associated with treatment failure in CML patients. Variants not only included single nucleotide variants and small insertions/deletions but also fusion transcripts and partial or whole gene deletions in addition to aberrant splicing. In fact, at the time of BP-CML, all patients had at least one additional genetic abnormality detectable in conjunction with the standard *BCR::ABL1* translocation. A single cost-effective method for variant detection was required as detection of all variants required a combination of WES, RNA-Seq and copy number variation.[9,16] A customized hybridization capture next-generation sequencing method that could be applied to either DNA or RNA was developed. Both DNA and RNA were able to detect SNVs, indels, focal gene deletions and genomic fusions involving the captured

genes. The concordance of variant detection between DNA and RNA was excellent however RNA had the added benefit of being able to interrogate the spliceosome and identify fusion transcripts.

The results of the development and validation of the customized next generation sequencing panel was published in the Journal of Molecular Diagnostics as "*RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia*", reference included. The supplementary files follow the main manuscript.

- Shanmuganathan N, Wadham C, Thomson D, et al. RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia. *J Mol Diagn*. 2022;24(7):802–822.

RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia

Shortened title: RNA-based targeted sequencing in CML

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Keywords: mutation, aberrant splicing, next generation sequencing, capture panel, chronic myeloid leukemia

5.1 Abstract

Mutation detection is increasingly used for the management of hematological malignancies. Prior whole transcriptome and whole exome sequencing studies using total RNA and DNA identified diverse mutation types in cancer-related genes associated with treatment failure in patients with chronic myeloid leukemia (CML). Variants included single nucleotide variants and small insertions/deletions, plus fusion transcripts and partial or whole gene deletions. The hypothesis that all of these mutation types could be detected by a single cost-effective hybridization capture next-generation sequencing method using total RNA was assessed. A method was developed that targeted 130 genes relevant for myeloid and lymphoid leukemia. Retrospective samples with 121 pre-characterized variants were tested using total RNA and/or DNA. Concordance of detection of pre-characterized variants using RNA or DNA was 96%, while the enhanced sensitivity identified additional variants. Comparison between 24 matched DNA and RNA samples demonstrated 95.3% of 170 variants detectable using DNA were detected using RNA, including all but one variant predicted to activate nonsense-mediated decay. RNA identified an additional 10 variants, including fusion transcripts. Furthermore, the true effect of splice variants on RNA splicing were only evident using RNA. In conclusion, capture sequencing using total RNA alone is suitable for detecting a range of variants relevant in CML and may be more broadly applied to other hematological malignancies where diverse variant types define risk groups.

5.2 Introduction

Chronic myeloid leukemia (CML) is characterized by the *BCR::ABL1* fusion oncogene,[1] which is formed by a translocation between chromosome 9 and 22 (Philadelphia [Ph] chromosome).[2] Other chimeric *ABL1* fusions have also been described in other hematological malignancies (e.g. *NUP214::ABL1* and *ETV6::ABL1* positive leukemias).[3] Tyrosine kinase inhibitor (TKI) therapies that target *BCR::ABL1* have dramatically improved survival in chronic phase CML.[4-7] However, ~20% of patients develop resistance to TKI therapy and 5-6%[5-7] progress to an acute leukemia, termed blast crisis. It has long been known that mutations within the kinase domain of *BCR::ABL1* are associated with TKI resistance and disease

progression. However, these mutations are only detected in ~50% of patients with acquired resistance[8] and are not responsible for primary resistance. Next-generation sequencing (NGS) is now unmasking the genomic complexities associated with resistance and disease progression in CML.[9-14] Mutations in well-defined cancer-related genes have been described, which not only include single nucleotide variants (SNVs) and small insertions and deletions (indels), but also gene deletions and fusions. Mutated genes identified in CML are often described in other hematological malignancies,[15] including acute myeloid and lymphoid leukemia. This is consistent with the myeloid and/or lymphoid phenotype of blast crisis. Lymphoid-associated variants are characterized by *RAG*-mediated gene deletions.[16] A novel class of rearrangement associated with the formation of the *BCR::ABL1* fusion has also been described in CML patients at diagnosis. These were termed Ph-associated rearrangements and were frequently complex events associated with inferior outcomes.[9,17]

Incorporation of genomic factors into patient management for a range of blood cancers has led to the implementation of high-throughput gene testing of DNA to detect clinically relevant SNVs/indels. However, a DNA-based method cannot detect fusion transcripts, although whole genome sequencing can detect gene fusion breakpoints. Furthermore, a DNA-based method cannot assess the effect of splice region variants, which are clinically relevant for patients with CML and other hematologic malignancies, such as acute myeloid leukemia (AML).[11,18] Specific genetic lesions, including fusion transcripts, SNVs and indels, risk stratify patients with AML.[15] Stored RNA is therefore a rich source of material for biomarker discovery in retrospective studies but the potential has been limited by RNA degradation with traditional RNA-seq poorly capturing degraded mRNA. Prior next-generation sequencing work demonstrated that using total RNA, as opposed to poly(A)-selection, was reliable for the detection of various mutation types in potentially degraded retrospective samples, some of which had been in storage for >10 years.[9] In the current study, a capture gene panel was designed with both DNA and RNA sequenced to confirm whether total RNA alone is suitable to detect the range of relevant mutation types in hematologic malignancies, including SNVs, indels, fusion transcripts, specific gene deletions, rearrangements and tandem duplications, in addition to altered RNA splicing associated with certain mutations. Samples included a

range of leukemic cell lines that are enriched for these variant types of interest in addition to previously characterized CML patient samples.

5.3 Materials and Methods

5.3.1 Samples

Cell lines and a spectrum of stored patient samples from chronic phase to blast crisis were selected for inclusion in this study. Samples were selected based on their mix of previously characterized variants and the availability of DNA and RNA. These included 21 CML patient samples with variants characterized and confirmed using one or more of the following techniques: whole exome sequencing (WES); RNA-seq; High Density SNP Array; RT-PCR, and Sanger sequencing.[9] Newly identified low-level *BCR::ABL1* kinase domain mutations using the targeted gene panel were confirmed using mass spectrometry.[19] Five uncharacterized patient samples were also tested. Some samples, including RNA, had been stored at -80°C for up to 15 years.

The 13 leukemic cell lines that were selected for this study were included specifically for known well characterized variants described in the Catalogue of Somatic Mutations in Cancer (COSMIC) Cell Lines Project (n=11),[20] and/or variants that have been reported in the literature. Cell lines were cultured in RPMI media and 10-20% fetal calf serum, and some were mixed for specific experiments. DNA and RNA were extracted from cell lines and patient blood samples as previously described.[9] The Seraseq Myeloid Mutation DNA Mix (SeraCare) was used to assess low level SNV and indel detection and was further diluted in DNA derived from the NA24385 reference cell line (Coriell Institute).

5.3.2 Hybridization capture panel design

A preliminary hybridization capture method was designed to assess its suitability to detect various mutation types relevant for CML[9] using DNA, including SNVs, indels, the p210 *BCR::ABL1* genomic fusion breakpoints and aberrant *RAG*-mediated *IKZF1* deletion breakpoints. Probes were designed to cover specific introns and exons of *BCR* and *ABL1*,

aiming to identify the genomic breakpoints of patients with the most common *BCR::ABL1* transcript types of CML, e13a2 and e14a2. To enhance detection of Ph-associated rearrangements, probe design also captured genes known to be fusion partners from historical work, which include genes adjacent to *ABL1* and *BCR* on chromosomes 9 and 22.[9] Detection of Ph-associated rearrangements will hinge on capture of at least one of the partner genes involved in the rearrangement, which is most frequently *ABL1* or *BCR*. Exons for all protein coding transcripts in the Ensembl database for all target genes were included in the capture panel design. The custom library of probes was designed using NimbleDesign software (Roche SeqCap EZ Prime Choice Probes ≤ 7Mb, hg19 Genome Build, Roche, Basel, Switzerland). The exon coordinates were downloaded from Ensembl Biomart release 75: Feb 2014 (GRCh37.p13). Intronic and intergenic coordinates were added manually. The least stringent masking option was selected in NimbleDesign to permit capture of low complexity intronic regions. Each region was covered by a minimum of 3 and a maximum of 20 overlapping probes.

IKZF1 exon deletions are among the most frequent mutations identified in CML lymphoid blast crisis and breakpoints commonly occur at intronic cryptic *RAG* recombination signal sequences.[16] Capture probes were included to specifically detect breakpoints at these signal sequences. After successful preliminary analysis using cell lines, the hybridization capture probes were supplemented to aid the detection of other *RAG*-mediated deletions of specific genes. These genes/regions were *PAX5*, *RB1*, *BTG1*, certain *CDKN2A*/*B* breakpoint regions and an *HBS1L::MYB* intergenic locus. Probes targeted *RAG* recombination signal sequence motifs within or surrounding these genes and covered sequences ranging in size from 75-375 kilobases (Kb), (Supplementary Table 1). Deletions that encompass the *CDKN2A*/*B* locus have been reported in solid tumors and myeloid and lymphoid leukemia. Some deletions are large (>6 Mb) and are mediated by various mechanisms.[21] Deletions of >400Kb surrounding the *CDKN2A*/*B* locus are not detectable using the strategy of targeting cryptic *RAG* recombination signal sequences. The final design targeted the exons of 130 genes relevant for myeloid and lymphoid leukemia.[9,22-24]

The genes encompassed relevant SNVs, indels, Ph-associated rearrangements, specific gene deletions and gene fusions. Capture probes that only target one gene are sufficient to detect a corresponding fusion partner even if the gene is not included in the design since sequenced fragments that span the fusion breakpoint will partially map to the partner. Previous versions of the panel design investigated the potential of gene expression analysis in CML for prognosis. While earlier results demonstrated that blast crisis had a specific differential gene expression clustering pattern, no specific signature was identified in chronic phase[9] and therefore was not a focus of the current study.

5.3.3 Target enrichment and sequencing

Approximately 500 ng of genomic DNA was sheared by ultra-sonication using a Covaris S220 Focused Ultrasonicator to achieve DNA fragments of 350-400 base pairs (bp). Sequencing libraries were prepared using the KAPA HyperPrep Library Kit according to manufacturer's instructions. Libraries for targeted RNA sequencing were prepared using the Illumina TruSeq Stranded Total RNA kit with ribosomal depletion using 1ug of DNAse treated RNA. Unique molecular identifiers were incorporated into the library construct to allow for detection and removal of fragments duplicated in library amplification steps. Target regions were enriched using the custom oligo capture probes. 150bp paired-end DNA and RNA sequencing was performed on an Illumina NextSeq 500 using the NextSeq 500/550 Mid Output Kit V2.5. The raw sequencing reads for DNA were aligned to the b37-decoy reference genome (Genome Analysis Toolkit (GATK) Resource bundle human_g1k_v37_decoy) using BWA-mem (v0.7.12).[25] For RNA samples, the raw sequencing reads were aligned to the same genome build using the STAR algorithm v2.7.2b and two pass mode for splice site definition. Sambamba[26] and UMI-tools[27] were used for removing PCR duplicates, for DNA and RNA panels, respectively.

5.3.4 Variant identification

Variant calling for both RNA and DNA was performed using the VarDict v1.8.2[28] and FreeBayes software v1.2.0.[29] Variants were annotated using an in-house pipeline with customized filtering strategies to identify relevant variants. Filters were set to call all variants with a

minimum of 5 and 10 quality reads using DNA and RNA, respectively, and the minimum variant allele frequency was set at 0.3%. The actual variant allele frequency (VAF) established as the limit of detection (LOD) is described in the section "Limit of detection and linearity assessments using DNA". The clinical relevance and pathogenicity of variants was assessed using strict criteria[9] with reference to guidelines.[24,30,31] The mutation type in specific genes was highly relevant and only those associated with hematological cancers were considered. These included non-synonymous SNVs and indels in protein-coding regions and essential splice-site SNVs. Missense-coding variants designated as deleterious were those predicted to be damaging by at least 3 of 4 functional prediction algorithms, had CADD scores[32] ≥20 and met minimum evolutionary conservation scores. Variants present in population databases, specifically the Genome Aggregation Database (gnomAD), 1000 Genomes Project, UK10K project, Exome Aggregation Consortium Version (ExAC), at allele frequency of >0.1% were excluded.[9] Variants that repeatedly occurred at low allele frequency in more than 10% of samples and were not located within a mutation hot-spot region or previously reported in the literature were suspected to be repeating artefacts and excluded from further analysis, as described in Thomas et al.[33] All variants of potential clinical relevance were reviewed in the Integrative Genomics Viewer (IGV)[34] v2.11.3 for confirmation of variant calls.[35]

The strategy to use total RNA, as opposed to poly(A)-selection, detected spliced fusion transcripts and genomic breakpoints from intron-retaining precursor mRNA that are usually absent in poly(A)-enriched RNA-seq.[9] For DNA samples, detection and characterization of the targeted *BCR::ABL1* genomic breaks and gene deletions (structural variants, SVs) from paired and split reads was achieved using Manta.[36] While reporting of SVs is generally qualitative, Manta provides a read pair count for each variant. For the purposes of the current analysis, read pair counts were normalized for individual samples by calculating the read pairs per million mapped read pairs.

Where RNA was sequenced, all variants in splice regions, defined as within 1-3 exonic bases from an exon boundary or within 1-8 intronic bases from an exon boundary, were assessed for their effect on exon splicing. These included synonymous and non-synonymous SNVs. Altered splicing was indicated by aberrant exon skipping, intron retention or novel splice site

usage. VarDict's realignment feature was not utilized using RNA due to the potential to miscall introns as deletions. Splicing was visualized in IGV by generating Sashimi plots and compared across multiple samples. Potential RNA splice variants were also investigated using *in silico* prediction tools (MaxEntScan,[37] NNSplice,[38] SpliceSiteFinder-like,[39] and GeneSplicer[40]) in Alamut Visual Plus (Sophia Genetics, Boston, MA).[41,42] The STAR aligner identified gene fusions and novel splice junctions in RNA samples using a catalogue of known splice boundaries.[43] *RAG*-mediated gene deletions can generate novel splice junctions between distant loci that has been previously reported for *IKZF1* deletions.[9] Gene fusions were called and filtered as previously described.[9]

5.3.4.1 Criteria for the assessment and assignment of variants as potential driver variants in CML (derived from Branford *et al.* 2018[9] and Branford *et al.* 2019[24])

Strict criteria were used to assess pathogenicity and variants that were predicted to change an amino acid or located at essential splice (donor or acceptor) sites were retained if their CADD score was ≥20 and were not present in population databases at an allele frequency of >0.1%, unless the variant was also present in the COSMIC database. This was to retain potentially rare variants that could be associated with myeloid malignancies. All missense variants additionally had to be predicted as damaging by at least 3 of the 4 functional prediction algorithms. This indicates that these variants likely modify protein function. The GERP and phyloP conservation scores were required to satisfy ≥2.0 and ≥1.0 respectively.
Retained variants, including SNVs, indels, fusions and deletions were further assessed and considered potentially clinically relevant if they were:

- *BCR::ABL1* kinase domain mutations
- Gene fusions recurrently reported in hematologic malignancies
- Known pathogenic germline mutations implicated in leukemia
- Variants in genes in the COSMIC Cancer Gene Census[20] if listed as either directly causally implicated in hematologic malignancy or if somatic mutations occur in the gene in more than 1% of hematopoietic tissue samples, providing they met one of the following criteria:

- The variant was recurrent in COSMIC, such as *IDH1* R132H, *ASXL1* E635fs*15 or *U2AF1* Q157R (only frameshift or nonsense variants in *ASXL1* were considered relevant)
- Novel variants occurring in the same domains as known variants, such as *RUNX1* Runt domain missense variants or *GATA2* zinc finger variants
- Truncating variants (nonsense, frameshift indels and essential splice site variants)
- Known and novel gene fusion transcripts where at least one of the fusion partners is a known gene fusion partner in hematologic malignancy, such as *KMT2A*, *RUNX1* and *MECOM*
- Deletions involving genes where deletion is a pathogenic mechanism, such as *IKZF1*, *PAX5* and *CDKN2A*, or occurring in genes associated with hematologic malignancy, such as *RUNX1*
- Ph-associated rearrangements

5.3.5 Capture panel validation using DNA

The performance characteristics of the final design of the gene panel was validated in 5 separate sequencing runs for the detection of SNVs, indels, genomic fusions and gene deletions using DNA (Supplementary Table 2). Inter- and intra-assay variation, limit of detection (LOD) and linearity were determined using cell line and patient samples. The target LOD for SNVs and indels was a variant allele frequency (VAF) of 5%. Based on data of the Association for Molecular Pathology and College of American Pathologists, the panel was estimated to have >98% power to detect variants present at ≥5% with at least 250 total sequencing reads, and variants present at 10% with at least 100 total sequencing reads.[44] Linearity of known variants was established using the model of Thomas et al,[33] where likely germline heterozygous (VAF 45-55%) and homozygous (VAF >95%) variants were identified. Retrospective analysis was performed for 11 patient samples with variants previously characterized from prior NGS studies[9] and the Myeloid Mutation DNA Mix.

Intra-assay repeatability involved analysis of 3 cell line 1:1 dilution mixes tested in triplicate in one run and 3 patient samples tested in triplicate in another run. Inter-assay reproducibility involved these 6 samples run in an additional 2 runs each. LOD experiments for SNVs and indels involved titration dilutions of the cell lines BV173 and REH at 1 in 10, 1 in 20 and 1 in 30, and the Myeloid Mutation DNA Mix reference sample and dilutions thereof.

The cell lines BV173 and REH were used for the linearity study with initial sequencing focusing on the undiluted cell lines to identify the variants present in BV173 and absent in REH. BV173 was diluted into REH at ratios of 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 30. For this analysis, variants identified within the captured regions with total read depths of >250 were retained. For the dilutions, variants called with ≥5 reads were considered detected.

5.3.6 Capture panel validation using RNA

After validation of the gene capture panel using DNA, RNA was used to assess the reliability of the method to detect the variants identified using DNA, plus fusion transcripts. RT-PCR and Sanger sequencing was used to confirm the observed splicing pattern of a representative synonymous splice region variant and others were verified from published data. Eleven retrospective patient samples and 11 cell lines/cell line mixes were compared. Three patient samples with sufficient RNA were assessed in duplicate in a single run for intra-assay repeatability while another 3 patient samples were assessed independently in 2 separate runs for inter-assay variation. Eight additional RNA samples with variants previously characterized from prior NGS studies were tested.

5.3.7 Statistical analysis

Linear regression analysis was used to assess the agreement between the VAFs of DNA and RNA for SNV and indel detection. The average of the individual coefficients of variation (CV) for the replicate samples performed for the intra- and inter-assay variation was reported for the overall CV for these analyses. The positive percentage agreement and positive predictive value between variants identified using RNA and DNA were calculated to assess the concordance of variant detection.[31]

5.4 Results

5.4.1 Test performance characteristics using DNA

Variants and their VAFs called by the VarDict and FreeBayes variant callers were highly concordant. For the purposes of this work, the VarDict calls are reported. Run performance metrics are detailed in Supplementary Table 2. The mean read depth of the samples was 1135 and the on-target rate for all samples was >94%.

5.4.1.1 Retrospective sample variant recall rate using DNA.

The variant recall rate of 15 retrospective patient samples with pre-characterized variants[9] and the Myeloid Mutation DNA Mix was established (Supplementary Table 3 and 4). These samples had 77 pre-characterized variants detectable using DNA, including 33 SNVs and 18 indels in 21 genes, 18 genomic gene fusion breakpoints (not including the corresponding reciprocal rearrangements) and 8 deletions involving 6 genes. All SNVs and 17/18 frameshift indels were detected. An *NPM1* frameshift (predicted VAF 5%) from the Myeloid DNA Mutation mix was not detected but had consistently poor coverage spanning this related exon, explaining the lack of detection. In total, 41 of the SNVs and indels were previously characterized using WES and there was high concordance of VAFs between WES and the gene panel (Figure 1A). All 26 SVs were detected, including 14 *BCR::ABL1* genomic fusion breakpoints. The panel detected an additional 6 variants that were not previously detected. The recall rate of variants is summarized in Table 1.

When comparing the previously characterized variants and the variants identified in the gene panel using DNA, the positive percentage agreement was 99% and the positive predictive value was 93%.

Figure 1: Comparison of VAFs between SNVs and indels in retrospective whole exome sequencing, RNA-Seq and capture panel testing using DNA and RNA

Dashed red line has a slope of 1 and is used for comparison in all panels. Solid black line indicates the linear regression line. A. DNA VAF%: Comparison of VAFs of SNVs and indels called by the capture panel using matched DNA samples compared with whole-exome sequencing (n=41); B. RNA VAF%:

Comparison of VAFs of SNVs and indels called by the capture panel using matched RNA samples compared with RNA-seq (n=21); C: DNA vs RNA VAF%: VAFs of all SNVs and indels (n=112) in matched DNA and RNA samples using the capture panel. D. Observed VAF% between DNA and RNA for 18 matched frameshift and nonsense variants predicted to activate nonsense mediated decay (NMD) using matched DNA and RNA samples. The VAF was on average 2-fold lower using RNA, consistent with NMD; E. Observed VAF% between DNA and RNA for 23 matched frameshift and nonsense variants predicted to escape NMD in matched DNA and RNA samples; F. Observed VAF% between DNA and RNA for 5 matched splice site/region variants predicted to activate NMD. The VAF was on average 1.5-fold higher using RNA; G. Observed VAF% between DNA and RNA for 59 matched missense SNVs (n=59); H. Observed VAF% between DNA and RNA for 13 matched ABL1 kinase domain mutations combining all panel data, whole exome sequencing (WES) and RNA-seq demonstrating overexpression using RNA. Orange points indicate data collated from either WES or RNA-seq; I. Observed VAF% between DNA and RNA for 40 matched missense SNVs with exclusion of ABL1 kinase domain mutations and variants with a second mutation in the same allele predicted to activate NMD (nonsense/frameshift variants).

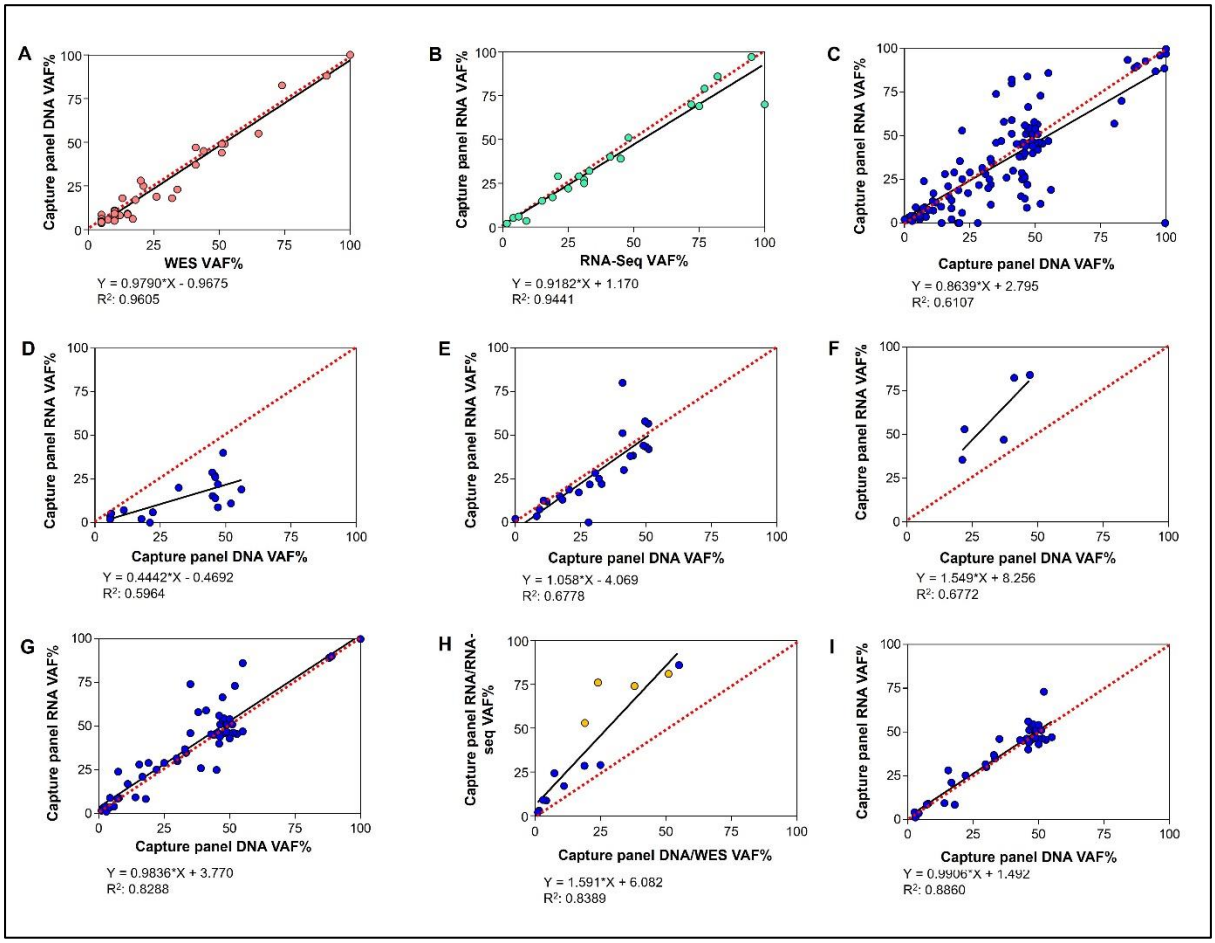


Table 1: Summary of recall rate in patient samples and cell lines in the retrospective study using DNA

Total number of matched samples for comparison = 16		Pre-characterized variants	Panel: detected using DNA	% recalled
Number of variants detected		77	82	
SNVs/indels pre-characterized, n=51	Missense Nonsense Splice donor site Frameshift indels	27 5 1 18	27 5 1 17	100% 100% 100% 94%
*Structural variants pre-characterized, n=26	Fusions Deletions	18 8	18 8	100% 100%
Other variants only detected using panel and DNA, n=6	Missense †Other SNV that altered RNA splicing Deletion	0 0 0	4 1 1	
ǂ Positive percentage agreement = 99% § Positive predictive value = 93% # F1 score = 0.96				

*Some primary structural variants, such as the *BCR::ABL1* fusion transcript generated from a chromosome translocation, may have multiple rearrangements associated with this primary disease-causing lesion. These include the *BCR::ABL1* genomic breakpoints and the reciprocal breaks and fusion transcript derived from the derivative chromosome. The concordance of detection of the pre-characterized structural variant and the gene panel using DNA was considered concordant if at least 1 of the associated rearrangements was detected. Each primary rearrangement is counted as one structural variant, including Ph-associated rearrangements.

ǂ Positive Percentage Agreement: number of variants detected using the panel and DNA that were pre-characterized divided by the (number of variants detected using the panel and DNA + number of pre-characterized variants that were not detected using the panel and DNA).

§ Positive Predictive Value: number of variants detected using the panel and DNA that were pre-characterized divided by the (number of variants detected using the panel and DNA + additional variants detected using the panel and DNA only).

*F*1 score = 2 x ((recall x positive predictive value)/(recall + positive predictive value)). This score evaluated the overall performance of the gene panel.

5.4.1.2 Intra-assay repeatability using DNA.

Sixty-eight SNVs and 26 indels were called in 3 cell line mixes and 3 patient samples analyzed in triplicate for the intra-assay analysis across 40 genes. Sixty-six SNVs (97%) were called in all three replicates of each sample. The two exceptions were low-level *ABL1* kinase domain mutations with VAFs of 2.2% and 1.8% secondary to lower sequencing depth over the target regions in one replicate. Twenty-five of 26 indels were detected in all replicates and the lowest VAF was 1.5%. The exception was a *SETD1B* indel which occurred in a region with poorer sequencing coverage in one replicate. Overall, 91 of the total 94 SNVs and indels (97%) were called in each of the replicates with highly concordant VAFs (Supplementary Table 5 and Supplementary Figure 1A). The average CV for these variants was 9.1%, range 0% to 61%.

Of the 28 SVs, 26 (93%) were detected in each of the replicate runs (Supplementary Table 6). Only two low level SVs were not detected in each run, which were low-level *ABL1*::*BCR* reciprocal genomic fusions with very low read counts and reduced sequencing depth. The average CV of the normalized read counts for the SVs detected in all 3 replicates was 15.4%, range 1.3% to 47%. Overall, 119/123 variants (97%) were detected in each of the 3 replicates.

5.4.1.3 Inter-assay reproducibility using DNA.

The same cell line mixes and patient samples used for the intra-assay analysis were assessed for inter-assay reproducibility in three separate runs. Overall, 90/94 of SNVs/indels (96%) were called in all three runs with highly concordant VAFs (Supplementary Table 5 and Supplementary Figure 1B). All variants with a VAF ≥5% were detected in all three runs and the variants not called had low VAF with reduced sequencing depth over the relevant regions. The average CV for the SNVs/indels called in each run was 11.8%, range 1% to 60%.

Of the 28 SVs, 24 (86%) were detected in each run (Supplementary Table 6). The average CV of the normalized read counts for the 24 SVs detected in all 3 runs was 36.2%, range 6.6% to 67%. All but one of the variants not detected in each run were low-level and were likely not

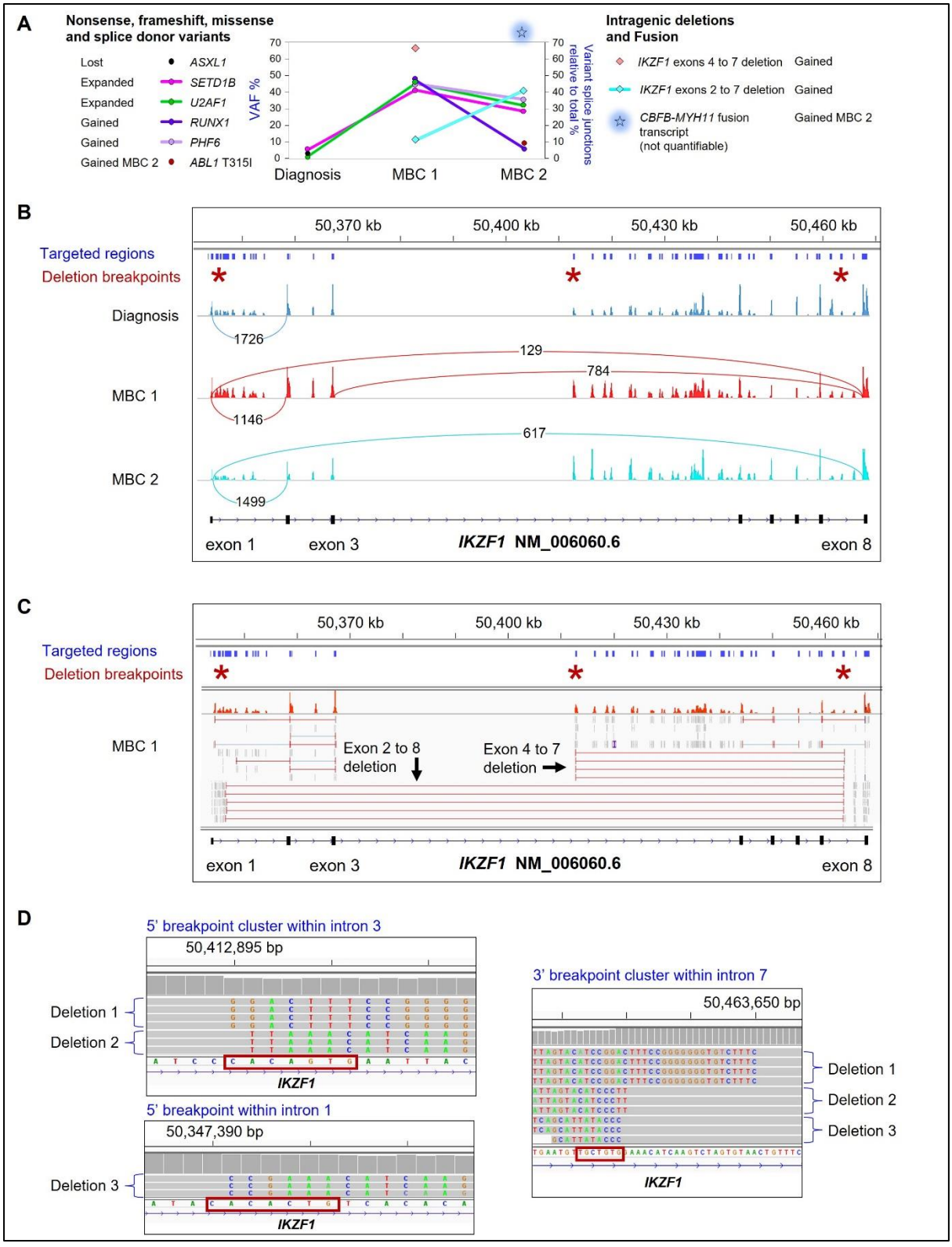
detected due to reduced coverage over the regions of interest. The remaining variant was a 118Kb *IKZF1* genomic inversion, which was clearly visible on manual inspection in IGV and the reason for the miscall is not known.

5.4.1.4 Limit of detection and linearity assessments using DNA.

The linearity and LOD of SNVs and indels was calculated from the cell line dilution series. Fifty-two homozygous SNVs/indels unique to BV173 and absent from the REH diluent were identified (48 SNVs and four indels), and 66 heterozygous SNVs (Supplementary Table 7, Supplementary Figure 2A-B). All variants with an expected VAF of ≥5% were called and 95% of variants with an expected VAF of ≥2.5% were called.

Figure 2: RNA sequencing revealed multiple types of variants during disease progression in patient 2.

A. Patient 2 was known to have multiple mutant clones from prior whole exome and whole transcriptome sequencing. Enhanced sensitivity of the targeted gene panel revealed earlier detection of some mutants, including a hot spot U2AF1 variant at diagnosis that subsequently expanded. Targeted RNA sequencing identified all the variant types for this patient, including IKZF1 deletions and a CBFB-MYH11 fusion transcript. The relative abundance of the IKZF1 deletions at each timepoint was calculated by comparing the atypical splice junction reads with the normal splice junction reads between exons 1 and 2. B. IKZF1 intragenic deletions revealed by atypical RNA splicing. Sashimi plots revealed atypical splicing of the IKZF1 gene that was consistent with intragenic deletions acquired at blast crisis. Normal splicing from exon 1 to 2 is shown, and atypical splicing from exons 1 to 8 and exons 3 to 8. Deletion breakpoints within IKZF1 typically occur at cryptic RAG recombination signal sequences where the consensus sequence motif is CACAGTG. These sequence motifs were specifically targeted by probes in order to capture deletion breakpoints within pre-spliced RNA. The blue colored regions indicate the targeted regions. The red asterisks indicate the location of the deletion breakpoints. C. Intronic split RNA sequencing reads indicated the IKZF1 intronic deletion breakpoint regions. The captured deletion breakpoints generate sequencing reads that are split across the deleted region. The connecting lines between the intronic reads indicate the boundaries of the deletions. Normal split reads between exons are also shown. D: RNA sequencing reads that spanned the genomic breakpoints revealed 3 IKZF1 deletions by revealed clustering close to cryptic RAG recombination signal sequences that are boxed. Two deletions spanning exons 4 to 7 were identified. Each deletion had unique random sequence at the deletion junction, which is consistent with RAG-mediated deletion.



The expected and observed variants of the Myeloid Mutation DNA Mix and dilutions are detailed in Supplementary Table 8. Of the 21 expected variants in the undiluted sample, 20 (95%) were called (expected VAF 5-15%). The variant not called was the *NPM1* indel with a VAF of 5%. Importantly, variants in genes frequently mutated in CML, such as *ABL1* and *ASXL1,*

were called in the undiluted and diluted samples to an expected VAF of 2.5%. All 11 SNVs with an expected VAF of 5% and 2.5% were called, whereas 82% (9/11) with an expected VAF of 1.25% were called. In total, there were 14 indels examined in the BV173 cell line and Myeloid Mutation DNA Mix dilutions. Thirteen of these 14 indels were called at an expected VAF of 5%. The exception was the *NPM1* variant.

The data from the linearity and Myeloid Mutation DNA Mix dilutions indicate that the desirable LOD of 5% was achieved with high confidence for SNVs and indels. 99% (138/139) were detectable at an expected VAF of 5%. With sufficient coverage, variants of lower VAF were reliably detected.

Linearity of the *BCR::ABL1* genomic breakpoint normalized reads of the CML cell line BV173 diluted in the *BCR::ABL1* negative REH cell line was also assessed (Supplementary Table 9,[45] Supplementary Figure 2C). This exploratory analysis demonstrated that SVs were detectable with high confidence up to a 1 in 30 dilution. Three *IKZF1* deletions were detected. The normalized read counts established that the largest deletion (95Kb) was the predominant clone which demonstrated a linear decline upon dilution (Supplementary Table 9, Supplementary Figure 2D).

5.4.2 Comparison of variants detected using DNA versus RNA

DNA sequencing cannot generally detect gene fusions unless the genomic breakpoint coordinates are encompassed by the probe design. Furthermore, DNA cannot assess the effect of splice region variants that are relevant for the genomic assessment of CML patients. Therefore, RNA sequencing was investigated for suitability for detecting the DNA-identifiable SNVs, indels and SVs, in addition to fusions and novel splicing. Twenty-four matched DNA and RNA samples (11 patient samples and 13 cell lines/cell line mixes) were assessed for concordance of variant detection (Supplementary Table 10). One hundred and one variants were detected in cell lines and 69 in patient samples using DNA. *CEBPA* can be a challenging target to sequence[46,47] and sequencing reads were assessed across the gene at 3 hot spot mutation sites (Supplementary Table 11) and were deemed acceptable.

5.4.2.1 SNVs, indels and SVs: DNA versus RNA.

In total, 117 SNVs (n=83) and indels (n=34) were called using DNA in 21 genes. Of these, 112 were also called using RNA, 97% (Supplementary Tables 10). The variants not called using RNA included 2 *TP53* deletions of 26 and 47bp. These deletions were erroneously interpreted as RNA splice junctions by the STAR aligner, using default parameters, and were also not interpreted as indels by the variant callers (see section Aberrant RNA splicing associated with SNVs and indels). These 2 variants were clearly visible when viewing their RNA read alignments in IGV and had adequate read depth. An *ASXL1* frameshift was not detectable using RNA due to poor coverage in this region for that sample. A *NOTCH1* nonsense variant was also not called using RNA, likely related to nonsense mediated decay. A *SETBP1* missense SNV was not detected using RNA due to low *SETBP1* expression. A low-level *CREBBP* nonsense mutation was called using RNA with VAF of 2% but was not called using DNA.

The overall variability in VAF between DNA and RNA is shown in Figure 1C and was greater than observed between replicate analysis of DNA and RNA samples (see Retrospective sample variant recall rate using RNA). Factors associated with differences of VAF% between DNA and RNA were investigated. The average total reads for variants detected using DNA and RNA was >950 and coverage was not considered a factor that contributed to VAF% differences. Variants that generate premature termination codons could activate nonsense-mediated decay (NMD), which is a translation-dependent mechanism that degrades RNA. Heterozygous variants that generated premature termination codons were divided into those predicted to activate NMD (Figure 1D) and those predicted to escape NMD (Figure 1E). Those predicted to escape NMD were located in the last exon or within 50 nucleotides upstream of the last exon junction, less than 150 nucleotides from the first exon and variants within large exons of greater than 400 nucleotides.[48] RNA splice altering variants predicted to activate NMD were analyzed separately. The frameshift and nonsense variants predicted to escape NMD showed good concordance of VAF% between DNA and RNA, with one exception. The frameshift and nonsense variants predicted to activate NMD had a VAF% using RNA on average 2-fold lower than DNA, consistent with NMD. Interestingly, the 5 splice site/region variants predicted to activate NMD had on average 1.5-fold higher VAF% using RNA compared with DNA (Figure 1F). Missense variants in general had good concordance of VAF% (Figure 1G), although

outliers were evident and investigated. The VAF% of *ABL1* kinase domain variants detected in samples derived from CML patients were in general higher using RNA than DNA (Figure 1H). These variants were located on the *BCR::ABL1* allele and expression was driven by the *BCR* promotor. *BCR* has a higher expression than wild-type *ABL1*, which could account for the discordance although this may be an example of allele specific expression. Interestingly, discordance in VAF% was observed for missense variants where an NMD activating variant occurred in the same gene: under-expression for NMD activating frameshift/nonsense variants and over-expression for splice site and splice region variants. This data indicates that the VAF of some variants may be influenced by other variants within the same gene. Exclusion of both these variant groups (missense variants where a second NMD activating variant is present in the same gene and *BCR::ABL1* kinase domain mutations) from the missense variants permitted closer concordance between the DNA and the RNA VAF%.

SVs detected using DNA included genomic gene fusions (n=23), deletions (n=28 in 9 genes), a tandem duplication and an inversion (total SVs=53, Supplementary tables 10). Gene deletions in specific genes are a frequent occurrence in blast crisis CML, in particular *RAG*-mediated deletions in lymphoid blast crisis.[16] Prior work demonstrated that many gene deletions generate novel splice junctions using RNA-seq.[9] Furthermore, when the genomic breakpoint regions occur within captured regions, gene deletions generate split and spanning reads when viewed in IGV that allow nucleotide level resolution of the breakpoint junctions (Figure 2). All the SVs detected using DNA were detected using RNA, except for 3 of 28 deletions. These were two *HBS1L::MYB* intergenic locus deletions and one *IKZF1* deletion involving deletion of the first non-coding exon. The lack of detection of these deletions was likely due to lack of expression of these events. Nine additional SVs were detected using RNA where there was no evidence identified for their presence using DNA. These were mostly fusion transcripts other than *BCR::ABL1* where the corresponding genomic breakpoints were not detected using DNA. The genomic breakpoint regions of all of these fusions occur over a very large region throughout the genome and were not specifically captured in the capture gene panel.

There was high overall concordance of variants detected using DNA compared with RNA (Figure 3). The positive percentage agreement was 95% and the positive predictive value was 94% (Table 2).

Figure 3: Comparison of variant identification between the matched DNA and RNA samples demonstrating the higher yield of variant subtypes using RNA.
There was high concordance of detection of SNVs and indels between DNA and RNA. All BCR::ABL1 genomic breakpoints were detectable using both DNA and RNA. However, fusion transcripts were only identified using RNA, which also enabled the detection of atypical BCR::ABL1 and novel fusion transcripts. Novel splicing events were also only identified using RNA.

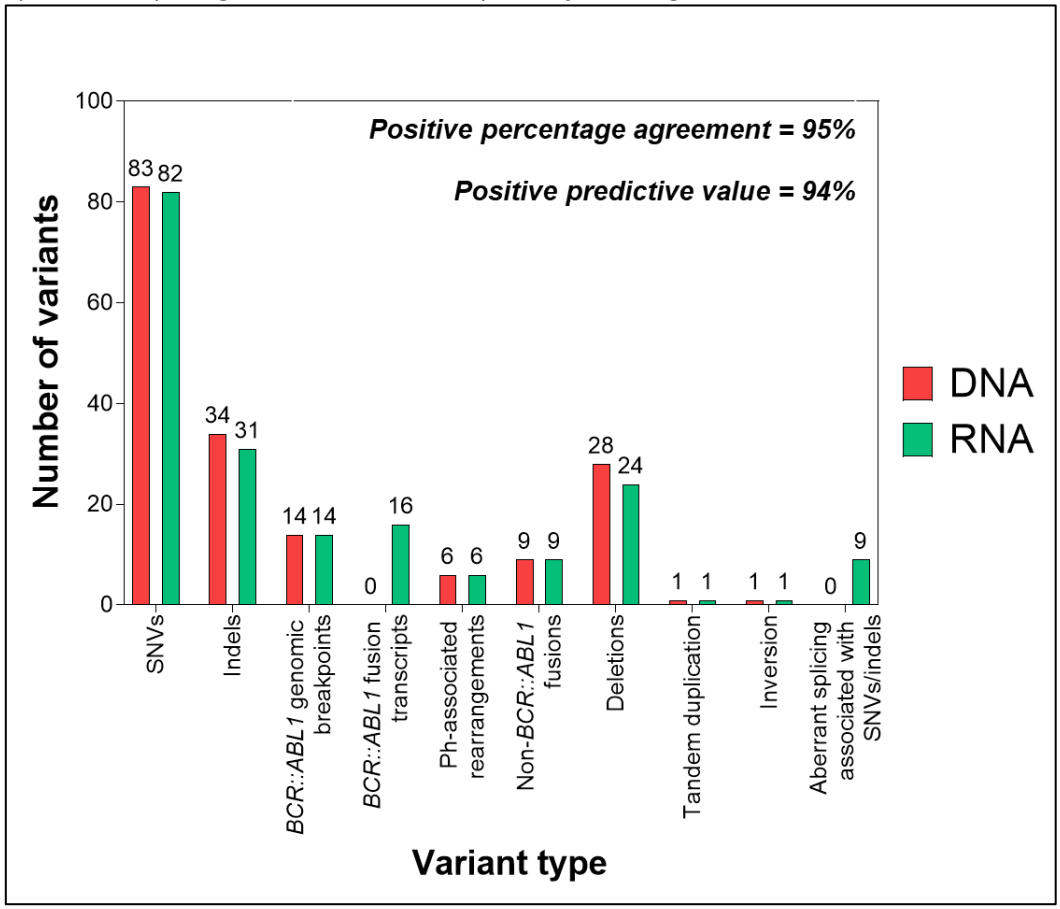


Table 2: Summary of DNA versus RNA results

Total number of matched samples for comparison = 24		Variants identified using DNA	Variants identified using RNA	% recalled
Total number of variants detected		170	172	
SNVs/indels detected using DNA, n=116	Missense	59	58	98%
	Nonsense	19	18	95%
	Splice donor site	4	3	75%
	†Splice region that altered RNA splicing	4	4	100%
	Frameshift indels	28	26	93%
	Non-frameshift indels	3	3	100%
*Structural variants detected using DNA, n=53	Fusions	23	23	100%
	Deletions	28	25	89%
	Tandem duplication	1	1	100%
	Inversion	1	1	100%
Other structural variants only detected using RNA, n=10	Nonsense	0	1	
	Fusion transcripts	0	9	
ǂ Positive percentage agreement = 95% § Positive predictive value = 94% # F1 score = 0.95				

*Some primary structural variants, such as the *BCR::ABL1* fusion transcript generated from a chromosome translocation, may have multiple rearrangements associated with this primary disease-causing lesion. These include the *BCR::ABL1* genomic breakpoints and the reciprocal breaks and fusion transcript derived from the derivative chromosome. The concordance of detection of the structural variant using DNA and RNA was considered concordant if at least 1 of the associated rearrangements was detected. For example, if a genomic fusion breakpoint was detected using DNA and the corresponding fusion transcript was detected using RNA, it was considered concordant. Each primary rearrangement is counted as one structural variant, including Ph-associated rearrangements.

† Only RNA can detect the aberrant splicing associated with these variants

ǂ Positive Percentage Agreement: number of variants detected using RNA that were detected using DNA divided by the (number of variants detected using RNA + number of variants detected using DNA that were not detected using RNA)

§ Positive Predictive Value: number of variants detected using RNA that were detected using DNA divided by the (number of variants detected using RNA that were detected using DNA + additional variants detected using RNA only)

*F*1 score = 2 x ((recall x positive predictive value)/(recall + positive predictive value)). This score evaluated the overall performance of the gene panel.

Ph-associated rearrangements. The strategy of specifically capturing the *BCR::ABL1* genomic breakpoint region within *BCR* introns 13 and 14 was assessed for suitability to identify patients with one or more Ph-associated rearrangements, irrespective of whether either DNA or RNA was used for sequencing. The majority of the Ph-associated rearrangements involved genomic fusions and fusion transcripts between *BCR* or *ABL1* and other genes. The panel design also specifically captured genes on chromosomes 9q and 22q that prior NGS analysis identified as fusion partners. Six samples with known Ph-associated rearrangements were tested using DNA and RNA. One or more Ph-associated rearrangements were confirmed for all samples using DNA and RNA. Supplementary Figure 3 contrasts the Ph-associated rearrangements detectable using DNA or RNA. Some rearrangements were highly complex, involving inversions and potential chromosomal fragmentation, as described for other cancers.[49] For example, the Ph-associated rearrangement of K562[50] involves a novel fusion between *NUP214* on chromosome 9 and *XKR3* on chromosome 22 (Supplementary Figure 4). The original location of the genes and the orientation of the novel fusion transcript indicate that a complex series of events was required for its formation, including sequence fragmentation, inversion and repositioning of the genes. Sequence was also potentially deleted.

5.4.2.2 Aberrant RNA splicing associated with SNVs and indels

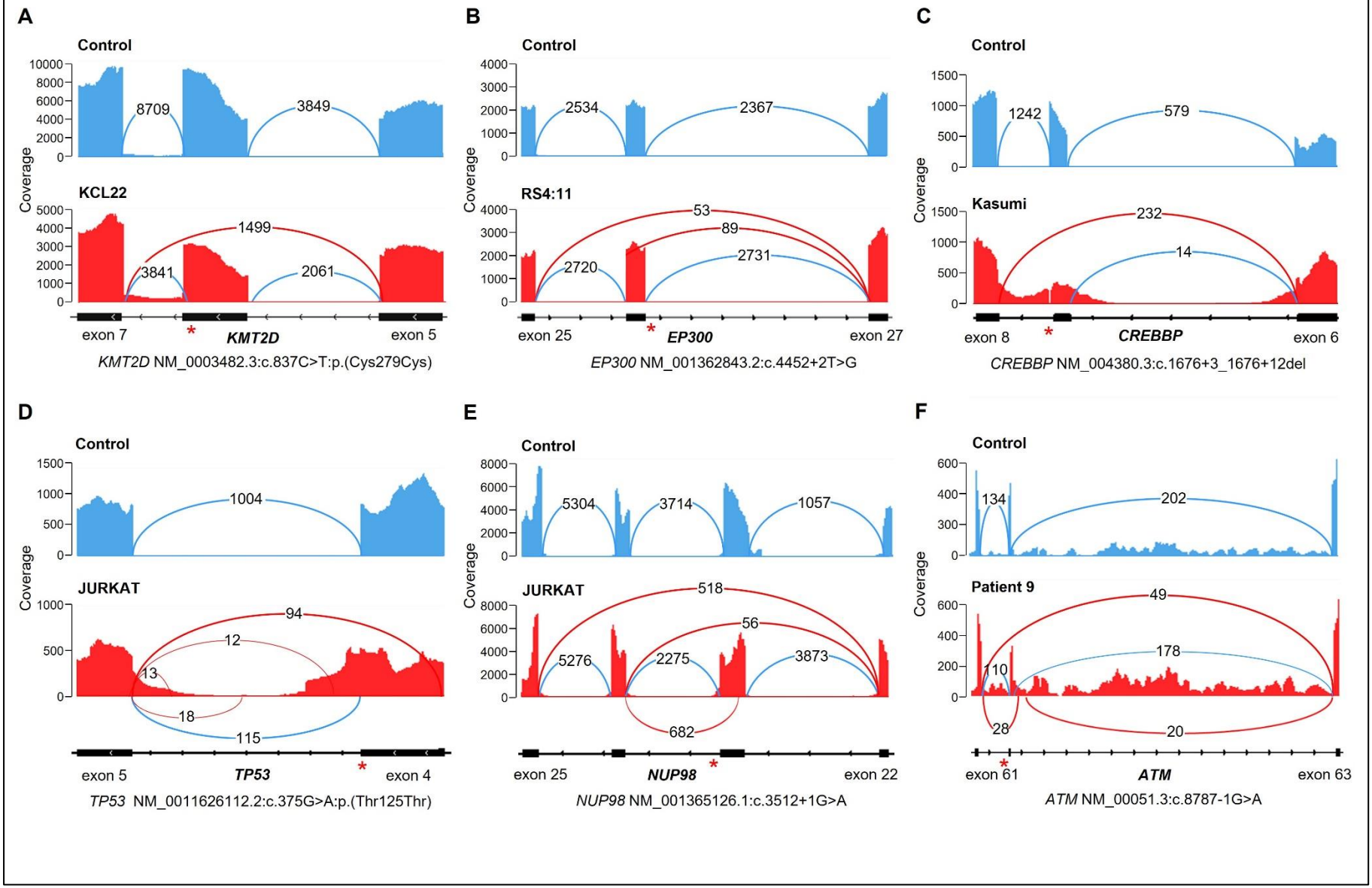
While *in silico* models can make predictions for RNA splicing consequences from splice region variants detected using DNA, analysis of RNA using RT-PCR or NGS is required to determine the actual effect on splicing. Normal splicing involves recognition of consensus splice sites at

the intron-exon border. Disruption of a consensus splice site can result in loss of recognition of the specific motif demarcating the intron-exon border, leading to unexpected transcriptomic alterations. Moreover, splicing variants can introduce or activate cryptic splice sites and alter normal transcription. Some variants will result in premature termination, either activating NMD or generating truncated proteins. The effect of variants on splicing using RNA and NGS was evaluated. Splice junctions were visualized with sashimi plots using IGV. Gene deletions were detected in most cases by novel splice junctions between distant loci where exons adjacent to the breakpoints were ligated. In some cases splicing occurred between an exon and an intergenic region due to activation of cryptic splice donor or acceptor sites adjacent to an intergenic breakpoint.[9]

All rare SNVs and indels annotated in the pipeline as splice donor (first 2 intronic nucleotides adjacent to the end of an exon), splice acceptor (last 2 intronic nucleotides before the start of an exon) or splice region variants for the 37 samples tested using RNA were assessed for their impact on normal RNA splicing (Figure 4, Supplementary Table 12). A splice region was within 1-3 exonic bases from the exon boundary or within 3-8 intronic bases from the exon boundary. Rare variants were defined as those with a population frequency of <0.1%. The variants included low impact intronic and synonymous variants. An additional sample was included in the analysis for illustration purposes where only DNA was available for sequencing, however prior RNA-seq was performed (Figure 4F). The *in silico* prediction models (MaxEntScan, NNSplice, SpliceSiteFinder-like and GeneSplicer) did not predict the complexity of aberrant splicing that was observed (Supplementary Table 12).

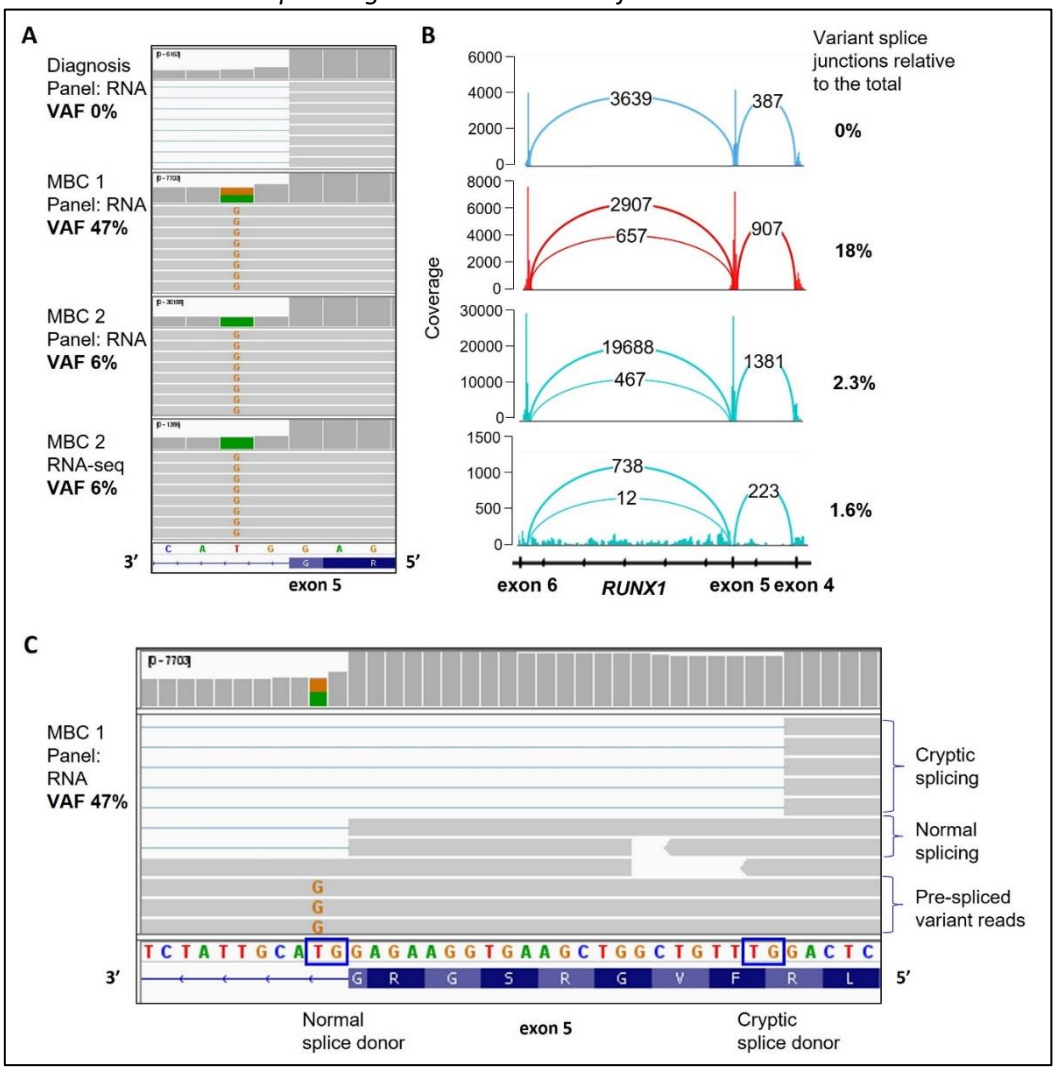
Figure 4: Atypical splicing related to essential splice site or splice region variants

The red asterisks indicate the variant sites. Control samples demonstrating normal splicing are shown in blue with normal splicing in the samples containing variants indicated by blue arcs. A. Splice region synonymous variant. Exon skipping associated with a synonymous splice region variant in KMT2D, in-frame. B. Splice donor site variant. Exon skipping and activation of an exonic cryptic splice site variant associated with a splice donor variant in EP300, premature termination. C. Splice region intronic variant. Exon skipping and intron retention associated with a 10bp homozygous intronic splice region variant in CREBBP, premature termination. D. Splice region synonymous variant. Intron retention and activation of multiple exonic and intronic cryptic splice sites associated with a synonymous splice region variant in TP53, premature termination. The variant is reported in Li-Fraumeni syndrome.26 E. Splice donor site variant. Single and two exon skipping, plus activation of an exonic cryptic splice site associated with a splice donor variant in NUP98. Note that all samples had alternate splicing between exon 23 and 25, which is not shown for simplicity. F. Splice acceptor site variant. Exon skipping and activation of intronic cryptic splice donor and acceptor sites associated with a splice acceptor site variant in ATM, premature termination. Cryptic splice site activation led to inclusion of 180bp of intronic sequence as a pseudo-exon. This sample was sequenced using RNA-seq and the intronic reads are high relative to the exonic reads and are derived from pre-spliced RNA.


A
Control
8709
3849
KCL22
1499
3841
2061
Coverage
exon 7
KMT2D
exon 5
KMT2D NM_0003482.3:c.837C>T:p.(Cys279Cys)
B
Control
2534
2367
RS4:11
53
89
2731
2720
exon 25
EP300
exon 27
EP300 NM_001362843.2:c.4452+2T>G
C
Control
1242
579
Kasumi
232
14
exon 8
CREBBP
exon 6
CREBBP NM_004380.3:c.1676+3_1676+12del
D
Control
1004
JURKAT
94
12
13
18
115
exon 5
TP53
exon 4
TP53 NM_0011626112.2:c.375G>A:p.(Thr125Thr)
E
Control
5304
3714
1057
JURKAT
518
56
5276
2275
3873
682
exon 25
NUP98
exon 22
NUP98 NM_001365126.1:c.3512+1G>A
F
Control
134
202
Patient 9
49
178
110
28
20
exon 61
ATM
exon 63
ATM NM_00051.3:c.8787-1G>A

Four splice donor and 1 splice acceptor variants were identified in patients and cell lines. A *RUNX1* splice donor variant resulted in loss of the canonical splice donor site of exon 5, which activated a cryptic splice site 23 nucleotides upstream within exon 5 (Figure 5). The same aberrant splicing pattern was observed in a different patient at blast crisis transformation where sequencing was performed previously (Supplementary Figure 5).[9] This cryptic splice donor within exon 5 has been reported to be activated in a family with a familial platelet disorder and a constitutional *RUNX1* splice region variant in the adjacent nucleotide.[51,52] This provides further verification of the validity of the findings observed in patients. Exon skipping was predicted for an *EP300* splice donor variant, which was confirmed by the splicing pattern observed using the panel sequencing and RNA. However, an additional unpredicted splicing event was observed related to activation of an exonic cryptic splice donor (Figure 4B). A *NUP98* splice donor variant was associated with exon skipping of 1 or 2 exons in addition to activation of an exonic cryptic splice site (Figure 4E). An *ATM* splice acceptor site variant resulted in exon skipping and formation of a pseudo-exon (Figure 4F). The *TP53* 47bp deletion that was not called using RNA generated aberrant splicing since the splice donor site of exon 7 was lost. Exon skipping and intron retention was observed, which predicted premature termination (Figure 6).

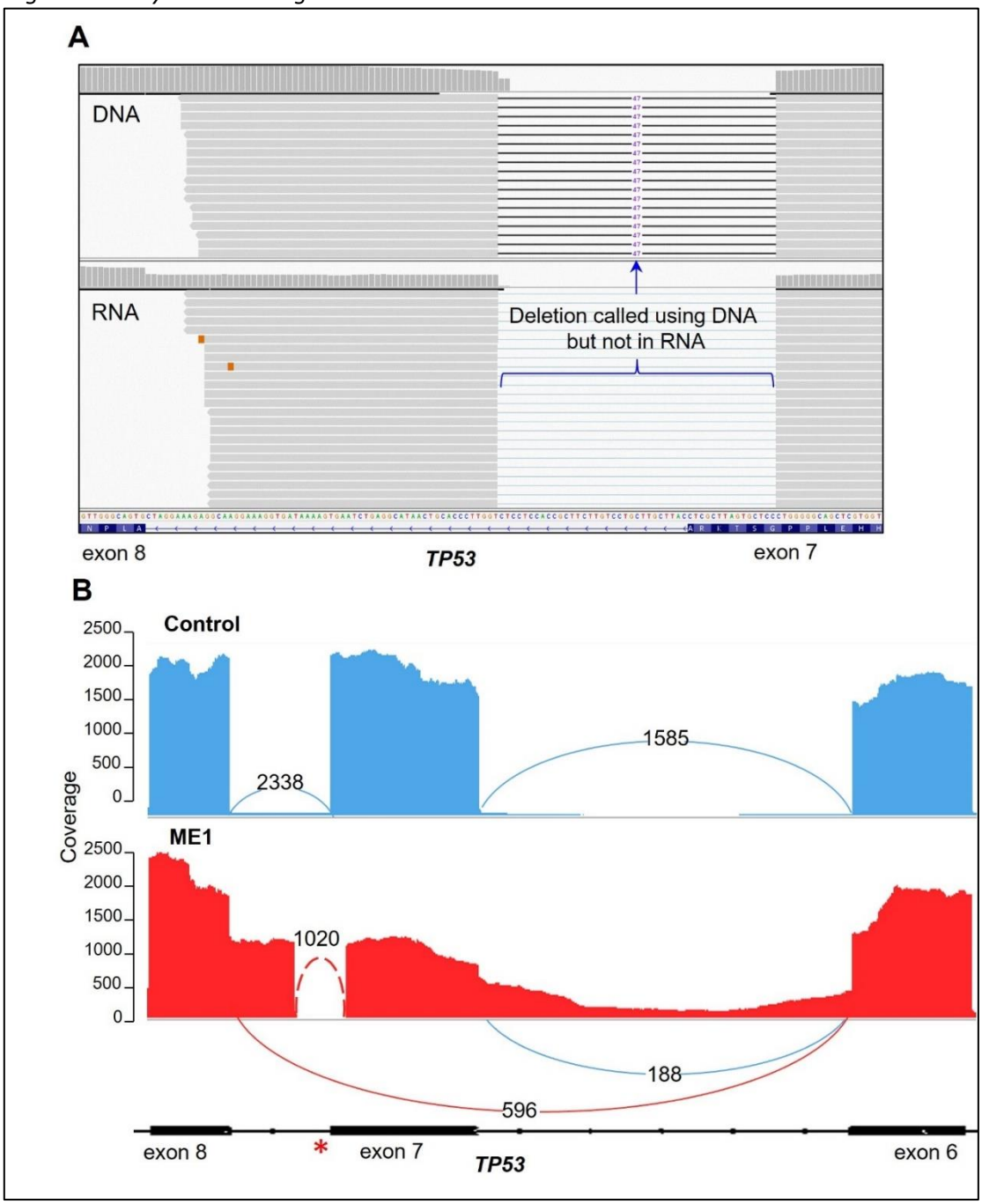
Figure 5: Atypical splicing associated with a RUNX1 splice donor mutant.
Patient 2 had multiple competing somatic mutant clones over the course of disease, including a RUNX1 splice donor variant where the VAF reduced at the time of a second MBC. A. IGV screenshot showing the RUNX1 mutant (NM_001754.5:c.508+2T>C) detectable by targeted gene panel testing or RNA-seq from pre-spliced sequencing reads using total RNA. The variant allele frequency from the targeted gene panel and RNA-seq were concordant for the second MBC sample, however, the number of reads were substantially higher using targeted sequencing. B. Sashimi plots revealed atypical splicing between RUNX1 exons 5 and 6 associated with the splice donor variant. The arcs represent splice junctions that connect exons and the number of splice junction reads are indicated. Atypical splicing was only present in the samples where the mutation was detected. The relative number of atypical splice junction reads correlated with the variant allele frequency of the mutant. C. Sequencing reads within exon 5 revealed activation of a cryptic splice donor site within exon 5 that excludes 23 nucleotides. The atypical splicing leads to a frameshift and premature termination. The same cryptic donor site was identified in a family with a constitutional RUNX1 splice region variant in the adjacent nucleotide.[51,52]



Eight splice region variants were detected that were classified as low impact by the SnpEff annotation tool,[53] including synonymous SNVs and intronic deletions (Supplementary Table 12). Three of these affected normal splicing (Figure 4). A *KMT2D* splice region variant was associated with exon skipping (Figure 4A), which was confirmed by RT-PCR (Supplementary Figure 6) but was not predicted by the *in silico* models utilized by Alamut. An intronic splice region *CREBBP* 10bp deletion resulted in the predicted exon skipping but also unpredicted intron retention (Figure 4C). A *TP53* synonymous variant had a profound impact on splicing, including intron retention and activation of multiple cryptic splice sites (Figure 4D). This variant is associated with Li-Fraumeni syndrome[54] and the observed splicing pattern was consistent with the published splicing pattern resolved using RT-PCR, however, capture RNA sequencing revealed additional novel splicing.

Figure 6: Atypical splicing enabling detection of the TP53 47bp deletion in ME1

A 47bp homozygous deletion that encompassed the splice donor site of exon 7 was not called using RNA since the STAR aligner processed the deletion as a splice junction. A. IGV screenshot showing the deletion in TP53 (TP53 NM_0011626112.2:p.Gly302_Arg306del/c.905_919+32del) clearly visible in both DNA and RNA. B A sashimi plot revealed an artificial splice generated at the deletion site (dashed arc), aberrant skipping of exon 7 and retention of introns 6 and intron 7. The blue arc indicates normal splicing and the red solid arc indicates abnormal splicing. The dashed red arc indicates an artificial splice generated by the STAR aligner at the deletion site. The red asterisks indicate the variant sites.

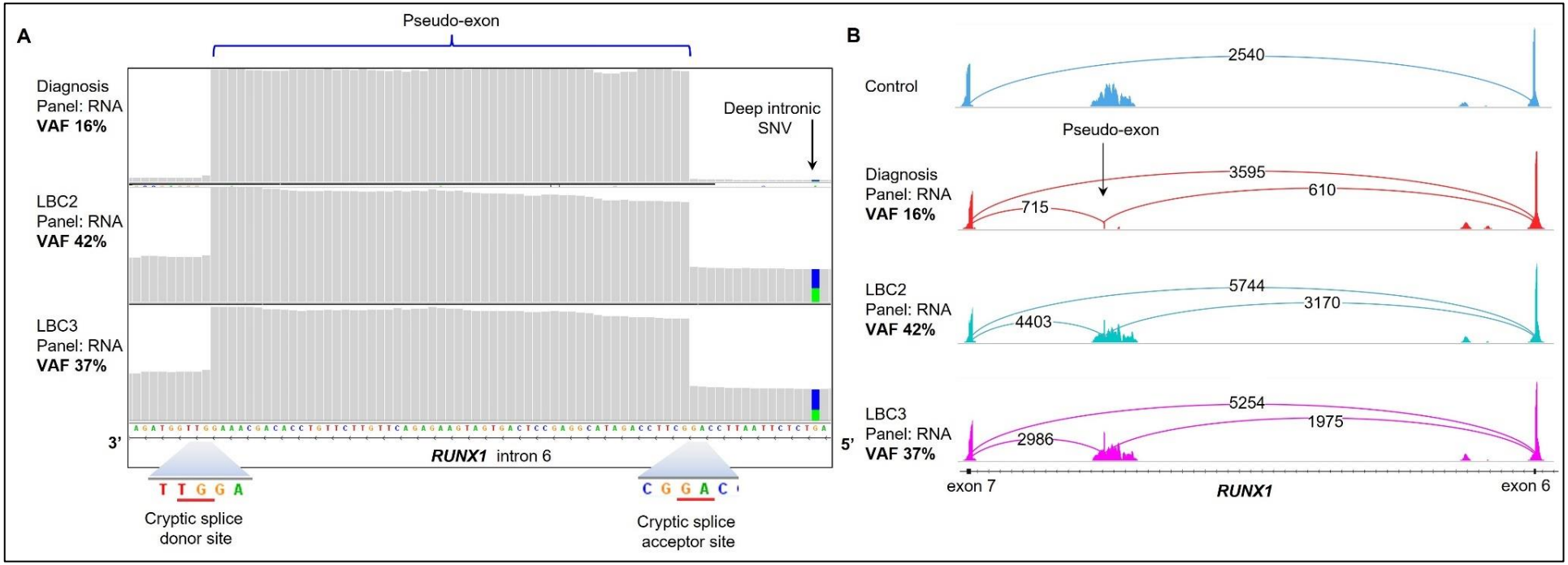


Atypical splicing of *RUNX1* was observed for Pt3 at the time of lymphoid blast crisis by prior RNA-seq, which was associated with retention of an intronic sequence and incorporation of a 55bp pseudo-exon (Figure 7). Inspection of the region surrounding the pseudo-exon

identified a deep intronic SNV that was gained at lymphoid blast crisis. The SNV was not detected by WES using matched DNA. Enhanced sensitivity of detection using the gene panel confirmed the presence of the variant and atypical splicing at the time of diagnosis using RNA. The mechanism of activation of the cryptic splice sites related to this deep intronic variant is not known.

Figure 7: Deep intronic variant that generated a variant activated pseudo-exon in RUNX1

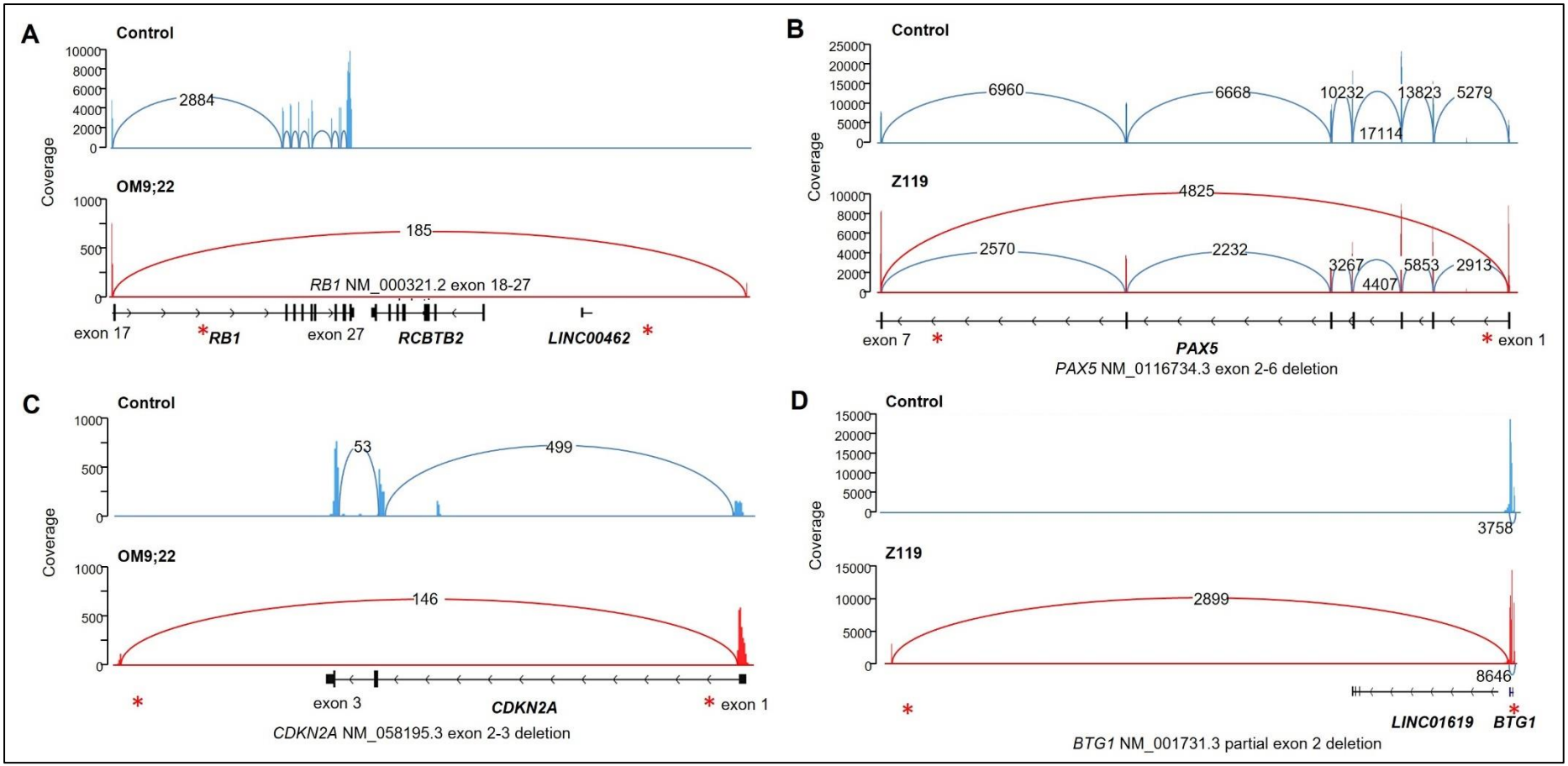
On evaluation of splicing of key genes, patient 3 had a deep intronic variant that generated a pseudo-exon between exons 6 and 7. Multiple timepoints were sequenced and once the variant was identified, the panel coverage was modified to include specific capture of this region to aid the detection of the variant if it proved to be recurrent in other patients. The somatic variant has so far not been detected in other patients. A. IGV screenshot showing the deep intronic mutant (RUNX1 NM_001754.5:c.614-5860G>T) detectable by targeted gene panel testing at diagnosis, second and third lymphoid blast crisis. While this variant was not located at a canonical splice site, a splicing element may have been impacted, which enabled activation of cryptic splice sites surrounding the pseudo-exon. At diagnosis, the VAF was 16%, which increased at disease progression. The mutation is predicted to lead to premature termination. RNA-seq of the first lymphoid blast crisis sample confirmed the presence of the SNV, which was below the level of detection by RNA-seq at diagnosis. WES was unable to detect this variant. The higher sensitivity of the gene panel enabled the detection of the SNV at diagnosis using RNA B. Sashimi plots revealed atypical splicing between RUNX1 exons 6 and 7 associated with the deep intronic variant and creation of the pseudo-exon. The diagnosis sample was sequenced by the original panel design without specific targeting of the region involving the deep intronic variant, whereas all other samples, including the control, were sequenced with the modified design with specific capture of this region. This accounts for the increased background reads at the pseudo-exon site. LBC, lymphoid blast crisis.



Atypical splicing was also identified for most *RAG*-mediated gene deletions which is demonstrated in Figure 8. Similar to the *IKZF1* deletions highlighted in Pt2 (Figure 2), gene deletions in *RB1, PAX5 CDKN2A* and *BTG1* of <200Kb were identified through specific targeting of sequences subject to aberrant *RAG*-mediated recombination using RNA.

Figure 8: Atypical splicing associated with RAG-mediated gene deletions.

Each of the deletions detected by atypical splicing in the *BCR::ABL1* positive acute lymphoblastic cell lines had non-template sequence inserted at the breakpoints, which is characteristic of *RAG*-mediated deletions. The splice junctions for the *RB1, CDKN2A* and *BTG1* deletions are associated with activation of cryptic splice donor and acceptor sites. The asterisks indicate the breakpoint locations of each deleted region, which occurred at cryptic *RAG* recombination signal sequences. Blue arcs are normal splice junctions. Red arcs are atypical splice junctions. Splice junctions are not shown for adjacent genes. A. *RB1* atypical splicing associated with a 192 kb homozygous deletion. Splice junction reads are not shown for the Control sample for *RB1* exons 18 to 27: the junctions reads ranged from 2499 to 3886. B. *PAX5* atypical splicing associated with a 122 kb heterozygous deletion. C. *CDKN2A* atypical splicing associated with a 43 kb homozygous deletion. D. *BTG1* atypical splicing associated with a 132 kb homozygous deletion


A
Control
Coverage
2884
OM9;22
185
RB1 NM_000321.2 exon 18-27
exon 17
RB1
exon 27
RCBTB2
LINC00462
B
Control
6960
6668
10232
13823
5279
17114
Z119
4825
2570
2232
3267
5853
2913
4407
exon 7
PAX5
exon 1
PAX5 NM_0116734.3 exon 2-6 deletion
C
Control
53
499
OM9;22
146
exon 3
CDKN2A
exon 1
CDKN2A NM_058195.3 exon 2-3 deletion
D
Control
3758
Z119
2899
8646
LINC01619
BTG1
BTG1 NM_001731.3 partial exon 2 deletion

5.4.3 Test performance characteristics using RNA

As RNA was proven to be a reliable medium for mutation detection in CML, 19 RNA samples with pre-characterized variants[9] were re-sequenced using the gene panel (Supplementary Tables 13-15). These samples had 78 pre-characterized variants detectable using RNA, including 32 SNVs, 7 indels, 27 fusions and 12 gene deletions. Three patient samples with available RNA were analyzed in duplicate to assess the intra-assay reproducibility and 3 samples were tested in two separate runs for inter-assay repeatability (Supplementary Tables 14 and 15). Germline variants were included in the analysis to supplement the clinically relevant variants. A total of 94 variants were assessed in the intra-assay analysis. All variants were detected and with high concordance of VAFs for SNVs and indels. For the inter-assay analysis, all 87 variants were detected in each run (Supplementary Tables 14 and 15, Supplementary Figure 7) and with high concordance of VAFs for SNVs and indels. The average CV for SNVs/indels for the intra-assay and inter-assay analysis was 3.8% and 6.4%, respectively.

5.4.3.1 Retrospective sample variant recall rate using RNA.

All SNVs and indels previously identified using whole-transcriptome RNA-seq were detected and the VAFs were highly concordant (Supplementary Table 13 and Figure 1B). Eight additional low-level variants (VAF 1.2%-7.9%) were detected and 1 deep intronic splice altering variant (Figure 7), which demonstrates the enhanced sensitivity of the capture gene panel. Pt2 had multiple timepoints tested using capture RNA sequencing and multiple variant types, including missense, frameshift, nonsense, splice site, *IKZF1* deletions and a *CBFB::MYH11* fusion. The complexity of the clonal evolution of variants for this patient is shown in Figure 2. Enhanced sensitivity allowed the detection of an *IKZF1* deletion at an earlier timepoint than by prior WES.

All previously detected *BCR::ABL1* transcripts were identified using the panel. Ph-associated rearrangements were confirmed for all 5 patients with previously characterized Ph-associated rearrangements. There were 3 gene deletions that were not detected. These were *HBS1L::MYB* intergenic locus deletions that were likely due to lack of expression of these

events. One *IKZF1* deletion was newly detected using the gene panel that was not previously detected. This deletion was also subsequently detected using whole transcriptome RNA-seq when the patient progressed to blast crisis and demonstrates the enhanced sensitivity of the capture gene panel.

Overall, when comparing the previously characterized variants and the variants identified using RNA, the positive percentage agreement was 96% and the positive predictive value was 88% (Table 3).

Table 3: Summary of recall rate in patient samples in the retrospective study using RNA

Total number of matched samples for comparison = 19		Pre-characterized variants	Panel: detected using RNA	% recalled
Total number of variants detected		77	84	
SNVs/indels pre-characterized, n=39	Missense	23	23	100%
	Nonsense	5	5	100%
	Splice donor site	2	2	100%
	Frameshift indels	6	6	100%
	In-frame deletion	1	1	100%
	Intronic splice altering	2	2	100%
*Structural variants pre-characterized, n=39	Fusions	26	26	100%
	Deletions	12	9	75%
Other variants only detected using panel and RNA, n=10	Missense	0	7	
	Nonsense	0	1	
	Intronic splice altering	0	1	
	Deletion	0	1	

ǂ Positive percentage agreement = 96%
§ Positive predictive value = 88%
# F1 score = 0.92

*Some primary structural variants, such as the *BCR::ABL1* fusion transcript generated from a chromosome translocation, may have multiple rearrangements associated with this primary disease-causing lesion. These include the *BCR::ABL1* genomic breakpoints and the reciprocal breaks and fusion transcript derived from the derivative chromosome. The concordance of detection of the pre-characterized structural variant and the gene panel using RNA was considered concordant if at least 1 of the associated rearrangements was detected. Each primary rearrangement is counted as one structural variant, including Ph-associated rearrangements.

ǂ Positive Percentage Agreement: number of variants detected using the panel and RNA that were pre-characterized divided by the (number of variants detected using the panel and RNA + number of pre-characterized variants that were not detected using the panel and RNA).

§ Positive Predictive Value: number of variants detected using the panel and RNA that were pre-characterized divided by the (number of variants detected using the panel and RNA + additional variants detected using the panel and RNA only).
# *F*1 score = 2 x ((recall x positive predictive value)/(recall + positive predictive value)). This score evaluated the overall performance of the gene panel.

5.5 Discussion

The rapid rate of NGS development has resulted in an explosion of knowledge regarding the genomic landscape of various malignancies. The search for mutations using panels of genes has generally focused on identification of SNVs and indels. However, additional mutational types, including known and novel fusion transcripts and deletions, have been identified in CML and likely contribute to disease progression. These variants may be missed by conventional DNA-based NGS technologies. Consequently, development and validation of a sensitive method to reliably identify SNVs, indels, gene deletions, fusions (including Ph-associated rearrangements) and RNA splice altering variants is critical when investigating patients with CML.

This data demonstrated that a capture gene panel can reliably and sensitively detect clinically relevant genomic events in CML, using DNA or total RNA. 97% of SNVs and indels called by the variant callers using DNA were also called using RNA. Utilizing total RNA as opposed to standard poly(A) enriched RNA-Seq as a strategy has proven capable of identifying sense and antisense spliced fusion transcripts as well as genomic breakpoints from intron-retaining precursor mRNA. This enabled the detection of additional variants using RNA, such as intronic splice altering variants that are frequently spliced out of mRNA transcripts due to their functional impact on splicing. Probes designed to selectively target annotated exons were utilized. This enriches for sequences that extend beyond the captured region, including splice sites in intron retaining pre-spliced RNA. Moreover, targeting only part of a transcript is sufficient to capture the full-length transcript, including novel spliced exons within the same transcript, and novel fusion partners. A major strength of an RNA-based gene panel is the ability to assess the consequences of potential splice altering variants. The data demonstrated

that the effects of splice site and splice region variants were readily apparent using RNA. These included variants predicted to have a low impact, such as splice region synonymous and intronic deletion variants. Some of these had profound and unpredicted effects on splicing. Despite use of *in silico* prediction models, the true consequence of the event cannot be concluded based on DNA sequencing alone without RT-PCR or RNA-based NGS analysis.

Comparison of the VAFs for DNA and RNA demonstrated that RNA can provide a reliable indication of the variant burden for clonal tracking. However, key differences were noted for some variants. While frameshift and nonsense variants were detectable using both RNA and DNA, NMD may have influenced the concordance of VAFs. Escape from NMD is predicted for premature termination codons that occur in the last exon or within 50 nucleotides of the last exon junction, in large exons (≳400 nucleotides) or 150 nucleotides of the start codon.[48,55] The VAFs between RNA and DNA for these variants were highly concordant. Importantly, variants predicted to activate NMD were detected using RNA, with only one exception. However, the VAF was lower using RNA. The strategy of using total RNA, where intron-retaining precursor mRNA was sequenced, may have contributed to the detection of some intronic NMD activating variants using RNA, particularly those that altered splicing. The VAFs of missense variants were generally concordant in the absence of a second NMD activating variant in the same gene. *BCR-ABL1* kinase domain mutations were generally overexpressed using RNA, which may be related to the activity of the *BCR* promoter. RNA in long-term storage was also demonstrated to generate good quality results. This should allow the retrospective assessment of large patient cohorts with well curated clinical data for the detection of genomic prognostic biomarkers.

Specific genomic breakpoints were detectable using DNA and total RNA by targeting known intronic breakpoint regions. The *BCR::ABL1* genomic breakpoints are unique to each patient and have been used to monitor patients for minimal residual disease.[56] All *BCR::ABL1* genomic breakpoints within the targeted regions were detected using DNA and RNA. However, only RNA can demonstrate the generation of fusion transcripts, including atypical *BCR::ABL1* fusions. Detection of Ph-associated rearrangements, recently demonstrated to have prognostic potential,[9] is also possible using DNA and RNA. All patients with previously

characterized Ph-associated rearrangements were correctly identified as having a Ph-associated rearrangement using the gene panel, whether using DNA or RNA. However, the full spectrum of rearrangements were not detected in all cases, while additional rearrangements were detected in others. Some events may not be detectable if they involve fusions between genes that are not included in the capture panel, as was observed for a single patient.

Targeting of specific sequences subject to aberrant *RAG*-mediated recombination allowed the characterization of deletions in key genes relevant in lymphoid blast crisis of CML, such as *IKZF1*. This was effective when using DNA or total RNA. In many cases aberrant RNA splicing aided the detection of these events using RNA. However, larger deletions involving breakpoints located in non-targeted regions will not be detected using this strategy. A limitation of this method is that some deletions involving *CDKN2A/B* will not be detected if the mechanism of deletion does not involve aberrant activation of *RAG*.[21] Similarly, where breakpoints are located outside of the captured *RAG* recombination signal sequences, deletions will not be detected. The breakpoints of a *RAG*-mediated deletion involving *RUNX1*[16] were incidentally detected because one of the breakpoints occurred within a captured exon. *RAG*-mediated deletions involving *RUNX1* have not previously been reported. *RAG*-mediated deletions have been identified in patients who progressed to lymphoid blast crisis and identifying these events is important to future risk prediction.

The customized panel can be readily deployed at the first sign of therapy failure, increasing the diagnostic yield by sequencing a high-risk cohort, or even at the time of a new diagnosis of CML as a screening tool. Preliminary work has already identified an early signal deeming Ph-associated rearrangements and cancer associated gene mutations as having an inferior prognostic association.[9] Ph-associated rearrangements may not have a direct causal role in progression but could be a marker of genomic instability and the propensity to acquire additional mutations that could drive progression. Other variants have a strong causal link in progression, such as the mutations described for *ASXL1, RUNX1* and *IKZF1*. The International CML Foundation Genomics Alliance has provided guidance on how to assign clinical relevance to genes and variants in CML.[24] This includes careful assessment of the evidence for clinical

relevance. *ASXL1*, *RUNX1* and *IKZF1* are frequently mutated in CML and these genes, in addition to others such as *BCORL1* and *TP53*, have already been identified as important in CML disease progression [11,14,18] *ASXL1*, *RUNX1* and *TP53* are known drivers of leukemia while *TP53* is a key tumor suppressor gene that is involved in a number of malignancies. *RUNX1* is heavily implicated in both myeloid and lymphoid malignancies and *RUNX1*-mutated AML has even been incorporated as a provisional entity in the recent edition of the WHO classification of myeloid neoplasms and acute leukemias.[2] Furthermore, *RUNX1* mutations contribute to impaired myeloid differentiation, inducing progression to blast crisis CML.[57] Mutated *ASXL1* is associated with inferior prognosis in many myeloid malignancies and has been specifically linked to progression to blast crisis CML following TKI failure.[58,59] Abnormalities in *IKZF1*, heavily involved in lymphoid development and maturation, have been associated with reprogramming lymphoblasts towards oncogenesis.[60] Patients harboring genomic events that are considered a negative impact on outcome could be considered for either upfront therapy with more potent TKIs and perhaps early preparation for an allogeneic stem cell transplant. Substantial work is required to clarify the true portent of these variants before the panel can be readily translated into the clinic to inform treatment decisions.

In conclusion, the customized capture gene panel is suitable for use with both DNA and RNA. The vast majority of variants implicated in treatment failure of CML, including SNVs, indels, p210 *BCR::ABL1* related SVs and specific *RAG*-mediated gene deletions were detectable using DNA or RNA. The added benefit of RNA is that it enables the detection of known and novel fusion transcripts and reveals aberrant splicing with potential pathogenic effect. Furthermore, older RNA samples in long-term storage were successfully sequenced with this customized method. Improvements in bioinformatic strategies will enable the detection of more complex variants such as small deletions that generate novel splice junctions or duplications of >50bp. These proved problematic for the variant callers using RNA. This data, in combination with work from others,[61] suggest that total RNA sequencing may be appropriate to detect multiple types of mutations reported in patients with CML, where some are not detectable using DNA alone. This could prove beneficial when the cost of sequencing is prohibitive using both DNA and RNA. RNA capture sequencing using total RNA also has the

potential to be expanded into other hematological malignancies, for both prospective and retrospective applications.

5.6 Authorship Contributions

NS collected and processed the DNA and RNA samples, analyzed the data and prepared the manuscript. VS collected samples. CW designed the probes for the capture panel, prepared samples and analyzed the data. NHS assisted with sample processing. ML, RRK and RMK assisted with NGS processing. AWS, DT, JF, PW and CHK provided bioinformatic assistance. SB conceptualized the paper, analyzed and collated data and reviewed the manuscript. All authors reviewed the manuscript and provided analytical support.

5.7 Disclosure of Conflicts of Interest

NS received travel and accommodation expenses from Novartis, Gilead, Amgen, and Janssen. TPH is a member of the advisory boards and has received research funding and honoraria from Novartis and Bristol-Myers Squibb. SB is a member of the advisory boards of Qiagen, Novartis and Cepheid, received honoraria from Qiagen, Novartis, Bristol-Myers Squibb, Incyte and Cepheid, and research funding from Novartis and Cepheid.

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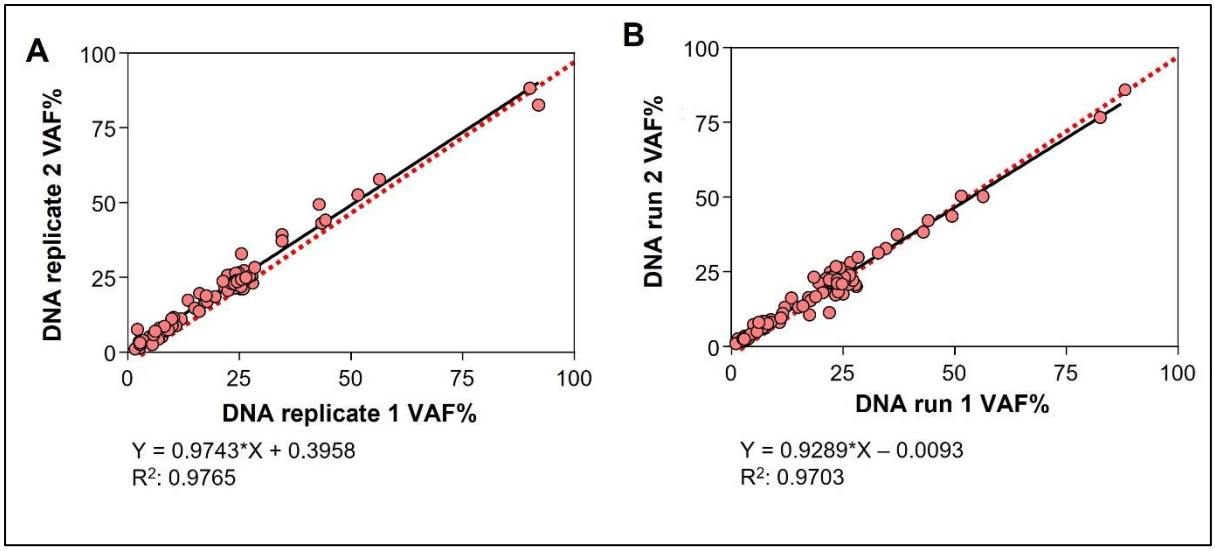
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Supplement

Supplemental Figures and Tables to Shanmuganathan N, Wadham C, Thomson D, et al. RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia. J Mol Diagn. 2022;24(7):802–822. (Chapter 5)

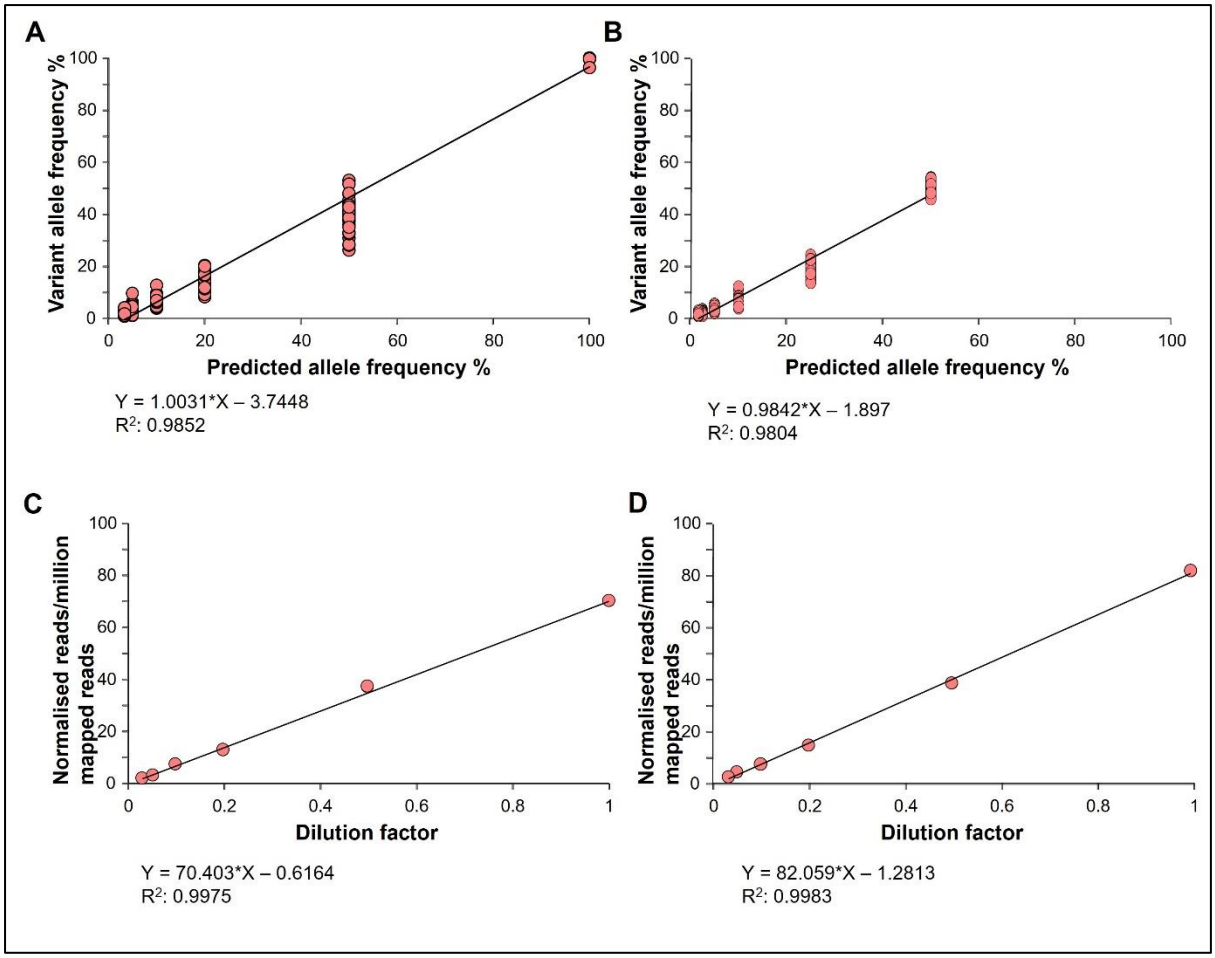
Supplementary Figures

Supplementary Figure 1: Representative examples of the precision analyses using DNA
Dashed red line has a slope of 1 and is used for comparison in all panels. Solid black line indicates the linear regression line. A. DNA intra-assay analysis: Comparison of VAFs of all clinically relevant SNVs and indels (n=91) between identical samples (replicate 1 vs replicate 2) run in duplicate within the same run (intra-assay repeatability). B. DNA inter-assay analysis: Comparison of VAFs of all matched clinically relevant variants (n=92) between identical samples on two different runs (run 1 vs run 2, inter-assay reproducibility).



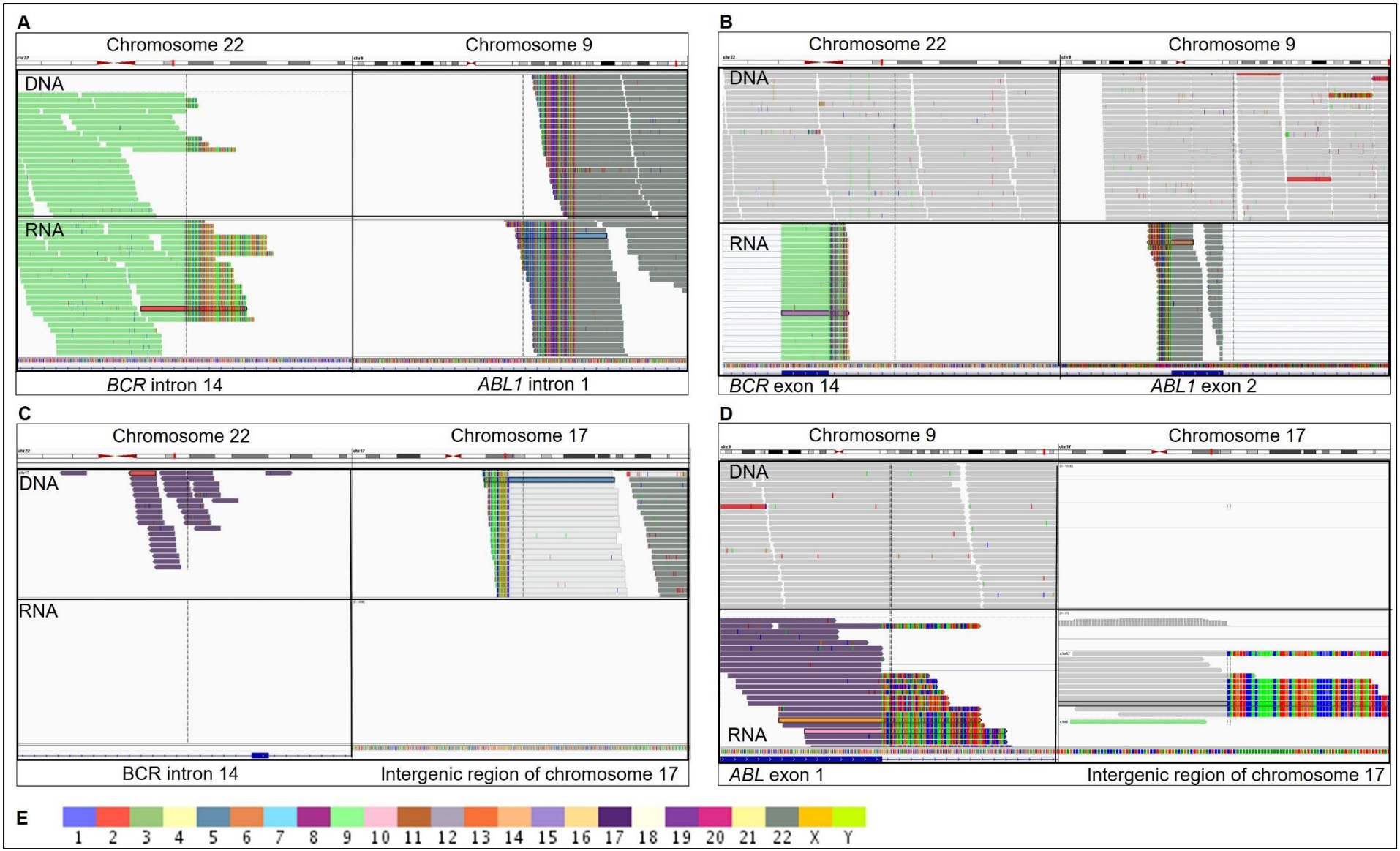
Supplementary Figure 2: Linearity and limit of detection analysis based upon the BV173 cell line dilution series.

Solid black line indicates the linear regression line. A. Linearity curves generated from the homozygous SNVs/indels. B. Linearity curves generated from heterozygous SNVs/indels; C. Linear decline of normalized read counts for the BCR::ABL1 genomic breakpoint. D. Linear decline of normalized read counts for the IKZF1 exon 2-3 deletion (95 Kb) across the dilution series



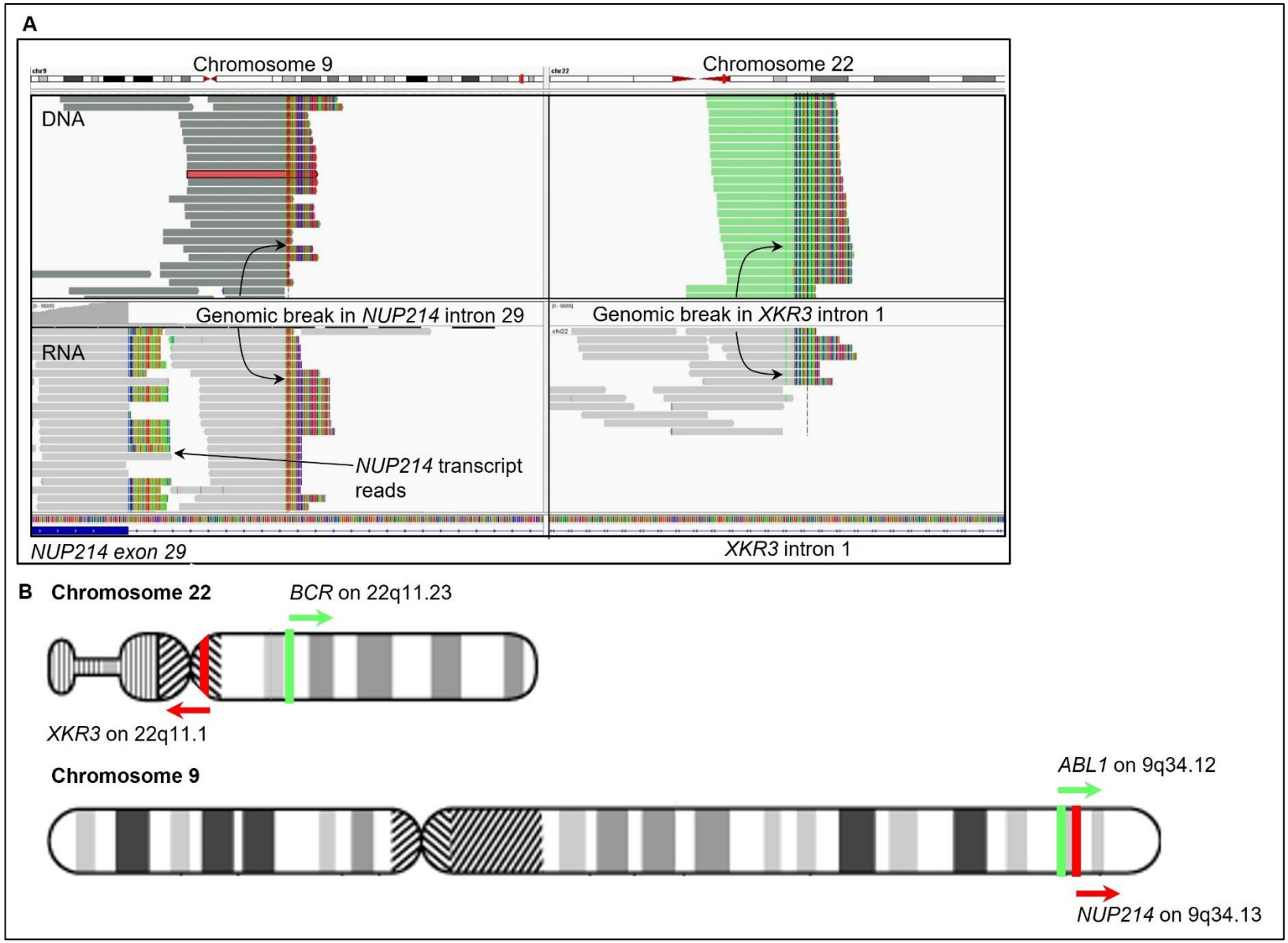
Supplementary Figure 3: Comparison between DNA and RNA in Pt1 for identification of the BCR::ABL1 fusion and Ph-associated rearrangements.

A. IGV screenshots showing the genomic breakpoints of the BCR::ABL1 fusion within intron 14 of BCR and intron 1 of ABL1, which were detectable using both DNA and RNA. Colored reads indicate where the chimeric reads derived from the corresponding fusion partner map: light green indicates reads derived from chromosome 9; and dark green for reads derived from chromosome 22. B. The e14a2 transcript was only detectable using RNA as observed by the fusion chimeric reads at the end of exon 14 of BCR and exon 2 of ABL1. C. The genomic breakpoints within BCR intron 14 and chromosome 17 were only observed using DNA and indicates that the genomic fusion does not lead to an expressed event. The chimeric reads arising from exon 1 of ABL1 map to chromosome 17 (dark purple). D. In contrast, a transcript formed by a fusion and inversion between exon 1 of ABL1 and an intergenic region within chromosome 17 was only detectable using RNA. E. Color code of the chromosomes in the Integrative Genomics Viewer.[34-35]


A
Chromosome 22
Chromosome 9
DNA
RNA
BCR intron 14
ABL1 intron 1
B
Chromosome 22
Chromosome 9
DNA
RNA
BCR exon 14
ABL1 exon 2
C
Chromosome 22
Chromosome 17
DNA
RNA
BCR intron 14
Intergenic region of chromosome 17
D
Chromosome 9
Chromosome 17
DNA
RNA
ABL exon 1
Intergenic region of chromosome 17
E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y

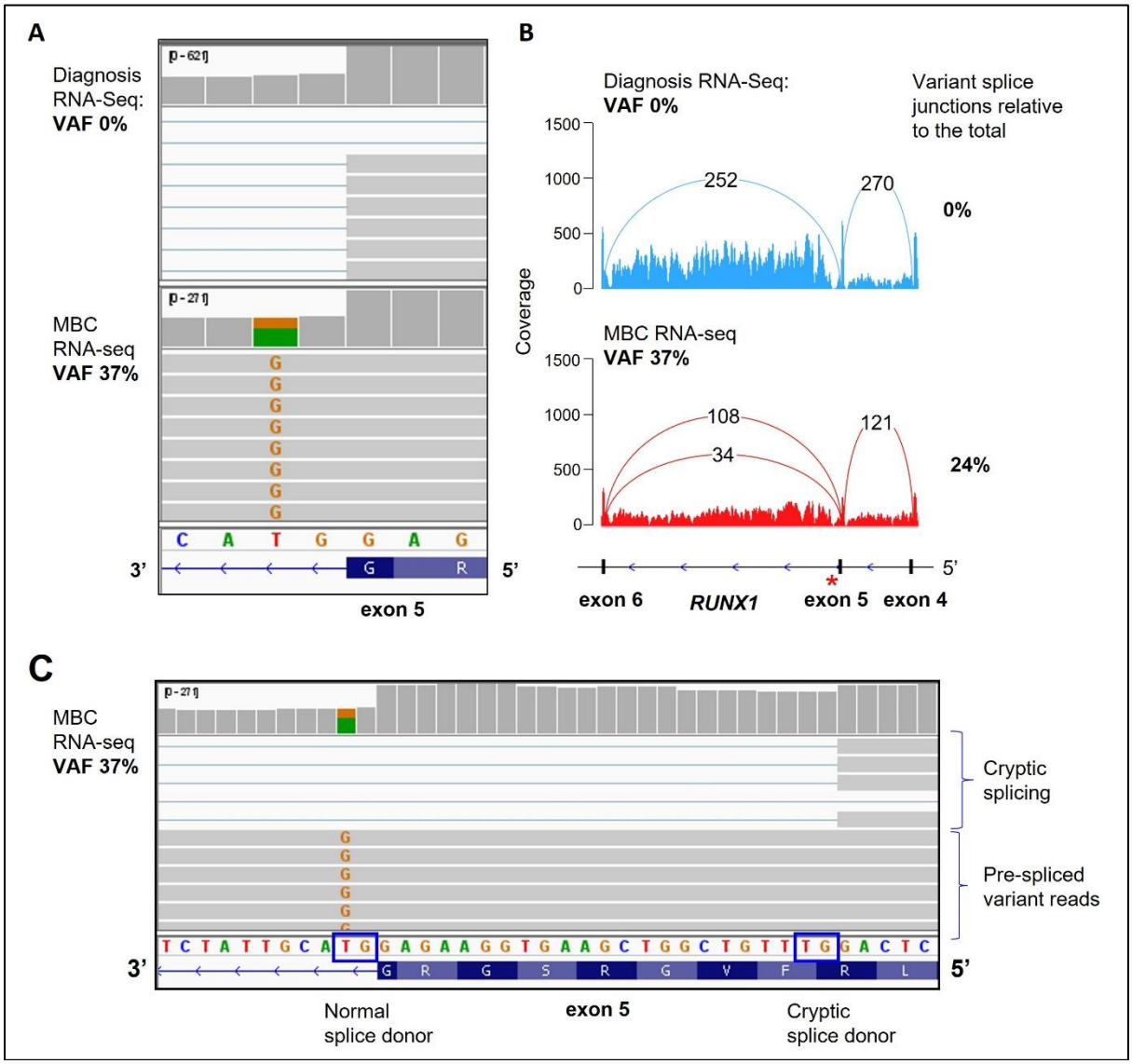
Supplementary Figure 4: Ph-associated rearrangement in K562 involving NUP214::XKR3

The 9;22 NUP214::XKR3 fusion was generated by a complex sequence of events which includes sequence fragmentation, inversion and random reassembly. XKR3 was not targeted by the capture panel but as the fusion partner NUP214 was, sequencing reads spanned the fusion breakpoint and mapped to the novel partner. Moreover, the genomic breakpoint of the NUP214 fusion was not specifically targeted but as it was located adjacent to the captured exon, it was identified. A. The genomic fusion between NUP214 on chromosome 9 and XKR3 on chromosome 22 were detectable using RNA and DNA. XKR3 is transcribed on the minus strand and reads are naturally in the antisense direction. The alignment of reads between NUP214 and XKR3 in the same transcriptional orientation could only occur due to an inversion. The corresponding fusion transcript was detected using RNA. B. Location of BCR and ABL1 relative to NUP214 and XKR3 on chromosomes 9 and 22. Arrows indicate direction of transcription.


A
Chromosome 9
Chromosome 22
DNA
Genomic break in NUP214 intron 29
Genomic break in XKR3 intron 1
RNA
NUP214 transcript reads
NUP214 exon 29
XKR3 intron 1
B
Chromosome 22
BCR on 22q11.23
XKR3 on 22q11.1
Chromosome 9
ABL1 on 9q34.12
NUP214 on 9q34.13

Supplementary Figure 5:Atypical splicing secondary to a RUNX1 splice donor variant in a historical patient sequenced using RNA-Seq

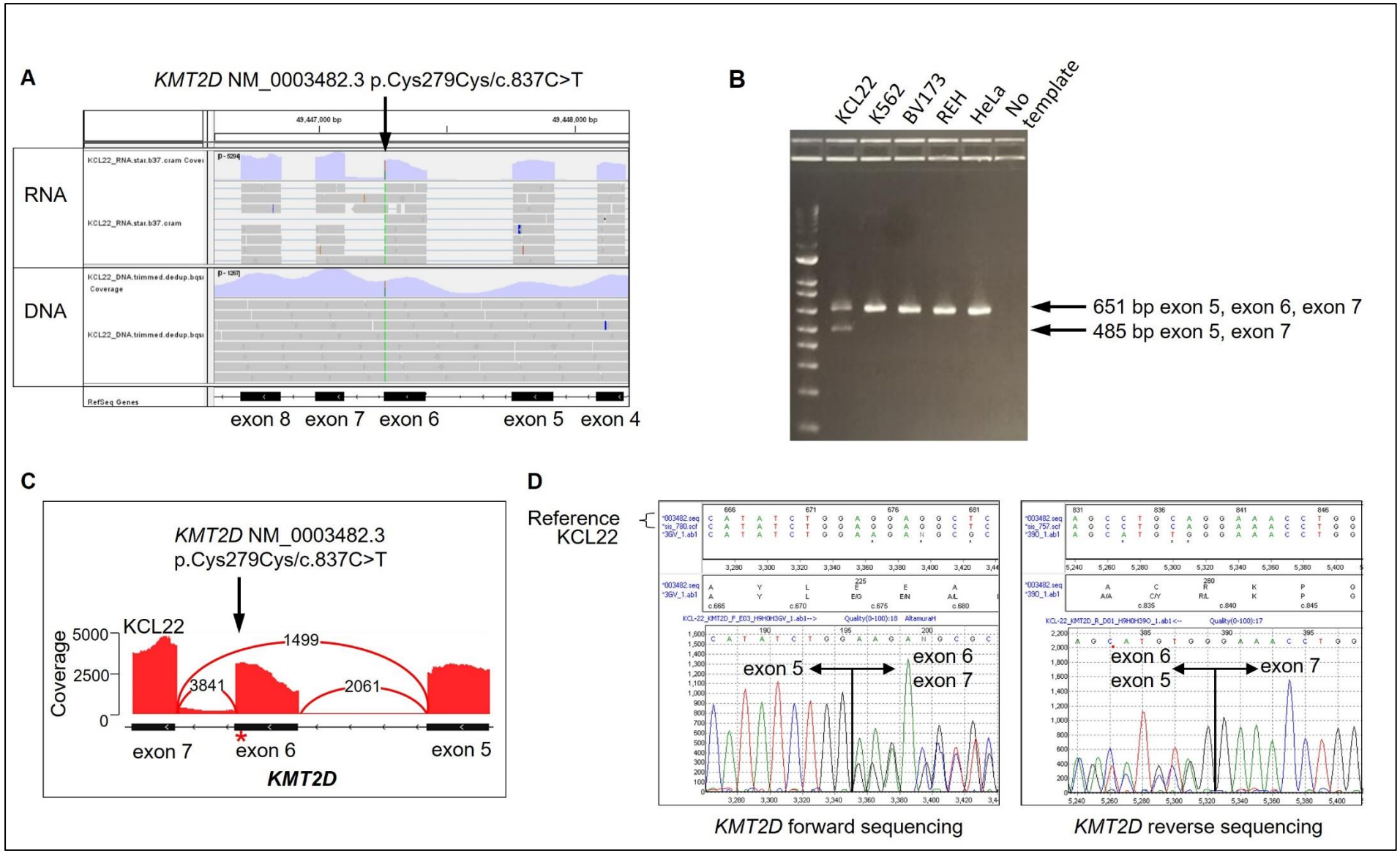
This historical patient[9] had the same RUNX1 splice donor variant (RUNX1 NM_001754.5:c.508+2T>C) as Pt2 that evolved at the time of myeloid blast crisis (MBC). A. IGV screenshot of the variant site at diagnosis and MBC. The variant was only detected at MBC with a VAF% of 37%. B. Sashimi plots revealed atypical splicing between RUNX1 exons 5 and 6 associated with the splice donor variant. The arcs represent splice junctions that connect exons and the number of splice junction reads are indicated. Atypical splicing was only present in the samples where the mutation was detected. The relative number of atypical splice junction reads correlated with the variant allele frequency of the mutant. C. Sequencing reads within exon 5 revealed activation of a cryptic splice donor site within exon 5 that excludes 23 nucleotides. The atypical splicing leads to a frameshift and premature termination. The same cryptic donor site was also utilized in Pt2.



Supplementary Figure 6: RT-PCR confirmation of exon 6 skipping associated with a KMT2D synonymous splice region variant in the KCL22 cell line

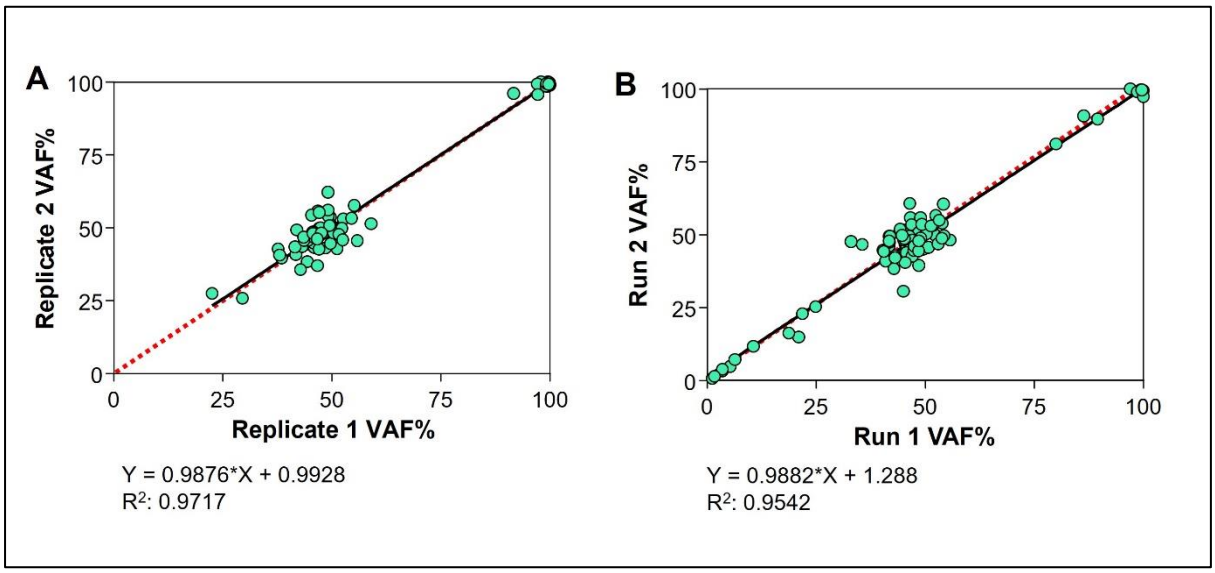
Novel skipping of exon 6 of the KMT2D gene in KCL22 was observed using RNA. The only variant located within the surrounding region, including introns 5 and 6 and exons 5, 6 and 7 was an exon 6 splice region synonymous variant Cys279Cys. This variant is absent from population databases but is listed once as a pathogenic somatic variant in COSMIC (COSV56418405). The nucleotide exchange occurred at the third base from the exon boundary. RT-PCR validated the splicing pattern observed by panel sequencing using RNA. KCL22 is a blast crisis CML cell line with a myeloid phenotype. Additional high impact variants were detected in cancer genes, including a second KMT2D variant (frameshift), frameshift variants in TP53, ASXL1 and CREBBP, and nonsense variants in CREBBP and CUX1. Interestingly, frameshift/nonsense variants in TP53 and ASXL1 were also detected in the 2 other CML myeloid blast crisis phenotype cell lines studied, K562 and LAMA84.

A. IGV screenshot of the region of KMT2D surrounding the skipped exon. The top panel is RNA and the bottom panel is DNA. High sequence coverage ranging from 180 to >1,800 reads within the intronic regions using DNA revealed a sole variant. B. RT-PCR using primers within exons 4 and 8 confirmed KCL22 novel splicing of KMT2D. C. Sashimi plot revealed exon 6 skipping using RNA targeted NGS sequencing. The red asterisk indicates the location of the variant. The blue splice junctions indicate normal splicing. D. Bulk PCR product Sanger sequencing confirmed exon 6 skipping.


A
KMT2D NM_0003482.3 p.Cys279Cys/c.837C>T
49,447,000 bp
49,448,000 bp
RNA
DNA
KCL22_RNA.star.b37.cram
KCL22_DNA.trimmed.dedup.bqsr
Coverage
RefSeq Genes
exon 8
exon 7
exon 6
exon 5
exon 4
B
KCL22
K562
BV173
REH
HeLa
No template
651 bp exon 5, exon 6, exon 7
485 bp exon 5, exon 7
C
KMT2D NM_0003482.3
p.Cys279Cys/c.837C>T
KCL22
Coverage
5000
2500
0
1499
3841
2061
exon 7
exon 6
exon 5
KMT2D
D
Reference
KCL22
exon 5
exon 6
exon 7
KMT2D forward sequencing
exon 6
exon 5
exon 7
KMT2D reverse sequencing

Supplementary Figure 7:Precision analysis using RNA

Dashed red line has a slope of 1 and is used for comparison in all panels. Solid black line indicates the linear regression line. A. Comparison of VAFs of all germline and clinically relevant variants (n=94) between identical samples (replicate 1 vs replicate 2) run in duplicate within the same panel run using RNA (intra-assay precision analysis); B. Comparison of VAFs of all matched germline and clinically relevant variants (n=87) between identical samples on two different runs (run 1 vs run 2, inter-assay precision).



Supplementary Tables

Supplementary Table 1: Captured genes with additional captured intronic regions

Blue text indicates the genes added to the final gene panel design
Genes included to detect fusion transcripts are indicated, including Ph-associated rearrangements on chromosomes 9 and 22

Genes included to detect RAG-mediated deletions or to aid their detection are indicated (Deletion)
Some genes are mutated by various mechanisms (including fusions, deletions and indels), such as *IKZF1, PAX5* and *RUNX1*

Gene captured	Chromosome	Comment
ABL1	9	Fusion
AFF1	4	Fusion
AFF4	7	Fusion
AIF1L	9	Fusion
AKT1	14	
ASS1	9	Fusion
ASXL1	20	
ATM	11	
BAP1	3	
BCOR	X	
BCORL1	X	
BCR	22	Fusion
BTG1	12	Deletion
BTK	X	
CABIN1	22	Fusion
CACNA1B	9	Fusion
CALR	5	
CBFB	16	Fusion
CBL	11	
CDKN2A	9	Deletion
CDKN2B	9	Deletion
CDKN2B-AS1	9	Deletion
CEBPA	19	
CREBBP	16	
CRLF2	X	Fusion
CSF3R	1	
CUX1	7	
DNMT3A	2	
EED	11	
ELL	19	Fusion
EP300	22	
ERG	21	Fusion
ETV6	12	Fusion
EXOSC2	9	Fusion
EZH2	7	

FAT1	4	
FAT3	5	
FBXW7	4	
FIGNL1	7	Deletion
FLT3	13	
FOCAD	9	Deletion
GATA2	3	
GGT1	22	Fusion
HBS1L	6	Deletion
HMCN2	9	Fusion
IDH1	2	
IDH2	15	
IKZF1	7	Deletion/Fusion
IL7R	5	
JAK1	1	
JAK2	9	
JAK3	19	
KAT6A	8	Fusion
KDM5A	12	Fusion
KDM6A	X	
KIT	4	
KMT2A	11	Fusion
KMT2D	12	
KRAS	12	
MBNL1	3	Fusion
MECOM	3	Fusion
MEF2D	1	Fusion
MICAL3	22	Fusion
MLLT1	19	Fusion
MLLT10	10	Fusion
MLLT3	9	Fusion
MLLT4	6	Fusion
MLLT6	17	Fusion
MPL	1	
MSI2	17	Fusion
MTAP	9	Deletion
MYB	6	Fusion
MYC	8	Fusion
MYH11	16	Fusion
MYH9	22	Fusion
NCOR1	17	
NEK6	9	Fusion
NF1	17	
NOTCH1	9	
NPDC1	9	Fusion
NPM1	19	
NRAS	1	

NSD2	4	Fusion
NT5C2	10	
NUP188	9	Fusion
NUP214	9	Fusion
NUP98	11	Fusion
PAX5	9	Deletion/Fusion
PDGFRB	5	Fusion
PHF6	X	
PPM1D	17	
PPM1F	22	Fusion
PTEN	10	
PTPN11	12	
RAD21	8	
RAD51	15	Fusion
RARA	17	Fusion
RB1	13	Deletion
RCBTB2	13	Deletion
RUNX1	21	Fusion
RUNX1T1	8	Fusion
SETBP1	18	
SETD1B	12	
SETD2	3	
SF3B1	2	
SH2B3	12	
SHANK3	22	Fusion
SMARCA4	19	
SMARCB1	22	Fusion
SMC1A	X	
SMC3	10	
SPECC1L	22	Fusion
SRSF2	17	
STAG2	X	
STAT5B	17	
SUZ12	17	Fusion
TCF3	19	Fusion
TET2	4	
TNRC6B	22	Fusion
TP53	17	
TXNRD2	22	Fusion
U2AF1	21	
UBE2A	X	
USP20	9	Fusion
VPREB1	22	Fusion
WT1	11	
XPO1	2	
ZCCHC7	9	Fusion
ZNF384	12	Fusion

ZRSR2	X	

Additional intergenic/intronic captured regions to detect specific focal deletions and the p210 *BCR-ABL1* genomic breakpoints

Chromosome	Start	End	Associated gene
22	23631623	23633120	*BCR* p210 breakpoint cluster region, intron 13, 14 and 15
13	48856820	48856971	*RB1*
13	48858675	48858990	*RB1*
13	48860525	48860748	*RB1*
13	48867360	48867576	*RB1*
13	48868330	48868511	*RB1*
13	48875705	48875934	*RB1*
13	48881095	48881272	*RB1*
13	48886125	48886222	*RB1*
13	48896420	48896614	*RB1*
13	48896685	48896876	*RB1*
13	48918535	48918754	*RB1*
13	48922545	48922716	*RB1*
13	48932310	48932530	*RB1*
13	48935915	48936052	*RB1*
13	48950005	48950129	*RB1*
13	48955670	48955842	*RB1*
13	48966980	48967229	*RB1*
13	48968650	48968728	*RB1*
13	48968760	48968835	*RB1*
13	48973245	48973461	*RB1*
13	48984480	48984709	*RB1*
13	48986460	48986668	*RB1*
13	48997285	48997403	*RB1*
13	49000080	49000301	*RB1*
13	49004490	49004606	*RB1*
13	49008150	49008251	*RB1*
13	49009995	49010172	*RB1*
13	49013975	49014106	*RB1*
13	49018115	49018301	*RB1*
13	49020730	49020954	*RB1*
13	49028130	49028314	*RB1*
13	49033545	49033721	*RB1*
13	49056460	49056659	*RB1*
13	49061545	49061761	*RB1*
13	49074900	49075083	*RB1*
21	36212278	36214100	*RUNX1*
6	135365878	135366126	*HBS1L-MYB* intergenic region
6	135366538	135366683	*HBS1L-MYB* intergenic region
6	135423673	135423800	*HBS1L-MYB* intergenic region

6	135423813	135423913	*HBS1L-MYB* intergenic region
6	135437513	135437758	*HBS1L-MYB* intergenic region
7	50168194	50168365	*IKZF1*
7	50345069	50345619	*IKZF1*
7	50345869	50346147	*IKZF1*
7	50346419	50347403	*IKZF1*
7	50347404	50347584	*IKZF1*
7	50350309	50350704	*IKZF1*
7	50351569	50351850	*IKZF1*
7	50352074	50352258	*IKZF1*
7	50352339	50352485	*IKZF1*
7	50352639	50352858	*IKZF1*
7	50354139	50354398	*IKZF1*
7	50359029	50359205	*IKZF1*
7	50363499	50363703	*IKZF1*
7	50412736	50413150	*IKZF1*
7	50416336	50416614	*IKZF1*
7	50418576	50418960	*IKZF1*
7	50419816	50420171	*IKZF1*
7	50423391	50423820	*IKZF1*
7	50424386	50424626	*IKZF1*
7	50427026	50427653	*IKZF1*
7	50429151	50429298	*IKZF1*
7	50429581	50429746	*IKZF1*
7	50431561	50431745	*IKZF1*
7	50432141	50432679	*IKZF1*
7	50433961	50434179	*IKZF1*
7	50434831	50435001	*IKZF1*
7	50435026	50435353	*IKZF1*
7	50438286	50438558	*IKZF1*
7	50440361	50441112	*IKZF1*
7	50441786	50442077	*IKZF1*
7	50442701	50442846	*IKZF1*
7	50445936	50446250	*IKZF1*
7	50446851	50447143	*IKZF1*
7	50457031	50457321	*IKZF1*
7	50458861	50459143	*IKZF1*
7	50461346	50461632	*IKZF1*
7	50461651	50461896	*IKZF1*
7	50463446	50463792	*IKZF1*
7	50465816	50466062	*IKZF1*
7	50471416	50471665	*IKZF1*
7	50471696	50471885	*IKZF1*
7	50472961	50473175	*IKZF1*
7	50474036	50474245	*IKZF1*
7	50477941	50478212	*IKZF1*
7	50481221	50481503	*IKZF1*

7	50483291	50483526	*IKZF1*
7	50483761	50484190	*IKZF1*
7	50484201	50484293	*IKZF1*
7	50484316	50484678	*IKZF1*
7	50486726	50486998	*IKZF1*
7	50489341	50489599	*IKZF1*
7	50496801	50497012	*IKZF1*
7	50517171	50517353	*IKZF1*
9	21827569	21827777	*CDKN2A/B*
9	21902059	21902240	*CDKN2A/B*
9	21967164	21967369	*CDKN2A/B*
9	21967864	21968006	*CDKN2A/B*
9	21970099	21970432	*CDKN2A/B*
9	21971944	21972040	*CDKN2A/B*
9	21975709	21975910	*CDKN2A/B*
9	21981199	21981481	*CDKN2A/B*
9	21982954	21983141	*CDKN2A/B*
9	21984224	21984437	*CDKN2A/B*
9	21985899	21986085	*CDKN2A/B*
9	21988794	21988930	*CDKN2A/B*
9	21990329	21990543	*CDKN2A/B*
9	21990909	21991257	*CDKN2A/B*
9	21991774	21991977	*CDKN2A/B*
9	21992704	21992866	*CDKN2A/B*
9	22008189	22008436	*CDKN2A/B*
9	36802954	36803129	*PAX5*
9	36806444	36806663	*PAX5*
9	36807204	36807457	*PAX5*
9	36808164	36808383	*PAX5*
9	36825269	36825476	*PAX5*
9	36828324	36828529	*PAX5*
9	36830839	36831052	*PAX5*
9	36831849	36832058	*PAX5*
9	36832159	36832387	*PAX5*
9	36833714	36833853	*PAX5*
9	36835399	36835581	*PAX5*
9	36839144	36839326	*PAX5*
9	36840879	36841009	*PAX5*
9	36841019	36841117	*PAX5*
9	36849184	36849360	*PAX5*
9	36854969	36855142	*PAX5*
9	36857259	36857485	*PAX5*
9	36857784	36857978	*PAX5*
9	36858969	36859189	*PAX5*
9	36859504	36859683	*PAX5*
9	36861964	36862234	*PAX5*
9	36863419	36863531	*PAX5*

9	36867249	36867458	*PAX5*
9	36868344	36868548	*PAX5*
9	36875204	36875425	*PAX5*
9	36876944	36877057	*PAX5*
9	36877384	36877594	*PAX5*
9	36880869	36880974	*PAX5*
9	36882489	36882701	*PAX5*
9	36885144	36885373	*PAX5*
9	36885659	36886042	*PAX5*
9	36892769	36892970	*PAX5*
9	36894534	36894743	*PAX5*
9	36895884	36896065	*PAX5*
9	36896184	36896398	*PAX5*
9	36911864	36912079	*PAX5*
9	36912169	36912361	*PAX5*
9	36913384	36913612	*PAX5*
9	36914019	36914232	*PAX5*
9	36914334	36914554	*PAX5*
9	36914559	36914777	*PAX5*
9	36921529	36921735	*PAX5*
9	36922184	36922408	*PAX5*
9	36925694	36925902	*PAX5*
9	36926149	36926353	*PAX5*
9	36928944	36929114	*PAX5*
9	36930509	36930715	*PAX5*
9	36931889	36932016	*PAX5*
9	36933854	36934064	*PAX5*
9	36942344	36942516	*PAX5*
9	36943559	36943675	*PAX5*
9	36945524	36945625	*PAX5*
9	36946769	36946970	*PAX5*
9	36947159	36947366	*PAX5*
9	36963449	36963659	*PAX5*
9	36967114	36967325	*PAX5*
9	36970584	36970967	*PAX5*
9	36970989	36971401	*PAX5*
9	36975164	36975378	*PAX5*
9	36977089	36977247	*PAX5*
9	36980524	36980744	*PAX5*
9	36981539	36981745	*PAX5*
9	36981779	36981997	*PAX5*
9	37007129	37007342	*PAX5*
9	37009124	37009305	*PAX5*
9	37011789	37012015	*PAX5*
9	37014584	37014752	*PAX5*
9	37018584	37018795	*PAX5*
9	37022529	37022711	*PAX5*

9	37022919	37023274	*PAX5*
9	37024539	37024751	*PAX5*
9	37024764	37024941	*PAX5*
9	37026559	37026787	*PAX5*
9	37027609	37027740	*PAX5*
9	37030544	37030792	*PAX5*
9	37032619	37032842	*PAX5*
9	37032844	37033002	*PAX5*
9	37033009	37033107	*PAX5*
9	37034614	37034798	*PAX5*
9	37042229	37042439	*PAX5*
9	37048214	37048379	*PAX5*
9	37052179	37052449	*PAX5*

Supplementary Table 2: DNA Sequencing coverage for each of 5 DNA runs to establish the gene panel performance characteristics

	Run 1		Run 2		Run 3		Run 4		Run 5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Reads	26,468,757	7,063,651	26,083,015	9,566,204	24,750,624	9,791,697	25,307,840	3,142,548	25,761,406	6,862,960
* %Reads	8.33	2.1	8.33	3.1	8.33	3.3	8.33	1	8.33	2.2
† Uniformity of coverage	95.3	1.5	97.5	0.7	92.3	2.1	86.8	0.9	97.4	2.2
Median Insert size	322	38.8	334	44	375	60	376	12	368	28
%Duplication	36	3.6	48	13	48	1.4	56	10	60	8
‡ Ts/Tv	2.47	0.09	2.42	0.04	2.5	0.06	2.41	0.05	2.43	0.08
§ Mean Read Depth	1391	364	1259	448	1182	176	912	291	749	187
‖ bp100x %	98.3	1.8	99.1	0.2	94.7	1.1	96.5	1.2	98.5	1.2
¶ %bp250x %	94.5	6.4	96.5	2.7	85.8	2.5	85.8	5.7	92.8	5
** % of reads mapping on target%	95.2	0.4	95	0.35	96.1	0.45	96.2	0.5	94.3	0.74
*** Chimeric alignments%	0.27	0.07	0.26	0.02	0.25	0.04	0.28	0.04	0.38	0.11

* %Reads: Mean percentage of reads per sample.

† Uniformity of Coverage: % of bases with coverage within 20% of the mean depth.

‡ Ratio of transitions to transversions: ideally 2-3.

§ Mean read depth of the samples in each run that were included in the validation study.

‖ bp100x, % of targeted bases that are covered by at least 100 unique reads for samples included in the validation study.

¶ bp250x, % of targeted bases covered by at least 250 unique reads for samples included in the validation study.

** Read enrichment: % of reads mapping on target for the samples included in the validation study.

*** Chimeric alignments: % of reads that cannot be represented as a linear alignment. High % >2 can be due to low quality of DNA.

Supplementary Table 3: Retrospective samples utilized for gene panel validation

Pre-characterized variants for the samples used to establish the panel performance characteristics from whole exome sequencing, RNA-Seq, High Density SNP Array and Sanger sequencing

Abbreviations

Pt = Patient
S = Sample
Chr = Chromosome
VAF = Variant allele frequency
WES = Whole exome sequencing
ND = Not detected
MBC = Myeloid blast crisis
LBC = Lymphoid blast crisis

† Variant detected in prior NGS studies but not reported as the VAF% was too low for Sanger confirmation

Note that the VAF for ABL1 kinase domain mutations called by Sanger sequencing refers to amplification of the single BCR-ABL1 mutated allele and the VAFs cannot be directly compared with values generated by the gene panel

A blank cell for VAF indicates that the DNA or RNA sample was not tested

											***BCR-ABL1* genomic breakpoints and Ph-associated rearrangements**				
Sample ID	**Disease phase**	**Analyses**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**VAF% pre-characterized using DNA**	**VAF% pre-characterized using RNA**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**
Pt1	LBC	1. Intra-assay repeatability: DNA	*BCORL1*	X	129147354	ENST00000218147	Frameshift	p.(Ile203Trpfs*52)/c.606_607insTGGGC	74	100	*BCR::ABL1*	*BCR*	22	23634010	
		2. Inter-assay reproducibility: DNA	*ABL1*	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.756G>C	26	29		*ABL1*	9	133641245	
		3. DNA versus RNA	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	10	19					
		4. Ph-associated rearrangement: DNA/RNA	*ABL1*	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	15 (Sanger)	15 (Sanger)	17q12::*BCR*	17q12	17	36315618	Ph-associated
		5. Recall rate of variants: DNA	*ABL1*	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.763G>A	20 (Sanger)	20 (Sanger)		*BCR*	22	23634101	inversion
		6. Recall rate of variants: RNA	*SMC3*	10	112361923	ENST00000361804	Nonsense	p.(Leu1031*)/c.3092T>A	7.5†	1.5†					
Pt2-S1	Germline	5. Recall rate of variants: DNA													
Pt2-S2	Diagnosis	1. Intra-assay repeatability: DNA	*ASXL1*	20	31024043	ENST00000375687	Frameshift	p.(Leu1177Profs*5)/c.3529dup	12	9	*BCR::ABL1*	*BCR*	22	23633088	
		2. Inter-assay reproducibility: DNA	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	17	4		*ABL1*	9	133704830	
		3. DNA vs RNA													
		5. Recall rate of variants: DNA									*ABL1::BCR*	*ABL1*	9	133704834	
		6. Recall rate of variants: RNA										*BCR*	22	23633094	
		7. Inter-assay reproducibility: RNA													
Pt2-S3	MBC - 1st	1. Intra-assay repeatability DNA/RNA	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	52		*BCR::ABL1*	*BCR*	22	23633088	
		2. Inter-assay reproducibility DNA	*U2AF1*	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	51			*ABL1*	9	133704830	
		3. DNA vs RNA	*PHF6*	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	91						
		5. Recall rate of variants: DNA	*RUNX1*	21	36252852	ENST00000300305	Splice donor	c.508+2T>C	41		*ABL1::BCR*	*ABL1*	9	133704834	
		6. Recall rate of variants: RNA										*BCR*	22	23633094	
Pt2-S4	MBC - 2nd	6. Recall rate of variants: RNA	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T		31	*BCR::ABL1*	*BCR*	22	23633088	
			U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G		33		*ABL1*	9	133704830	
			PHF6	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A		75					
			RUNX1	21	36252852	ENST00000300305	Splice donor	c.508+2T>C		6	*ABL1::BCR*	*ABL1*	9	133704834	
												BCR	22	23633094	
Pt3-S1	Diagnosis	3. DNA versus RNA									*BCR::ABL1*	*BCR*	22	23632899	
		5. Recall rate of variants: DNA										*ABL1*	9	133597820	
		6. Recall rate of variants: RNA													
											ABL1::BCR	*ABL1*	9	133597823	
												BCR	22	23632899	
Pt3-S2	LBC - 2nd	6. Recall rate of variants: RNA	*ABL1*	9	133755488	ENST00000318560	Missense	p.(Phe486Ser)/c.1514T>C	32		*BCR::ABL1*	*BCR*	22	23632899	
			ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>T	57			*ABL1*	9	133597820	
			ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>C	10						
			RUNX1	21	36212758	ENST00000300305	Splice variant	c.614-5860G>T	44						
Pt3-S3	LBC - 3rd	6. Recall rate of variants: RNA	*ABL1*	9	133755488	ENST00000318560	Missense	p.(Phe486Ser)/c.1514T>C		100	*BCR::ABL1*	*BCR*	22	23632899	
		This sample was only pre-characterized for *BCR-ABL1* kinase domain mutations	*ABL1*	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>C		40		*ABL1*	9	133597820	
			ABL1	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.821A>T		75	Data from WES and RNA-seq analysis at diagnosis of CML				
			ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.813G>C		20					

											***BCR-ABL1* genomic breakpoints and Ph-associated rearrangements**				
Sample ID	**Disease phase**	**Analyses**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**VAF% pre-characterized using DNA**	**VAF% pre-characterized using RNA**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**
Pt4	CP	3. DNA versus RNA	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	21	51	*BCR::ABL1*	*BCR*	22	23634569	
	treatment	4. Ph-associated rearrangement: DNA/RNA	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	20	19		*ABL1*	9	133615099	
	failure	5. Recall rate of variants: DNA													
		6. Recall rate of variants: RNA									7q34::*BCR*	intergenic *TRBV12-1*	7	142221314	Ph-associated
												and *TRBV11* -1-i14			inversion
												BCR	22	23634640	
Pt5-S1	Diagnosis	5. Recall rate of variants: DNA									*BCR::ABL1*	*BCR*	22	23633455	
												ABL1	9	133685776	
Pt5-S2	LBC	3. DNA vs RNA	*ABL1*	X	129155104	ENST00000318560	Missense	p.(Gly250Glu)/c.749G>A	65	82	*BCR::ABL1*	*BCR*	22	23633455	
		5. Recall rate of variants: DNA	*BCORL1*	9	133738349	ENST00000218147	Missense	p.(Arg1196*)/c.3586C>T	100	95		*ABL1*	9	133685776	
		6. Recall rate of variants: RNA	*RUNX1*	21	36231860	ENST00000300305	Missense	p.(Leu175Gln)/c.524T>A	44	31					
		7. Inter-assay reproducibility: RNA	*RUNX1*	21	36231855	ENST00000300305	Frameshift	p.(Ile177Serfs*34)/c.528del	41	25					
Pt6	LBC	4. Ph-associated rearrangement: DNA	*ABL1*	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	13		*BCR::ABL1*	*BCR*	22	23633471	
		5. Recall rate of variants: DNA	*XPO1*	2	61719472	ENST00000401558	Missense	p.(Glu571Lys)/c.1711G>A	32			*ABL1*	9	133631569	
											PPM1F::SPECC1L	*PPM1F*	22	22304097	Ph-associated
												SPECC1L	22	24667107	inversion
											SPECC1L::TXNRD2	*SPECC1L*	22	24667004	Ph-associated
												TXNRD2	22	19909523	inversion
											COMT::NUP188	*COMT*	22	19931280	Ph-associated
												NUP188	9	131729274	inversion
Pt7	Diagnosis	4. Ph-associated rearrangement: DNA	*SETD1B*	12	122243806	ENST00000267197	Frameshift	p.(Ile114Asnfs*3)/c.340dup	18	17	*BCR::* q34.12	*BCR*	22	23632349	Ph-associated
		5. Recall rate of variants: DNA										Intergenic (5' of *ABL1*)	9	133583846	
											q34.12::*BCR*	Intergenic (5' of *ABL1*)	9	133583294	Ph-associated
												BCR	22	23632546	inversion
											BCR inversion	*BCR*	22	23632356	Ph-associated
												BCR	22	23632512	inversion
Pt8-S1	Diagnosis	5. Recall rate of variants: DNA									*BCR::ABL1*	*BCR*	22	23635575	
												ABL1	9	133698732	
											ABL1::BCR	*ABL1*	9	133698704	
												BCR	22	23634612	
Pt8-S2	LBC	5. Recall rate of variants: DNA	*BCORL1*	X	129149315	ENST00000218147	Frameshift	p.(Ala858Glyfs*29)/c.2572dup	51	57	*BCR::ABL1*	*BCR*	22	23635575	
			ABL1	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	34	76		*ABL1*	9	133698732	
											ABL1::BCR	*ABL1*	9	133698704	
												BCR	22	23634612	
Pt9	LBC	5. Recall rate of variants: DNA	*ABL1*	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.820G>A		53	*BCR::ABL1*	*BCR*	22	23634205	
			ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.813G>C		10		*ABL1*	9	133697850	
			ATM	11	108225537	ENST00000278616	Splice acceptor	c.8787-1G>A		76					
											ABL1::BCR	*ABL1*	9	133697843	
												BCR	22	23634216	

											***BCR-ABL1* genomic breakpoints and Ph-associated rearrangements**				
Sample ID	**Disease phase**	**Analyses**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**VAF% pre-characterized using DNA**	**VAF% pre-characterized using RNA**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**
Pt10	MBC	5. Recall rate of variants: DNA	*ASXL1*	20	31023300	ENST00000375687	Nonsense	p.(Glu929*)/c.2785G>T		14	*BCR::NUP214*	*BCR*	22	23665088	Ph-associated
			IDH1	2	209113113	ENST00000415913	Missense	p.(Arg132Cys)/c.394C>T		46		*NUP214*	9	134050572	
			RUNX1	21	36164627	ENST00000300305	Frameshift	p.(Phe416Leufs*185)/c.1244_1247dup		32					
											NUP214::BCR	*NUP214*	9	134050563	Ph-associated
												BCR	22	23657072	inversion
											NUP214::ABL1	*NUP214*	9	134051424	Ph-associated
												ABL1	9	133644990	
											BCR	*BCR*	22	23655029	*BCR* intergenic
													22	23679451	inversion
Pt11	MBC	5. Recall rate of variants: DNA	*ABL1*	9	133748414	ENST00000318560	Missense	p.(Phe378Val)/c.1132T>G		74	*BCR::ABL1*	*BCR*	22	23633790	
			ASXL1	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup		21		*ABL1*	9	133697671	
Pt12	Diagnosis	4. Ph-associated rearrangement: RNA									*BCR::ABL1* genomic fusion was not detected				
		6. Recall rate of variants: RNA													
											BCR::NFIX	*BCR*	22	23633428	Ph-associated
												NFIX	19	13113890	
Pt13	Diagnosis	4. Ph-associated rearrangement: RNA	*ASXL1*	20	31022592	ENST00000375687	Nonsense	p.(Arg693*)/c.2077C>T	40	45	*BCR::ABL1*	*BCR*	22	23631881	
		6. Recall rate of variants: RNA										*ABL1*	9	133730113	
											TNRC6B::NEK6	*TNRC6B*	22	40580457	Ph-associated
												NEK6	9	127114639	
											URM1::GGT1	*URM1*	22	25003751	Ph-associated
												GGT1	9	131150170	inversion
											ST13:: 9q34.11	*ST13*	22	41247301	Ph-associated
												Intergenic 5′ of *ASS1*	9	133313307	inversion
Pt14	Diagnosis	4. Ph-associated rearrangement: RNA									*BCR::ABL1*	*BCR*	22	23632144	
		6. Recall rate of variants: RNA										*ABL1*	9	133708168	
											ABL1::BCR	*ABL1*	9	133707916	Ph-associated
												BCR	22	23636599	inversion
											MYH9::BCR	*MYH9*	22	36703827	Ph-associated
												BCR	22	23636274	inversion
Pt15	Diagnosis	6. Recall rate of variants: RNA									*BCR::ABL1*	*BCR*	22	23633758	
												ABL1	9	133680224	
											ABL1::BCR	*ABL1*	9	133680221	
												BCR	22	23633758	
Pt16	Accelerated phase	6. Recall rate of variants: RNA (Sanger only)	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T		75 (Sanger)	*BCR::ABL1*	*BCR*	22	23633152	
		8. Intra-assay repeatability:RNA										*ABL1*	9	133589720	
											Data from RNA-seq analysis at diagnosis of CML				

											***BCR-ABL1* genomic breakpoints and Ph-associated rearrangements**				
Sample ID	**Disease phase**	**Analyses**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**VAF% pre-characterized using DNA**	**VAF% pre-characterized using RNA**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**
Pt17	Diagnosis	6. Recall rate of variants: RNA									*BCR::ABL1*	*BCR*	22	23634297	
												ABL1	9	133592378	
Pt18	Diagnosis	6. Recall rate of variants: RNA													
Pt19	Diagnosis	6. Recall rate of variants: RNA									*BCR::ABL1*	*BCR*	22	23631991	
												ABL1	9	133614844	
Pt20	LBC	6. Recall rate of variants: RNA	*ABL1*	9	133750330	ENST00000318560	Missense	p.(Leu387Phe)/c.1218G>T		48	*BCR::ABL1*	*BCR*	22	23634692	
			IDH2	15	90631934	ENST00000330062	Missense	p.(Arg140Gln)/c.419G>A		41		*ABL1*	9	133719753	
											ABL1::BCR	*ABL1*	9	133719747	
												BCR	22	23634686	
Pt21	MBC	6. Recall rate of variants: RNA	*GATA2*	3	128200116	ENST00000341105	Frameshift	p.(Asn397Thrfs*80)/c.1188del	52	77	*BCR::ABL1*	*BCR*	22	23633027	
			GATA2	3	128200766	ENST00000341105	In-frame deletion	p.(Ala341_Gly346del)/c.1021_1038del	28	42		*ABL1*	9	133652394	
			TET2	4	106180795	ENST00000540549	Missense	p.(Gly1275Arg)/c.3823G>C	ND	15					
Myeloid mutation DNA Mix		5. Recall rate of variants: DNA	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	10						
			ASXL1	20	31022402	ENST00000318560	Frameshift	p.(Gly634Glnfs*14)/c.1900_1922del	10						
			ASXL1	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	10						
			CALR	19	13054564	ENST00000316448	Frameshift	p.(Leu367Thrfs*46)/c.1092_1143del	5						
			CBL	11	119148919	ENST00000264033	Missense	p.(Arg420Gln)/c.1259G>A	10						
			CBL	11	119149251	ENST00000264033	Missense	p.(Leu380Pro)/c.1139T>C	10						
			CEBPA	19	33792381	ENST00000498907	Frameshift	p.(His24Alafs*84)/c.68_69insC	15						
			CEBPA	19	33793252	ENST00000498907	Frameshift	p.(Lys313dup)/c.939_940insAAG	15						
			CSF3R	1	36933434	ENST00000373103	Missense	p.(Thr618Ile)/c.1853C>T	5						
			FLT3	13	28608300	ENST00000241453	Frameshift	p.(Asn587_Asp600dup)/c.1759_1800dup	5						
			FLT3	13	28608250	ENST00000241453	Frameshift	p.(Lys602_Trp603insGlyAlaPheArgGluTyrGluT	10						
			FLT3	13	28592642	ENST00000241453	Missense	p.(Asp835Tyr)/c.2503G>T	10						
			IDH1	2	209113113	ENST00000415913	Missense	p.(Arg132Cys)/c.394C>T	5						
			JAK2	9	5073770	ENST00000381652	Missense	p.(Val617Phe)/c.1849G>T	5						
			JAK2	9	5070033	ENST00000381652	Inframe	p.(Asn542_Glu543del)/c.1624_1629del	10						
			MPL	1	43815009	ENST00000372470	Missense	p.(Trp515Leu)/c.1544G>T	5						
			NPM1	5	170837547	ENST00000296930	Frameshift	p.(Trp288Cysfs*12)/c.863_864dup	5						
			SF3B1	2	198266834	ENST00000335508	Missense	p.(Lys700Glu)/c.2098A>G	5						
			SF3B1	2	198267359	ENST00000335508	Missense	p.(Lys666Asn)/c.1998G>T	5						
			SRSF2	17	74732935	ENST00000392485	Frameshift	p.(Pro95_R102del)/c.284_307del	5						
			U2AF1	21	44524456	ENST00000291552	Missense	p.(Ser34Phe)/c.101C>T	10						

		Genomic breakpoints of focal deletions and other rearrangements						Additional fusion transcripts detectable only using RNA			
Sample ID	**Disease phase**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Fusion**	**Chr**	**Transcript type**	**Comment**
Pt1	LBC	*CLIC6::RUNX1*	*CLIC6*	21	36080470	34	deletion	*BCR::ABL1*	22 and 9	e14e2	
			RUNX1	21	36421160						
		HBS1L::MYB intergenic locus	*HBS1L*	6	135366696	71	deletion	*ABL1::* 17q12	9 and 17	e1-intergenic	Ph-associated inversion
			Intergenic	6	135437658						
								IKZF1:: 2p11.2	7 and 2	e1-intergenic IGKV3-7 and IGKV1-8	
		IKZF1::IGKV3-7	*IKZF1*	7	50348584		interchromosomal fusion				
			IGKV3-7	2	89277898						
		IGKV3-7::IKZF1	*IGKV3-7*	2	89277981		reciprocal interchromosomal fusion				
			IKZF1	7	50348584						
Pt2-S1	Germline										
Pt2-S2	Diagnosis							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt2-S3	MBC - 1st	*IKZF1*	*IKZF1*	7	50412895-50463650	51	Exon 4 to 7 deletion (predominant)	*BCR::ABL1*	22 and 9	e14e2	
		IKZF1	*IKZF1*	7	50412895-50463630	116	Exon 4 to 7 deletion (minor)	*ABL1::BCR*	9 and 22	e1e15	
Pt2-S4	MBC - 2nd	*IKZF1*	*IKZF1*	7	50347391-50463629	116	Exon 2 to 7 deletion	*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
								CBFB::MYH11	16	e5e34	Inversion
Pt3-S1	Diagnosis							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt3-S2	LBC - 2nd	*IKZF1*	*IKZF1*	7	50412884-50463636	51	Exon 4 to 7 deletion	*BCR::ABL1*	22 and 9	e14e2	
		HBS1L::MYB intergenic locus	*HBS1L*	6	135366118	72	intergenic deletion	*ABL1::BCR*	9 and 22	e1e15	
			Intergenic	6	135437668						
Pt3-S3	LBC - 3rd							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	

		Genomic breakpoints of focal deletions and other rearrangements						Additional fusion transcripts detectable only using RNA			
Sample ID	**Disease phase**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Fusion**	**Chr**	**Transcript type**	**Comment**
Pt4	CP							*BCR::ABL1*	22 and 9	e13e2 and e14e2	
	treatment										
	failure							*ABL1::TRBV5-5*	9 and 7	e1-within *TRBV5-5*	Ph-associated inversion
Pt5-S1	Diagnosis										
Pt5-S2	LBC	*IKZF1*	*IKZF1*	7	50412896-50463633	51	Exon 4 to 7 deletion	*BCR::ABL1*	22 and 9	e14e2	
		HBS1L::MYB intergenic locus	*HBS1L*	6	ND	65	Deletion				
			Intergenic	6	ND						
Pt6	LBC							*BCR::ABL1*	22 and 9	e13e2 and e14e2	
								PPM1F::SPECC1L	22	e1e2	Ph-associated inversion
								SPECC1L::TXNRD2	22	e1e3	Ph-associated inversion
Pt7	Diagnosis							*BCR::ABL1*	22 and 9	e13a2	
								ABL1:: 17q12	9 and 22	e1-intergenic	Ph-associated inversion
Pt8-S1	Diagnosis							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	

		Genomic breakpoints of focal deletions and other rearrangements						Additional fusion transcripts detectable only using RNA			
Sample ID	**Disease phase**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Fusion**	**Chr**	**Transcript type**	**Comment**
Pt8-S2	LBC							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt9	LBC	*IKZF1*	*IKZF1*	7	50412878-50463632	51	Exon 4 to 7 deletion	*BCR::ABL1*	22 and 9	e14e2	
		CDKN2A/B	*CDKN2A/B*	9	21827667-22008305	181	Deletion of *CDKN2A/B* extending into adjacent *MTAP* gene	*ABL1::BCR*	9 and 22	e1e15	
Pt10	MBC							*BCR::ABL1*	22 and 9	e19e2	
Pt11	MBC							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt12	Diagnosis							*BCR::ABL1*	22 and 9	e13e2 and e14e2	
								ABL1::LLGL2	9 and 17	e1e2	Ph-associated

		Genomic breakpoints of focal deletions and other rearrangements						Additional fusion transcripts detectable only using RNA			
Sample ID	**Disease phase**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Fusion**	**Chr**	**Transcript type**	**Comment**
Pt13	Diagnosis							*BCR::ABL1*	22 and 9	e13e3	
								TNRC6B::NEK6	22 and 9	e4e10	Ph-associated
								ST13::ASS1	22 and 9	e1e2	Ph-associated
											Inversion
Pt14	Diagnosis							*BCR::ABL1*	22 and 9	e13e2	
								BCR::ABL1	22 and 9	e14-intron 1	Ph-associated
											Inversion
								MYH9::BCR	22	e15e16	Ph-associated
											Inversion
Pt15	Diagnosis							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt16	Accelerated phase							*BCR::ABL1*	22 and 9	e14e2	
Pt17	Diagnosis							*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt18	Diagnosis							*BCR::ABL1*	22 and 9	e13e2	
								ABL1::BCR	9 and 22	e1e15	
Pt19	Diagnosis							*BCR::ABL1*	22 and 9	e13e2	
								ABL1::BCR	9 and 22	e1e14	

		Genomic breakpoints of focal deletions and other rearrangements						Additional fusion transcripts detectable only using RNA			
Sample ID	**Disease phase**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Fusion**	**Chr**	**Transcript type**	**Comment**
Pt20	LBC	*CDKN2A/B*	*CDKN2A/B*	9	21902166-22008313	106	Deletion of *CDKN2A/B*	*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Pt21	MBC	*RUNX1*	*RUNX1*	21	36392146-36201259	191	Exon 3 - 7 deletion	*BCR::ABL1*	22 and 9	e14e2	
								ABL1::BCR	9 and 22	e1e15	
Myeloid mutation DNA Mix											

Supplementary Table 4: Recall rate of variants using DNA
Additional variants detected using the gene panel are indicated in blue text
DNA panel results shaded in red

Abbreviations
Pt = Patient
S = Sample
Chr = Chromosome
NC = Not called
ND = Not detected
MBC = Myeloid blast crisis
LBC = Lymphoid blast crisis

† Variant detected in prior NGS studies but not reported as the VAF% was too low for Sanger confirmation
‡ Breakpoints were incidentally detected as the panel was not designed to detect BCR-ABL1 e19a2 genomic breaks and was only detected as these events were located adjacent to a captured region
§ RNA-seq data was compared with the panel data generated using DNA to verify variant detection
‖ ATM was not included in the original probe design and was not evaluable for variant detection
¶ Confirmed using mass spectrometry

Note that the VAF for ABL1 kinase domain mutations called by Sanger sequencing refers to amplification of the single BCR-ABL1 mutated allele and the VAFs cannot be directly compared with values generated by the gene panel

Sample ID	Disease phase	Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Expected variants	Pre-characterized VAF%	Gene panel VAF%	Fusion	Gene	Chr	Genomic coordinates (hg19)	Comment	Detected by gene panel
Pt1	LBC	*BCORL1*	X	129147354	ENST00000218147	Frameshift	p.(Ile203Trpfs*52)/c.606_607insTGGGC	74	82.6	*BCR::ABL1*	*BCR*	22	23634010		Yes
		ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.756G>C	26	18.9		*ABL1*	9	133641245		
		ABL1	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	10	11.1						
		ABL1	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	15 (Sanger)	4.3	17q12::*BCR*	17q12	17	36315618	Ph-associated	No
		ABL1	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.763G>A	20 (Sanger)	7.4		*BCR*	22	23634101	inversion	
		SMC3	10	112361923	ENST00000361804	Nonsense	p.(Leu1031*)/c.3092T>A	7.5†	5.8						
		ABL1	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	ND	1.6¶	17q12::*BCR*	Intergenic	17	36315503	Ph-associated	Additional
		ABL1	9	133738356	ENST00000318560	Missense	p.(Phe359Val)/c.10757>G	ND	1.1¶		*BCR*	22	23634047	inversion	
		EZH2	7	148506461	ENST00000320356	Missense	p.(Arg684His)/c.2051G>A	ND	2.6						
										ABL1:: 17q12	*ABL1*	9	133641030	Ph-associated	Additional
											Intergenic	17	36314782	inversion	
Pt2-S1	Germline														
Pt2-S2	Diagnosis	*ASXL1*	20	31024043	ENST00000375687	Frameshift	p.(Leu1177Profs*5)/c.3529dup	12	8.2	*BCR::ABL1*	*BCR*	22	23633088		Yes
		SETD1B	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	17	6.2		*ABL1*	9	133704830		
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	ND	2.5						
										ABL1::BCR	*ABL1*	9	133704834		Yes
											BCR	22	23633094		
Pt2-S3	MBC - 1st	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	52	49	*BCR::ABL1*	*BCR*	22	23633088		Yes
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	51	44		*ABL1*	9	133704830		
		PHF6	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	91	88						
		RUNX1	21	36252852	ENST00000300305	Splice donor	c.508+2T>C	41	37	*ABL1::BCR*	*ABL1*	9	133704834		Yes
											BCR	22	23633094		
Pt3-S1	Diagnosis	*RUNX1*	21	36212758	ENST00000300305	Intronic splice altering	c.614-5860G>T	ND	9	*BCR::ABL1*	*BCR*	22	23632899		Yes
											ABL1	9	133597820		
										ABL1::BCR	*ABL1*	9	133597823		Yes
											BCR	22	23632899		
Pt4	CP	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	21	25	*BCR::ABL1*	*BCR*	22	23634569		Yes
	treatment failure	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	20	28		*ABL1*	9	133615099		
										7q34::*BCR*	intergenic *TRBV12-1*	7	142221314	Ph-associated	Yes
											and *TRBV11-1* -i14			inversion	
											BCR	22	23634640		
Pt5-S1	Diagnosis									*BCR::ABL1*	*BCR*	22	23633455		Yes
											ABL1	9	133685776		
										ABL1::BCR	*ABL1*	9	133685777		Additional
											BCR	22	23633458		
Pt5-S2	LBC	*ABL1*	9	133738349	ENST00000318560	Missense	p.(Gly250Glu)/c.749G>A	65	55	*BCR::ABL1*	*BCR*	22	23633455		Yes
		BCORL1	X	129155104	ENST00000218147	Nonsense	p.(Arg1196*)/c.3586C>T	100	100		*ABL1*	9	133685776		
		RUNX1	21	36231860	ENST00000300305	Missense	p.(Leu175Gln)/c.524T>A	44	45						
		RUNX1	21	36231855	ENST00000300305	Frameshift	p.(Ile177Serfs*34)/c.528del	41	47	*ABL1::BCR*	*ABL1*	9	133685778		Additional
											BCR	22	23633457		

Sample ID	Disease phase	Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Expected variants	Pre-characterized VAF%	Gene panel VAF%	Fusion	Gene	Chr	Genomic coordinates (hg19)	Comment	Detected by gene panel
Pt6	LBC	*ABL1*	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	13	18	*BCR::ABL1*	*BCR*	22	23633471		Yes
		XPO1	2	61719472	ENST00000401558	Missense	p.(Glu571Lys)/c.1711G>A	32	18		*ABL1*	9	133631569		
										PPM1F::SPECC1L	*PPM1F*	22	22304097	Ph-associated	No
											SPECC1L	22	24667107	inversion	
										SPECC1L::TXNRD2	*SPECC1L*	22	24667004	Ph-associated	Yes
											TXNRD2	22	19909523	inversion	
										COMT::NUP188	*COMT*	22	19931280	Ph-associated	No
											NUP188	9	131729274	inversion	
Pt7	Diagnosis	*SETD1B*	12	122243806	ENST00000267197	Frameshift	p.(Ile114Asnfs*3)/c.340dup	18	17	*BCR* ::q34.12	*BCR*	22	23632349	Ph-associated	Yes
											Intergenic (5' of *ABL1*)	9	133583846		
										q34.12::*BCR*	Intergenic (5' of *ABL1*)	9	133583294	Ph-associated	Yes
											BCR	22	23632546	inversion	
										BCR inversion	*BCR*	22	23632356	Ph-associated	Yes
											BCR	22	23632512	inversion	
Pt8-S1	Diagnosis									*BCR::ABL1*	*BCR*	22	23635575		Yes
											ABL1	9	133698732		
										ABL1::BCR	*ABL1*	9	133698704		Yes
											BCR	22	23634612		
Pt8-S2	LBC	*BCORL1*	X	129149315	ENST00000218147	Frameshift	p.(Ala858Glyfs*29)/c.2572dup	51	49	*BCR::ABL1*	*BCR*	22	23635575		Yes
		ABL1	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	34	23		*ABL1*	9	133698732		
										ABL1::BCR	*ABL1*	9	133698709		Yes
											BCR	22	23634612		
Pt9[§]	LBC	*ABL1*	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.820G>A	53[§]	19	*BCR::ABL1*	*BCR*	22	23634205		Yes
		ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.813G>C	10[§]	3.0		*ABL1*	9	133697850		
		ATM	11	108225537	ENST00000278616	Splice acceptor	c.8787-1G>A	76[§]	Not evaluable[‖]						
										ABL1::BCR	*ABL1*	9	133697843		Yes
											BCR	22	23634216		
Pt10[§]	MBC	*ASXL1*	20	31023300	ENST00000375687	Nonsense	p.(Glu929*)/c.2785G>T	14[§]	13	*BCR::NUP214*	*BCR*	22	23665088	Ph-associated	Yes‡
		IDH1	2	209113113	ENST00000415913	Missense	p.(Arg132Cys)/c.394C>T	46[§]	33		*NUP214*	9	134050572		
		RUNX1	21	36164627	ENST00000300305	Frameshift	p.(Phe416Leufs*185)/c.1244_1247dup	32[§]	45						
										NUP214::BCR	*NUP214*	9	134050563	Ph-associated	Yes‡
											BCR	22	23657072	inversion	
										NUP214::ABL1	*NUP214*	9	134051424	Ph-associated	No
											ABL1	9	133644990		
										BCR	*BCR*	22	23655029	*BCR* intergenic	No
												22	23679451	inversion	

Sample ID	Disease phase	Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Expected variants	Pre-characterized VAF%	Gene panel VAF%	Fusion	Gene	Chr	Genomic coordinates (hg19)	Comment	Detected by gene panel
Pt11[§]	MBC	*ABL1*	9	133748414	ENST00000318560	Missense	p.(Phe378Val)/c.1132T>G	74[§]	37	*BCR::ABL1*	*BCR*	22	23633790		Yes
		ASXL1	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	21[§]	28		*ABL1*	9	133697671		
										ABL1::BCR	*ABL1*	9	133697672		Additional
											BCR	22	23633782		
Myeloid mutation DNA Mix		*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	10	11						
		ASXL1	20	31022402	ENST00000318560	Frameshift	p.(Gly634Glnfs*14)/c.1900_1922del	10	7.7						
		ASXL1	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	10	6.6						
		CALR	19	13054564	ENST00000316448	Frameshift	p.(Leu367Thrfs*46)/c.1092_1143del	5	5.1						
		CBL	11	119148919	ENST00000264033	Missense	p.(Arg420Gln)/c.1259G>A	10	11						
		CBL	11	119149251	ENST00000264033	Missense	p.(Leu380Pro)/c.1139T>C	10	11						
		CEBPA	19	33792381	ENST00000498907	Frameshift	p.(His24Alafs*84)/c.68_69insC	15	9.3						
		CEBPA	19	33793252	ENST00000498907	Frameshift	p.(Lys313dup)/c.939_940insAAG	15	8.8						
		CSF3R	1	36933434	ENST00000373103	Missense	p.(Thr618Ile)/c.1853C>T	5	5.3						
		FLT3	13	28608300	ENST00000241453	Frameshift	p.(Asn587_Asp600dup)/c.1759_1800dup	5	8.7						
		FLT3	13	28608250	ENST00000241453	Frameshift	p.(Lys602_Trp603insGlyAlaPheArgGluTyr	10	10.5						
		FLT3	13	28592642	ENST00000241453	Missense	p.(Asp835Tyr)/c.2503G>T	10	11.1						
		IDH1	2	209113113	ENST00000415913	Missense	p.(Arg132Cys)/c.394C>T	5	6.1						
		JAK2	9	5073770	ENST00000381652	Missense	p.(Val617Phe)/c.1849G>T	5	4.9						
		JAK2	9	5070033	ENST00000381652	Inframe	p.(Asn542_Glu543del)/c.1624_1629del	10	5.1						
		MPL	1	43815009	ENST00000372470	Missense	p.(Trp515Leu)/c.1544G>T	5	5						
		NPM1	5	170837547	ENST00000296930	Frameshift	p.(Trp288Cysfs*12)/c.863_864dup	5	ND						
		SF3B1	2	198266834	ENST00000335508	Missense	p.(Lys700Glu)/c.2098A>G	5	3.6						
		SF3B1	2	198267359	ENST00000335508	Missense	p.(Lys666Asn)/c.1998G>T	5	4.4						
		SRSF2	17	74732935	ENST00000392485	Frameshift	p.(Pro95_R102del)/c.284_307del	5	4.9						
		U2AF1	21	44524456	ENST00000291552	Missense	p.(Ser34Phe)/c.101C>T	10	9.2						

Sample ID	Disease phase	Deletion / Fusion	Gene	Chr	Genomic coordinates (hg19)	Size (Kb)	Comment	Detected by gene panel	Pre-characterized rearrangement	Rearrangement detected using DNA
Pt1	LBC	*CLIC6::RUNX1*	*CLIC6*	21	36080470	34	deletion	Yes	*BCR::ABL1* fusion	Yes
			RUNX1	21	36421160				Ph-associated rearrangement, inversion	Yes
									CLIC6::RUNX1 deletion	Yes
		HBS1L::MYB intergenic locus	*HBS1L*	6	135366696	71	deletion	Yes	*HBS1L::MYB* intergenic locus deletion	Yes
			Intergenic	6	135437658				*IKZF1::IGK* fusion	Yes
		IKZF1::IGKV3-7	*IKZF1*	7	50348584		interchromosomal fusion	Yes		
			IGKV3-7	2	89277898					
		IGKV3-7::IKZF1	*IGKV3-7*	2	89277981		reciprocal interchromosomal fusion	Yes		
			IKZF1	7	50348584					
Pt2-S1	Germline									
Pt2-S2	Diagnosis								*BCR::ABL1* fusion	Yes
Pt2-S3	MBC - 1st	*IKZF1*	*IKZF1*	7	50412895-50463650	51	Exon 4 to 7 deletion (predominant)	Yes	*BCR::ABL1* fusion	Yes
									IKZF1 exon 4-7 deletion	Yes
		IKZF1	*IKZF1*	7	50412895-50463630	116	Exon 4 to 7 deletion (minor)	Yes	*IKZF1* exon 4 to 7 deletion (minor)	Yes
		IKZF1	*IKZF1*	7	50347391-50463629	116	Exon 2 to 7 deletion	Additional	Additional	
									IKZF1 exon 2-7 deletion	
Pt3-S1	Diagnosis								*BCR::ABL1* fusion	Yes
Pt4	CP treatment failure								*BCR::ABL1* fusion	Yes
									Ph-associated rearrangement, inversion	Yes
Pt5-S1	Diagnosis								*BCR::ABL1* fusion	Yes
Pt5-S2	LBC	*IKZF1*	*IKZF1*	7	50412896-50463633	51	Exon 4 to 7 deletion	Yes	*BCR::ABL1* fusion	Yes
									IKZF1 exon 4-7 deletion	Yes
		HBS1L::MYB intergenic locus	*HBS1L*	6	ND	65	Deletion	Yes	*HBS1L::MYB* intergenic locus deletion	Yes
			Intergenic	6	ND					

Sample ID	Disease phase	Deletion / Fusion	Gene	Chr	Genomic coordinates (hg19)	Size (Kb)	Comment	Detected by gene panel	Pre-characterized rearrangement	Rearrangement detected using DNA
Pt6	LBC								*BCR::ABL1* fusion	Yes
									Ph-associated rearrangement, inversion	Yes
Pt7	Diagnosis								Ph-associated rearrangement, inversion	Yes
Pt8-S1	Diagnosis								*BCR::ABL1* fusion	Yes
Pt8-S2	LBC								*BCR::ABL1* fusion	Yes
Pt9[§]	LBC	*IKZF1*	*IKZF1*	7	50412878-50463632	51	Exon 4 to 7 deletion	Yes	*BCR::ABL1* fusion	Yes
									CDKN2A/B deletion	Yes
		CDKN2A/B	*CDKN2A/B*	9	21827667-22008305	181	Deletion of *CDKN2A/B* extending into adjacent *MTAP* gene	Yes		

Sample ID	Disease phase	Deletion / Fusion	Gene	Chr	Genomic coordinates (hg19)	Size (Kb)	Comment	Detected by gene panel	Pre-characterized rearrangement	Rearrangement detected using DNA
Pt10[§]	MBC								Ph-associated rearrangement, inversion	Yes
Pt11[§]	MBC								*BCR::ABL1* fusion	Yes
Myeloid mutation DNA Mix										

Supplementary Table 5: Intra- and inter-assay analysis using DNA: SNVs and indels

Abbreviations

ND = Not detected

Chr = Chromosome

Sample 1 of the intra-assay analysis was used for run 1 of the inter-assay analysis

For the purposes of the validation study, some variants detected in the cell line mixes did not meet all of our criteria for clinical relevance but were included to increase the number of variants under review.

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay repeatability						Inter-assay reproducibility					
							Allele frequency %			Mean	SD	CV%	Allele frequency %			Mean	SD	CV%
							1	2	3				1	2	3			
Kasumi-1 / K562	20	31022288	*ASXL1*	ENST00000375687	Nonsense	p.(Tyr591*)/c.1773C>A	17.3	16.7	19.1	17.7	1.2	7.1	17.3	16.4	18.7	17.5	1.2	6.6
Kasumi-1 / K562	20	31022441	*ASXL1*	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	12.0	11	12.3	11.8	0.7	5.8	12.0	13.1	14.9	13.3	1.5	11.0
Kasumi-1 / K562	16	3831192	*CREBBP*	ENST00000262367	Splice region	c.1676+3_1676+12del	22.4	22	26.2	23.5	2.3	9.9	22.4	18.8	25.5	22.2	3.4	15.1
Kasumi-1 / K562	4	55599340	*KIT*	ENST00000288135	Missense	p.(Asn822Lys)/c.2466T>A	51.5	52.5	54.7	52.9	1.6	3.1	51.5	50.4	55.5	52.5	2.7	5.1
Kasumi-1 / K562	9	139413165	*NOTCH1*	ENST00000651671	Missense	p.(Gly326Val)/c.977G>T	13.5	17.4	16.4	15.8	2.0	12.8	13.5	16.3	16.9	15.6	1.8	11.7
Kasumi-1 / K562	4	1941422	*NSD2*	ENST00000508803	Nonsense	p.(Arg600*)/c.1798C>T	17.5	18.4	16.6	17.5	0.9	5.1	17.5	10.6	17.1	15.1	3.9	25.7
Kasumi-1 / K562	8	117866656	*RAD21*	ENST00000297338	Frameshift	p.(Lys330Thrfs*6)/c.988_989insCGGG	17.8	16.9	17.7	17.5	0.5	2.8	17.8	15.4	20.8	18.0	2.7	15.0
Kasumi-1 / K562	19	11145745	*SMARCA4*	ENST00000344626	Frameshift	p.(Gly1370Alafs*14)/c.4109del	22.2	22.8	20.9	22.0	1.0	4.4	22.2	24.8	22.2	23.1	1.5	6.5
Kasumi-1 / K562	17	7577538	*TP53*	ENST00000269305	Missense	p.(Arg248Gln)/c.743G>A	34.6	39.2	36.1	36.6	2.3	6.4	34.6	32.8	39.3	35.6	3.4	9.4
Kasumi-1 / K562	17	7578523	*TP53*	ENST00000269305	Frameshift	p.(Gln136Profs*13)/c.406dup	56.4	57.8	55.5	56.6	1.2	2.0	56.4	50.2	61.3	56.0	5.6	9.9
RS4 and 11 / ME1	16	3801797	*CREBBP*	ENST00000262367	Missense	p.(Cys1237Arg)/c.3709T>C	15.1	14.7	13.6	14.5	0.8	5.4	15.1	13.1	10.1	12.8	2.5	19.7
RS4 and 11 / ME1	22	41566577	*EP300*	ENST00000263253	Splice donor	c.4452+2T>G	10.8	8.8	8.7	9.4	1.2	12.6	10.8	8	ND	9.4	2.0	21.1
RS4 and 11 / ME1	13	28592686	*FLT3*	ENST00000241453	Missense	p.(Thr820Asn)/c.2459C>A	25.1	25.9	24	25.0	1.0	3.8	25.1	17.6	31.2	24.6	6.8	27.7
RS4 and 11 / ME1	13	28644740	*FLT3*	ENST00000241453	Frameshift	p.(Ser18Phefs*24)/c.52dup	5.3	5.1	4.3	4.9	0.5	10.8	5.1	6.6	6.1	5.9	0.8	12.9
RS4 and 11 / ME1	15	90631874	*IDH2*	ENST00000330062	Missense	p.(Leu160Pro)/c.479T>C	6.1	4.5	6.4	5.7	1.0	18.0	6.1	5.4	5.7	5.7	0.4	6.1
RS4 and 11 / ME1	1	115256528	*NRAS*	ENST00000369535	Missense	p.(Gln61His)/c.183A>T	23.4	23.2	24	23.5	0.4	1.8	23.4	17.3	27.4	22.7	5.1	22.4
RS4 and 11 / ME1	1	115258748	*NRAS*	ENST00000369535	Missense	p.(Gly12Cys)/c.34G>T	16.1	13.7	13.7	14.5	1.4	9.6	16.1	13.5	12.8	14.1	1.7	12.3
RS4 and 11 / ME1	4	1962801	*NSD2*	ENST00000508803	Missense	p.(Glu1099Lys)/c.3295G>A	25.3	23.5	24.7	24.5	0.9	3.7	25.3	23.6	19.9	22.9	2.8	12.0
RS4 and 11 / ME1	18	42532264	*SETBP1*	ENST00000282030	Missense	p.(Arg987Trp)/c.2959C>T	8.9	7.8	8.5	8.4	0.6	6.6	8.9	9	5.9	7.9	1.8	22.2
RS4 and 11 / ME1	12	111856427	*SH2B3*	ENST00000341259	Missense	p.(Ala160Thr)/c.478G>A	26.7	26	23.9	25.5	1.5	5.7	26.7	28	19.8	24.8	4.4	17.7
RS4 and 11 / ME1	19	11097008	*SMARCA4*	ENST00000344626	Missense	p.(Arg167Trp)/c.499C>T	25.2	21.1	23.3	23.2	2.1	8.8	25.2	23.6	19.3	22.7	3.1	13.4
RS4 and 11 / ME1	19	11121057	*SMARCA4*	ENST00000344626	Frameshift	p.(Asn709Lysfs*8)/c.2126dup	2.7	1.4	1.5	1.9	0.7	38.8	2.7	1.6	0.7	1.7	1.0	60.1
RS4 and 11 / ME1	X	123179048	*STAG2*	ENST00000218089	Missense	p.(Pro166His)/c.497C>A	7.1	8.0	10.2	8.4	1.6	18.9	7.1	8.3	6.3	7.2	1.0	13.9
RS4 and 11 / ME1	17	7576986	*TP53*	ENST00000269305	Splice donor + inframe deletion	p.(Gly302_Arg306del)/c.905_919+32del	28	23	24.5	25.2	2.6	10.2	28	20	35	27.7	7.5	27.1
RS4 and 11 / ME1	11	32410674	*WT1*		Missense	p.(Arg495Gln)/c.1484G>A	27.8	25.5	25.6	26.3	1.3	4.9	27.8	20.6	28.9	25.8	4.5	17.5
RS4 and 11 / ME1	11	32413523	*WT1*	ENST00000332351	Missense	p.(Lys476Arg)/c.1427A>G	4.9	5	2.9	4.3	1.2	27.8	4.9	ND	3.9	4.4	0.7	16.1
RS4 and 11 / ME1	2	61719472	*XPO1*	ENST00000401558	Missense	p.(Glu571Lys)/c.1711G>A	26.1	24.5	24.3	25.0	1.0	4.0	26.1	24.6	20.5	23.7	2.9	12.2

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay repeatability						Inter-assay reproducibility					
							Allele frequency %			Mean	SD	CV%	Allele frequency %			Mean	SD	CV%
							1	2	3				1	2	3			
Z119 / Jurkat	11	108124743	*ATM*	ENST00000278616	Missense	p.(Leu701Ile)/c.2101C>A	23.2	25.8	22.8	23.9	1.6	6.8	25.8	23.8	23.1	24.2	1.4	5.8
Z119 / Jurkat	11	108159742	*ATM*	ENST00000278616	Missense	p.(Ser1383Leu)/c.4148C>T	22.6	22	23	22.5	0.5	2.2	22	11.4	25.5	19.6	7.3	37.4
Z119 / Jurkat	3	52441419	*BAP1*	ENST00000460680	Missense	p.(Ala145Thr)/c.433G>A	23.9	23.9	24.8	24.2	0.5	2.1	23.9	18.3	24.7	22.3	3.5	15.6
Z119 / Jurkat	16	3779587	*CREBBP*	ENST00000262367	Nonsense	p.(Gln1821*)/c.5461C>T	1.66	1.12	1.35	1.4	0.3	19.7	1.12	1.59	1.29	1.3	0.2	17.8
Z119 / Jurkat	16	3786784	*CREBBP*	ENST00000262367	Missense	p.(Pro1476Leu)/c.4427C>T	2.6	3.39	3.07	3.0	0.4	13.2	3.39	2.89	4.26	3.5	0.7	19.7
Z119 / Jurkat	16	3807988	*CREBBP*	ENST00000262367	Missense	p.(Thr1144Ile)/c.3431C>T	25.1	26.25	23.71	25.0	1.3	5.1	26.3	20.8	25.02	24.0	2.8	11.8
Z119 / Jurkat	16	3790458	*CREBBP*	ENST00000262367	Frameshift	p.(Phe1358Leufs*18)/c.4074del	22.63	25.04	24.02	23.9	1.2	5.1	25.04	22.34	25.19	24.2	1.6	6.6
Z119 / Jurkat	1	36938146	*CSF3R*	ENST00000373103	Missense	p.(Pro272Leu)/c.815C>T	25.43	26.12	26.17	25.9	0.4	1.6	26.1	23.43	25.5	25.0	1.4	5.6
Z119 / Jurkat	7	101845476	*CUX1*	ENST00000360264	Missense	p.(Gly978Arg)/c.2932G>A	23.89	24.97	25.4	24.8	0.8	3.1	24.97	22.19	22.31	23.2	1.6	6.8
Z119 / Jurkat	2	25470573	*DNMT3A*	ENST00000264709	Missense	p.(Arg301Trp)/c.901C>T	24.42	21.48	24.31	23.4	1.7	7.1	21.48	22.48	25.3	23.1	2.0	8.6
Z119 / Jurkat	22	41553308	*EP300*	ENST00000263253	Missense	p.(Arg1133Trp)/c.3397C>T	3.54	3.9	3.55	3.7	0.2	5.7	3.91	2.81	3.51	3.4	0.6	16.3
Z119 / Jurkat	11	92531583	*FAT3*	ENST00000298047	Missense	p.(Asp1802Asn)/c.5404G>A	2.78	2.5	2.06	2.4	0.4	14.8	2.5	1.9	2.61	2.3	0.4	16.6
Z119 / Jurkat	11	92568114	*FAT3*	ENST00000298047	Missense	p.(Gly3317Asp)/c.9950G>A	2.78	2.5	2.06	2.4	0.4	14.8	2.5	1.89	2.61	2.3	0.4	16.6
Z119 / Jurkat	11	92600334	*FAT3*	ENST00000298047	Missense	p.(Pro4029Leu)/c.12086C>T	2.26	7.62	3.69	4.5	2.8	61.4	7.62	6.1	6.29	6.7	0.8	12.2
Z119 / Jurkat	11	92616455	*FAT3*	ENST00000298047	Missense	p.(Arg4278Gln)/c.12833G>A	23.83	23.94	23.87	23.9	0.1	0.2	23.94	23.08	23.74	23.6	0.5	1.9
Z119 / Jurkat	11	92623876	*FAT3*	ENST00000298047	Missense	p.(Pro4456Leu)/c.13367C>T	23.68	22.39	23.65	23.2	0.7	3.2	22.39	21.1	23.63	22.4	1.3	5.7
Z119 / Jurkat	4	153247289	*FBXW7*	ENST00000281708	Missense	p.(Arg505Cys)/c.1513C>T	26	27.25	24.82	26.0	1.2	4.7	27.25	22.22	24.13	24.5	2.5	10.3
Z119 / Jurkat	12	417050	*KDM5A*	ENST00000399788	Missense	p.(Arg1167His)/c.3500G>A	23.09	23.87	25.11	24.0	1.0	4.2	23.87	23.1	25.61	24.2	1.3	5.4
Z119 / Jurkat	X	44928975	*KDM6A*	ENST00000377967	Frameshift	p.(Ala694Leufs*21)/c.2080del	2.82	3.09	2.7	2.9	0.2	7.0	3.09	3.44	2.97	3.2	0.2	7.7
Z119 / Jurkat	X	44870255	*KDM6A*	ENST00000377967	Frameshift	p.(Phe146Profs*7)/c.434_435insGCCT	7.67	5.02	6.15	6.3	1.3	21.2	5.02	7.29	5.97	6.1	1.1	18.7
Z119 / Jurkat	X	44966666	*KDM6A*	ENST00000377967	Frameshift	p.(Arg1298Lysfs*35)/c.3892dup	43.55	43.01	43.4	43.3	0.3	0.6	43.01	38.27	40.5	40.6	2.4	5.8
Z119 / Jurkat	12	49415917	*KMT2D*	ENST00000301067	Missense	p.(Cys5477Tyr)/c.16430G>A	22.38	25.62	24.87	24.3	1.7	7.0	25.62	22.47	24.86	24.3	1.6	6.8
Z119 / Jurkat	12	49447099	*KMT2D*	ENST00000301067	Missense	p.(Pro282Leu)/c.845C>T	24.21	24.46	24.06	24.2	0.2	0.8	24.46	23.7	25.79	24.7	1.1	4.3
Z119 / Jurkat	12	49426972	*KMT2D*	ENST00000301067	Frameshift	p.(Gln3839Serfs*42)/c.11515del	3.53	3.7	3.84	3.7	0.2	4.2	3.66	3.41	4.02	3.7	0.3	8.3
Z119 / Jurkat	12	49446154	*KMT2D*	ENST00000301067	Frameshift	p.(Met438Glyfs*8)/c.1312_1315delinsGGGGG	10.01	8.84	7.99	8.9	1.0	11.3	8.84	8.13	10.61	9.2	1.3	13.9
Z119 / Jurkat	12	49431873	*KMT2D*	ENST00000301067	Frameshift	p.(Val3089Trpfs*30)/c.9265del	21.73	21.5	22.91	22.0	0.8	3.4	21.5	22.9	24.5	23.0	1.5	6.5
Z119 / Jurkat	1	43814572	*MPL*	ENST00000372470	Missense	p.(Arg456His)/c.1367G>A	24.06	22.7	20.78	22.5	1.6	7.3	22.68	21.38	26.16	23.4	2.5	10.6
Z119 / Jurkat	9	139408998	*NOTCH1*	ENST00000651671	Missense	p.(Pro724Leu)/c.2171C>T	25.88	21.23	23.25	23.5	2.3	9.9	21.23	22.8	25.35	23.1	2.1	9.0
Z119 / Jurkat	9	139402693	*NOTCH1*	ENST00000651671	Nonsense	p.(Gln1106*)/c.3316C>T	8.31	7.19	6.6	7.4	0.9	11.8	7.19	6.48	6.1	6.6	0.5	8.3
Z119 / Jurkat	9	139396887	*NOTCH1*	ENST00000651671	Inframe insertion	p.(Ala1740_Ala1741insGlnAlaValGluProProProProAlaGlnLeuHisPheMetTyrValAla)/c.5220_5221insCAGGCCGTGGAGCCGCCCCCGCCGGCGCAGCTGCACTTCATGTACGTGGCG	16.16	19.65	19.68	18.5	2.0	10.9	19.65	21.33	21.06	20.7	0.9	4.4
Z119 / Jurkat	4	1980558	*NSD2*	ENST00000508803	Frameshift	p.(Pro1343Glnfs*49)/c.4028del	22.5	25.19	24.06	23.9	1.3	5.6	25.2	23.61	23.99	24.3	0.8	3.4
Z119 / Jurkat	11	3723692	*NUP98*	ENST00000533346	splice donor	c.3512+1G>A	24.8	26.5	23.88	25.1	1.3	5.3	26.5	23.86	26.1	25.5	1.4	5.6
Z119 / Jurkat	10	89717712	*PTEN*	ENST00000371953	Stop gained + inframe insertion	p.(Leu247*)/c.737_738insCTGAAGTTCATGTACTTTGAGTTCCCTCAGCCCTGGGTT	10.3	11.51	11.76	11.2	0.8	6.8	11.5	11.0	14.3	12.2	1.8	14.3
Z119 / Jurkat	10	89717673	*PTEN*	ENST00000371953	Frameshift	p.(Arg234fs)/c.698_699insGGCCC	22.41	20.53	20.84	21.3	1.0	4.7	20.53	17.97	25.98	21.5	4.1	19.0
Z119 / Jurkat	21	36252917	*RUNX1*	ENST00000300305	Missense	p.(Ala149Thr)/c.445G>A	27.31	25.36	24.56	25.7	1.4	5.5	25.36	23.21	26.86	25.1	1.8	7.3
Z119 / Jurkat	12	122252549	*SETD1B*	ENST00000267197	Nonsense	p.(Gln810*)/c.2428C>T	24.27	26.37	23.93	24.9	1.3	5.3	26.37	23.75	27.94	26.0	2.1	8.1
Z119 / Jurkat	12	122265461	*SETD1B*	ENST00000267197	Missense	p.(Arg1749Trp)/c.5245C>T	26.43	24.3	23.6	24.8	1.5	6.0	24.32	26.27	25.41	25.3	1.0	3.9

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay repeatability						Inter-assay reproducibility					
							Allele frequency %			Mean	SD	CV%	Allele frequency %			Mean	SD	CV%
							1	2	3				1	2	3			
Z119 / Jurkat	12	122242657	*SETD1B*	ENST00000267197	Frameshift	p.(His8Thrfs*27)/c.22del	ND	20.86	24.37	22.6	2.5	11.0	20.86	ND	16.27	18.6	3.2	17.5
Z119 / Jurkat	2	198260937	*SF3B1*	ENST00000335508	Missense	p.(Val1128Ile)/c.3382G>A	24.35	24.03	24	24.1	0.2	0.8	24.03	21.33	23.61	23.0	1.5	6.3
Z119 / Jurkat	2	198263191	*SF3B1*	ENST00000335508	Missense	p.(Ala1043Val)/c.3128C>T	23.23	22.98	23.87	23.4	0.5	2.0	22.98	23.6	25.6	24.1	1.4	5.8
Z119 / Jurkat	19	11106911	*SMARCA4*	ENST00000344626	Missense	p.(Arg539His)/c.1616G>A	24.07	23.42	25.48	24.3	1.1	4.3	23.42	21.26	25.03	23.2	1.9	8.1
Z119 / Jurkat	19	11143994	*SMARCA4*	ENST00000344626	Missense	p.(Arg1192His)/c.3575G>A	23.99	22.77	26.77	24.5	2.1	8.4	22.77	23.24	23.37	23.1	0.3	1.4
Z119 / Jurkat	19	11144150	*SMARCA4*	ENST00000344626	Missense	p.(Arg1244His)/c.3731G>A	25.41	21.97	24.25	23.9	1.8	7.3	21.97	22.28	25.53	23.3	2.0	8.5
Z119 / Jurkat	10	112361730	*SMC3*	ENST00000361804	Nonsense	p.(Arg967*)/c.2899C>T	24.6	23.54	24.32	24.1	0.5	2.2	23.5	26.77	22.0	24.1	2.4	10.0
Z119 / Jurkat	17	40370266	*STAT5B*	ENST00000293328	Missense	p.(Gly358Arg)/c.1072G>A	25.51	24.28	22.17	24.0	1.7	7.0	24.28	21.38	23.4	23.0	1.5	6.5
Z119 / Jurkat	17	40371827	*STAT5B*	ENST00000293328	Frameshift	p.(Gln195Argfs*4)/c.583del	19.64	18.53	19.82	19.3	0.7	3.6	18.53	23.24	21.6	21.1	2.4	11.3
Z119 / Jurkat	17	40370235	*STAT5B*	ENST00000293328	Frameshift	p.(Gln368Argfs*2)/c.1102del	28.48	28.41	27.21	28.0	0.7	2.5	28.41	29.78	28.91	29.0	0.7	2.4
Z119 / Jurkat	17	7578263	*TP53*	ENST00000269305	Nonsense	p.(Arg196*)/c.586C>T	24.49	23.76	23.33	23.9	0.6	2.5	23.76	22.3	24.55	23.5	1.1	4.8
Z119 / Jurkat	17	7573943	*TP53*	ENST00000269305	Frameshift	p.(Ser362Alafs*8)/c.1083del	21.38	23.73	26.01	23.7	2.3	9.8	23.73	21.1	23.12	22.6	1.4	6.2
Z119 / Jurkat	17	7579312	*TP53*	ENST00000269305	Splice altering	p.(Thr125Thr)/c.375G>A	26.52	24.87	24.06	25.2	1.3	5.0	24.87	20.9	24.36	23.4	2.2	9.2
Z119 / Jurkat	X	15841230	*ZRSR2*	ENST00000307771	Inframe insertion	p.(Ser445_Arg448dup)/c.1332_1343dup	25.48	32.98	33.33	30.6	4.4	14.5	32.98	31.37	30.19	31.5	1.4	4.4
Pt1	9	133738356	*ABL1*	ENST00000318560	Missense	p.(Gln252His)/c.756G>C	17.63	18.9	18.4	18.3	0.6	3.5	18.9	16.7	19.7	18.4	1.6	8.4
Pt1	9	133738357	*ABL1*	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	6.8	4.3	3.8	5.0	1.6	32.4	4.3	4.1	3.7	4.0	0.3	7.6
Pt1	9	133738363	*ABL1*	ENST00000318560	Missense	p.(Glu255Lys)/c.763G>A	9.1	7.4	6.5	7.7	1.3	17.2	7.4	8.4	8.4	8.1	0.6	7.2
Pt1	9	133738364	*ABL1*	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	ND	1.6	3.1	2.4	1.1	45.1	1.6	2.6	2.0	2.1	0.5	24.4
Pt1	9	133748283	*ABL1*	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	9.9	11.1	9.1	10.0	1.0	10.0	11.1	9.5	7.6	9.4	1.8	18.6
Pt1	9	133748414	*ABL1*	ENST00000318560	Missense	p.(Phe359Val)/c.1075T>G	ND	1.1	1.1	1.1	0.0	0.0	1.1	1.0	ND	1.1	0.1	6.7
Pt1	X	129147354	*BCORL1*	ENST00000218147	Frameshift	p.(Ile203Trpfs*52)/c.606_607insTGGGC	92.0	82.6	82.1	85.6	5.6	6.5	82.6	76.6	79.3	79.5	3.0	3.8
Pt1	7	148506461	*EZH2*	ENST00000320356	Missense	p.(Arg684His)/c.2051G>A	5.5	2.6	3.2	3.8	1.5	40.6	2.6	2.6	3.5	2.9	0.5	17.9
Pt1	10	112361923	*SMC3*	ENST00000361804	Nonsense	p.(Leu1031*)/c.3092T>A	5.8	5.8	6	5.9	0.1	2.0	5.8	5.0	5.6	5.5	0.4	7.6
Pt2-S2	20	31024043	*ASXL1*	ENST00000375687	Frameshift	p.((Leu1177Profs*5))/c.3529dup	8.2	8.7	7.6	8.2	0.6	6.7	8.2	7.6	7.2	7.7	0.5	6.6
Pt2-S2	12	122255792	*SETD1B*	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	6.2	7.0	8.7	7.3	1.3	17.5	6.2	8.1	7.0	7.1	1.0	13.4
Pt2-S2	21	44514777	*U2AF1*	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	2.9	2.7	3.7	3.1	0.5	17.1	2.9	2.5	3.7	3.0	0.6	20.1
Pt2-S3	X	133551322	*PHF6*	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	90.1	88.2	91.8	90.0	1.8	2.0	88.2	85.9	94.0	89.4	4.2	4.7
Pt2-S3	21	36252852	*RUNX1*	ENST00000300305	Splice donor	c.508+2T>C	34.6	37.2	34.4	35.4	1.6	4.4	37.2	37.4	35.2	36.6	1.2	3.3
Pt2-S3	12	122255792	*SETD1B*	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	42.9	49.4	42.8	45.0	3.8	8.4	49.4	43.6	48.9	47.3	3.2	6.8
Pt2-S3	21	44514777	*U2AF1*	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	44.3	44.1	44.8	44.4	0.4	0.8	44.1	42.13	52.6	46.3	5.6	12.0
										Average CV		9.1				Average CV		11.8

Supplementary Table 6: Intra- and inter-assay analysis using DNA: Structural variants

For this analysis each structural variant was assessed individually, even if multiple structural variants were associated with the primary lesion, such as the primary and reciprocal fusions

Abbreviations

Chr = Chromosome

ND = Not detected

† The breakpoints of these SVs were not specifically captured by probes, however were detected since they occurred close to an exon junction that was within a captured region.

Sample 1 of the intra-assay analysis was used for run 1 of the inter-assay analysis

Sample ID	Gene/s	First break	Second break	Type	Predicted RNA effect	Intra-assay repeatability						Inter-assay reproducibility					
						Normalized read counts			Mean	SD	CV%	Normalized read counts			Mean	SD	CV%
						1	2	3				1	2	3			
Kasumi-1 / K562	*BCR::ABL1*	chr22:23632726	chr9:133607170	genomic fusion	*BCR::ABL1* e14a2 transcript	120.9	111.2	114.8	115.6	4	3.5	120.9	103	167.6	130.5	27.2	20.9
Kasumi-1 / K562	*NUP214::XKR3* †	chr22:17300238	chr9:134074583	genomic fusion inversion	*NUP214::XKR3* e29e2 transcript	18.1	15.1	13.6	15.6	1.9	11.9	18.1	39.8	22	26.6	9.5	35.8
RS4:11 / ME1	*MYC*	chr8:128752858	chr8:128753194	336 base tandem duplication		4.9	3.6	5.7	4.7	0.9	18	4.9	ND	ND			
RS4:11 / ME1	*KMT2A::AFF1* †	chr11:118355075	chr4:88001854	genomic fusion	*KMT2A::AFF1* e9e5 transcript	13.3	12.1	11.7	12.4	0.7	5.5	13.3	5.8	10.9	10.0	3.1	31.1
RS4:11 / ME1	*CBFB::MYH11* †	chr16:15815077	chr16:67132445	genomic fusion inversion	*CBFB::MYH11* e5e34 transcript	9.4	9.6	8.5	9.2	0.5	5.1	9.4	15.8	16.2	13.8	3.1	22.5
RS4:11 / ME1	*MYH11::CBFB* †	chr16:67132446	chr16:15815076	reciprocal genomic fusion inversion	*MYH11::CBFB* e35-e4 transcript	4.8	3.5	3.1	3.8	0.7	19.4	4.8	10.6	6.8	7.4	2.4	32.2
Z119 / Jurkat	*IKZF1*	chr7:50346004	chr7:50463629	117.6 Kb genomic inversion		50.1	57	45.8	51	4.6	9.1	50.1	20.8	ND			
Z119 / Jurkat	*IKZF1*	chr7:50346008	chr7:50463630	117.6 Kb reciprocal genomic inversion		33.3	39.7	35.2	36.1	2.7	7.4	33.3	6.5	13.4	17.8	11.4	64.1
Z119 / Jurkat	*IKZF1*	chr7:50362776	chr7:50484457	121.7 Kb deletion	*IKZF1* exon 2-8 deletion	10.9	16.5	13	13.4	2.3	17.2	10.9	12.5	13.4	12.3	1.1	8.6
Z119 / Jurkat	*CDKN2A/B*	chr9:21973635	chr9:22008306	34.7 Kb deletion	*CDKN2A* exon 1 deletion, *CDKN2B* exon 2 deletion	18.3	24.5	20.6	21.1	2.6	12.2	18.3	4.2	15.6	12.7	6.1	48.3
Z119 / Jurkat	*CDKN2A/B*	chr9:21902179	chr9:22008304	106.1 Kb deletion	*CDKN2A* exon 1-3 deletion, *CDKN2B* exon 2 deletion	8.8	9.9	7.1	8.6	1.2	13.7	8.8	2.6	8.1	6.5	2.8	42.9
Z119 / Jurkat	*PAX5*	chr9:36929054	chr9:37030697	101.6 Kb deletion	*PAX5* exon 2-5 deletion	13	15.1	11.5	13.2	1.5	11.5	13	8	12.5	11.2	2.2	19.9
Z119 / Jurkat	*BTG1*	chr12:92205595	chr12:92538065	332.5 Kb deletion		12.4	13.4	11.6	12.5	0.7	5.8	12.4	10.6	11.9	11.7	0.8	6.6
Z119 / Jurkat	*RB1*	chr13:48984562	chr13:49074960	90.4 Kb deletion	*RB1* exon 18-27 deletion	10.2	10.8	10.9	10.6	0.3	2.8	10.2	3.8	8.5	7.5	2.7	36.5
Z119 / Jurkat	*RB1*	chr13:48984555	chr13:49176754	192.2 Kb deletion	*RB1* exon 18-27 deletion	2.9	2.9	3	2.9	0	1.3	2.9	0.3	3.3	2.2	1.3	60.1
Pt1	*BCR::ABL1*	chr22:23634009	chr9:133641246	genomic fusion	*BCR::ABL1* e14a2 transcript	10.1	41.4	33.5	28.3	13.3	46.8	10.1	43.5	60.6	38.1	21	55
Pt1	chr17q12::*BCR*	chr17:36315503	chr22:23634047	Ph-associated genomic fusion inversion		3	6.6	7.6	5.8	2	34.2	3	5.7	7.7	5.5	1.9	35
Pt1	*ABL1::* chr17q12	chr9:133641030	chr17: 36314782	Ph-associated genomic fusion inversion		0.3	0.2	0.4	0.3	0.1	24.7	0.3	ND	0.6			
Pt1	*CLIC6::RUNX1*	chr21:36080469	chr21:36421160	340.7 Kb deletion	*RUNX1* exon 3-9 deletion	7.4	17.5	15.9	13.6	4.4	32.4	7.4	10.8	15.7	11.3	3.4	30
Pt1	*IKZF1::IGKV3-7*	chr7:50348583	chr2:89277989	genomic fusion	*IKZF1::IGKV3-7* fusion transcript	5.7	18	14.7	12.8	5.2	40.5	5.7	21.3	15.5	14.2	6.4	45.4
Pt1	*IGKV3-7::IKZF1*	chr7:50348585	chr2:89277980	reciprocal genomic fusion	*IGKV3-7::IKZF1* fusion transcript	1.4	2	1.4	1.6	0.3	17.9	1.4	4.9	2.6	3	1.5	49
Pt1	*HBS1L::MYB* intergenic locus	chr6:135366695	chr6:135437658	71 Kb deletion		12.5	23.7	20.7	19	4.7	25	12.5	6.2	22.2	13.6	6.6	48.4
Pt2-S2	*BCR::ABL1*	chr22:23633087	chr9:133704831	genomic fusion	*BCR::ABL1* e14a2 transcript	14.1	16.1	15.1	15.1	0.8	5.4	14.1	16.16	9.8	13.3	2.6	19.8
Pt2-S2	*ABL1::BCR*	chr9:133704832	chr22:23633093	reciprocal genomic fusion	*ABL1::BCR* e1e15 transcript	0.4	ND	0.3				0.4	0.32	0.4	0.4	0	6.6
Pt2-S3	*BCR::ABL1*	chr22:23633087	chr9:133704831	genomic fusion	*BCR::ABL1* e14a2 transcript	17.9	16.8	15.3	16.7	1.1	6.5	17.9	20.8	6.8	15.2	6	39.8
Pt2-S3	*ABL1::BCR*	chr9:133704832	chr22:23633093	reciprocal genomic fusion	*ABL1::BCR* e1e15 transcript	0.4	ND	0.4				0.4	ND	0.4			
Pt2-S3	*IKZF1*	chr7:50412894	chr7:50463650	50.7 Kb deletion	*IKZF1* exon 4-7 deletion	22.4	23.8	18.5	21.6	2.3	10.5	22.4	5.8	6.4	11.5	7.7	66.9
Pt2-S3	*IKZF1*	chr7:50347390	chr7:50463629	116.2 Kb deletion	*IKZF1* exon 2-7 deletion	14.3	14.6	10.9	13.3	1.7	12.7	14.3	7.1	3	8.1	4.7	57.4
									Average CV		15.4				Average CV		36.8

Supplementary Table 7: Linearity and limit of detection of the homozygous and heterozygous SNVs and indels

Abbreviations

VAF = Variant Allele Frequency

SD = Standard deviation

CV = Coefficient of variation

Cell line	Dilution	Homozygous variants, n=52							Heterozygous variants, n=66						
		Expected VAF%	Observed mean VAF%	SD	CV%	Number of variants called	Mean total read depth	Mean variant read depth	Expected VAF%	Observed mean VAF%	SD	CV%	Number of variants called	Mean total read depth	Mean variant read depth
BV173	Neat	100	99.9	0.5	0.5	52 (100%)	1023	1022	50	49.2	2	4.1	66 (100%)	817	399
BV173:REH	1/2	50	40.2	5.3	13.2	52 (100%)	778	314	25	19.1	2.3	12	66 (100%)	623	118
BV173:REH	1/5	20	14.1	2.7	19.1	52 (100%)	1141	161	10	7.0	1.6	22.9	66 (100%)	932	64
BV173:REH	1/10	10	6.8	1.6	23.5	52 (100%)	1018	68	5	3.4	0.7	20.6	66 (100%)	832	28
BV173:REH	1/20	5	3.4	1.5	44.1	52 (100%)	1213	40	2.5	1.8	0.5	27.8	63 (95%)	991	17
BV173:REH	1/30	3.33	2.2	0.8	36.4	49 (94%)	1234	27	1.67	1.2	0.4	33.3	50 (76%)	976	10
REH	Neat	0	0				1127	0	0	0				911	0

Supplementary Table 8: Myeloid Mutation DNA Mix dilution results

Abbreviations

Chr = Chromosome

NC = Not called

ND = Not detected

VAF = Variant allele frequency

Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Known Variants	Expected VAF%			Observed VAF%		
						Neat	1 in 2 dilution	1 in 4 dilution	Neat	1 in 2 dilution	1 in 4 dilution
ABL1	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	10	5	2.5	10.9	8.4	4.0
ASXL1	20	31022402	ENST00000318560	Frameshift	p.(Gly634Glnfs*14)/c.1900_1922del	10	5	2.5	7.6	5.5	3.8
ASXL1	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	10	5	2.5	5.3	4.2	1.9
CALR	19	13054564	ENST00000316448	Frameshift	p.(Leu367Thrfs*46)/c.1092_1143del	5	2.5	1.25	5.1	3.8	1.3
CBL	11	119148919	ENST00000264033	Missense	p.(Arg420Gln)/c.1259G>A	10	5	2.5	10.7	6.4	4.3
CBL	11	119149251	ENST00000264033	Missense	p.(Leu380Pro)/c.1139T>C	10	5	2.5	10.1	6.2	3.8
CEBPA	19	33792381	ENST00000498907	Frameshift	p.(His24Alafs*84)/c.68_69insC	15	7.5	3.8	9.7	5	3.9
CEBPA	19	33793252	ENST00000498907	Frameshift	p.(Lys313dup)/c.939_940insAAG	15	7.5	3.8	8.7	7.7	2.6
CSF3R	1	36933434	ENST00000373103	Missense	p.(Thr618Ile)/c.1853C>T	5	2.5	1.25	5.1	4.3	1.9
FLT3	13	28608300	ENST00000241453	Frameshift	p.(Asn587_Asp600dup)/c.1759_1800dup	5	2.5	1.25	4.3	3.6	1.9
FLT3	13	28608250	ENST00000241453	Frameshift	p.(Lys602_Trp603insGlyAlaPheArgGluTyrGluTyrAspLeuLys)/c.1806_1807insGGGGCTTTCAGAGAATATGAATATGATCTCAAA	10	5	2.5	8.7	3	3
FLT3	13	28592642	ENST00000241453	Missense	p.(Asp835Tyr)/c.2503G>T	10	5	2.5	11	4.3	4.1

IDH1	2	209113113	ENST00000415913	Missense	p.(Arg132Cys)/c.394C>T	5	2.5	1.25	6.2	2.7	2.5
JAK2	9	5073770	ENST00000381652	Missense	p.(Val617Phe)/c.1849G>T	5	2.5	1.25	4.1	2.3	ND
JAK2	9	5070033	ENST00000381652	Frameshift	p.(Asn542_Glu543del)/c.1624_1629del	10	5	2.5	5.1	ND	ND
MPL	1	43815009	ENST00000372470	Missense	p.(Trp515Leu)/c.1544G>T	5	2.5	1.25	4.2	2.3	1.5
NPM1	5	170837547	ENST00000296930	Frameshift	p.(Trp288Cysfs*12)/c.863_864dup	5	2.5	1.25	ND	ND	ND
SF3B1	2	198266834	ENST00000335508	Missense	p.(Lys700Glu)/c.2098A>G	5	2.5	1.25	3.6	2.6	2.5
SF3B1	2	198267359	ENST00000335508	Missense	p.(Lys666Asn)/c.1998G>T	5	2.5	1.25	3.7	4.4	2.3
SRSF2	17	74732935	ENST00000392485	Frameshift	p.(Pro95_R102del)/c.284_307del	5	2.5	1.25	3.5	1.5	1.2
U2AF1	21	44524456	ENST00000291552	Missense	p.(Ser34Phe)/c.101C>T	10	5	2.5	7.1	4.3	ND

Supplementary Table 9: Normalized read counts (reads per million mapped reads) for the BCR::ABL1 genomic fusion and gene deletions in the linearity study of BV173

ND = Not detected

BV173 cell line has an additional copy of the Ph chromosome which could lead to enhanced detection at higher dilutions[45]

Cell line	Dilution	*BCR-ABL1* genomic fusion	*IKZF1* focal deletions			*CDKN2A/B* deletions	
			Exon 2-3 deletion	Exon 4-7 deletion	Exon 1 deletion	35 Kb deletion	147 Kb deletion
BV173	Neat	69	82	6.8	4.2	7.7	3.3
BV173:REH	1/2	37	38	3.9	2.7	2.9	1.1
BV173:REH	1/5	12	14	1	0.6	0.8	0.6
BV173:REH	1/10	7	7	ND	0.4	0.2	0.2
BV173:REH	1/20	3	4	ND	0.3	0.4	0.1
BV173:REH	1/30	2	2	ND	0.2	ND	ND
R^2		0.9975	0.9983	0.9783	0.9709	0.9829	0.9681

Supplementary Table 10: Targeted gene panel variant detection: DNA versus RNA

Note that the cell lines were tested using the first probe design and some of the mutated genes subsequently detected for the intra- and inter-assay validation study were not included

For the purposes of the comparison, some variants detected in the cell line mixes did not meet all of our criteria for clinical relevance but were included to increase the number of variants under review.

Variants not detected using the relevant gene panel are indicated in blue text

DNA panel results shaded in red

RNA panel results shaded in green

Abbreviations

Pt = Patient

S = Sample

Chr = Chromosome

NC = Not called

VAF = Variant allele frequency

MBC = Myeloid blast crisis

LBC = Lymphoid blast crisis

NE = Not evaluable as panel probes did not cover the target region. Subsequent panel design covered this region.

AML = Acute myeloid leukemia

ALL = Acute lymphoblastic leukemia

SNVs and indels										*BCR-ABL1* genomic breakpoints and Ph-associated rearrangements						
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**DNA VAF%**	**RNA VAF%**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**
Pt1	LBC	*ABL1*	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.756G>C	19	29	*BCR::ABL1*	*BCR*	22	23634010	Fusion	Yes	Yes
		ABL1	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	11	17		*ABL1*	9	133641245			
		ABL1	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	4.3	9	17q12::*BCR*	Intergenic	17	36315503	Ph-associated	Yes	No
		ABL1	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.763G>A	7.4	24		*BCR*	22	23634047	inversion		
		ABL1	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	1.6	3.3	*ABL1*:: 17q12	*ABL1*	9	133641030	Ph-associated		
		BCORL1	X	129147354	ENST00000218147	Frameshift	p.(Ile203Trpfs*52)/c.606_607insTGGGC	70	83		Intergenic	17	36314782	inversion	Yes	No
		EZH2	7	148506461	ENST00000320356	Missense	p.(Arg684His)/c.2051G>A	4.2	2.6							
		SMC3	10	112361923	ENST00000361804	Nonsense	p.(Leu1031*)/c.3092T>A	5.8	2							
		ABL1	9	133738356	ENST00000318560	Missense	p.(Phe359Val)/c.10757>G	1.1	1.8							
Pt2-S2	Diagnosis	*ASXL1*	20	31024043	ENST00000375687	Frameshift	p.(Leu1177Profs*5)/c.3529dup	8.2	3.5	*BCR::ABL1*	*BCR*	22	23633088	Fusion	Yes	Yes
		SETD1B	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	6.2	5.3		*ABL1*	9	133704830			
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	2.9	1.2	*ABL1::BCR*	*ABL1*	9	133704834	Reciprocal	Yes	No
											BCR	22	23633094			
Pt2-S3	MBC - 1st	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	49	40	*BCR::ABL1*	*BCR*	22	23633088	Fusion	Yes	Yes
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	44	45		*ABL1*	9	133704830			
		PHF6	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	88	89	*ABL1::BCR*	*ABL1*	9	133704834	Reciprocal	Yes	No
		RUNX1	21	36252852	ENST00000300305	Splice donor	c.508+2T>C	37	47		*BCR*	22	23633094			
Pt3-S1	Diagnosis	*RUNX1*	21	36212758	ENST00000300305	Splice altering	c.614-5860G>T	NE	NE	*BCR::ABL1*	*BCR*	22	23632899	Fusion	Yes	Yes
											ABL1	9	133597820			
										ABL1::BCR	*ABL1*	9	133597823	Reciprocal	Yes	No
											BCR	22	23632899			
Pt4	CP	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	25	29	*BCR::ABL1*	*BCR*	22	23634569	Fusion	Yes	Yes
	treatment	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	28	NC		*ABL1*	9	133615099			
	failure									7q34::*BCR*	intergenic TRBV12-1 and TRBV11-1-i14	7	142221314	Ph-associated inversion	Yes	No
											BCR	22	23634640			
Pt5-S2	LBC	*ABL1*	9	133738349	ENST00000318560	Missense	p.(Gly250Glu)/c.749G>A	55	86	*BCR::ABL1*	*BCR*	22	23633455	Fusion	Yes	Yes
		BCORL1	X	129155104	ENST00000218147	Nonsense	p.(Arg1196*)/c.3586C>T	100	97		*ABL1*	9	133685776			
		RUNX1	21	36231860	ENST00000300305	Missense	p.(Leu175Gln)/c.524T>A	45	25	*ABL1::BCR*	*ABL1*	9	133685778	Reciprocal	Yes	Yes
		RUNX1	21	36231855	ENST00000300305	Frameshift	p.(Ile177Serfs*34)/c.528del	47	22		*BCR*	22	23633457			
Pt22	Diagnosis	*CUX1*	7	101843371	ENST00000360264	Nonsense	p.(Arg672*)/c.2014C>T	11	7.2	*BCR::ABL1*	*BCR*	22	23632880	Fusion	Yes	Yes
		CUX1	7	101847749	ENST00000360264	Nonsense	p.(Arg1007*)/c.3019C>T	5.9	3.8		*ABL1*	9	133629143			
		PHF6	X	133559247	ENST00000332070	Missense	p.(His329Tyr)/c.985C>T	89	90	*ABL1::BCR*	*ABL1*	9	133629021	Reciprocal	Yes	No
											BCR	22	23633065			
Pt23	Diagnosis									*BCR::ABL1*	*BCR*	22	23633041	Fusion	Yes	Yes
											ABL1	9	133715120			
										ABL1::BCR	*ABL1*	9	133713166	Reciprocal	Yes	No
											BCR	22	23633047			

SNVs and indels										*BCR-ABL1* genomic breakpoints and Ph-associated rearrangements						
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**DNA VAF%**	**RNA VAF%**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**
Pt24	Diagnosis	*ASXL1*	20	31022297	ENST00000375687	Nonsense	p.(Cys594*)/c.1782C>A	41	51	*BCR::EXOSC2*	*BCR*	22	23633220	Ph-associated	Yes	Yes
												9	133580125			
Pt25	Diagnosis	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	22	33	*BCR::* q34.12	*BCR*	22	23631771	Ph-associated	Yes	Yes
											Intergenic (5' of *ABL1*)	9	133581770			
Pt26	LBC									*BCR::ABL1*	*BCR*	22	23634214	Fusion	Yes	Yes
											ABL1	9	133589937			
K562	CML	*ASXL1*	20	31022288	ENST00000375687	Nonsense	p.(Tyr591*)/c.1773C>A	31	28	*BCR::ABL1*	*BCR*	22	23632737	Fusion	Yes	Yes
	Cell line	*NOTCH1*	9	139413165	ENST00000651671	Missense	p.(Gly326Val)/c.977G>T	30	30		*ABL1*	9	133607147			
		NSD2	4	1941422	ENST00000508803	Nonsense	p.(Arg600*)/c.1798C>T	32	20	*NUP214::XKR3*	*NUP214*	9	134074583	Ph-associated	Yes	Yes
		TP53	17	7578523	ENST00000269305	Frameshift	p.(Gln136Profs*13)/c.406dup	96	87		*XKR3*	22	17300238	inversion		
Kasumi-1	AML	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	28	20							
	Cell line	*CREBBP*	16	3831192	ENST00000262367	Splice region intronic	c.1676+3_1676+12del	100	100							
		RAD21	8	117866657	ENST00000297338	Frameshift	p.(Lys330Profs*6)/c.988delinsCCGGG	46	9							
		SMARCA4	19	11145745	ENST00000344626	Frameshift	p.(Gly1370Alafs*14)/c.4109del	40	13							
		TP53	17	7577538	ENST00000269305	Missense	p.(Arg248Gln)/c.743G>A	100	100							
KCL22	CML	*ASXL1*	20	31022471	ENST00000375687	Frameshift	p.(Ala654Glyfs*4)/c.1960dup	45	38	*BCR::ABL1*	*BCR*	22	23632117	Fusion	Yes	Yes
	Cell line	*CREBBP*	16	3778915	ENST00000262367	Nonsense	p.(Gln2045*)/c.6133C>T	51	57		*ABL1*	9	133664204			
		CREBBP	16	3779056	ENST00000262367	Frameshift	p.(Val1998*)/c.5991del	21	19	*ABL1::BCR*	*ABL1*	9	133664207	Reciprocal	Yes	Yes
		CUX1	7	101892173	ENST00000360264	Nonsense	p.(Gln1468*)/c.4402C>T	50	58		*BCR*	22	23632117			
		KMT2D	12	49428410	ENST00000301067	Frameshift	p.(Gly3465Aspfs*37)/c.10394del	46	27							
		KMT2D	12	49425227	ENST00000301067	Missense	p.(Glu4421Lys)/c.13261G>A	47	67							
		KMT2D	12	49447261	ENST00000301067	Splice altering	p.(Cys279Cys)/c.837C>T	51	46							
		NOTCH1	9	139401817	ENST00000651671	Missense	p.(Gly1195Arg)/c.3583G>A	33	35							
		SETD1B	12	122265857	ENST00000267197	Missense	p.(Glu1827Lys)/c.5479G>A	47	46							
		SETD2	3	47162434	ENST00000431180	Missense	p.(Gly1231Glu)/c.3692G>A	53	46							
		SH2B3	12	111856140	ENST00000341259	Missense	p.(Ser64Leu)/c.191C>T	49	46							
		TP53	17	7577035	ENST00000269305	Frameshift	p.(Pro301Glnfs*44)/c.902del	98	96							
ME1	AML	*FLT3*	13	28592686	ENST00000241453	Missense	p.(Thr820Asn)/c.2459C>A	46	51							
	Cell line	*NRAS*	1	115256528	ENST00000369535	Missense	p.(Gln61His)/c.183A>T	47	53							
		TP53	17	7576986	ENST00000269305	Splice donor + inframe deletio	p.(Gly302_Arg306del)/c.905_919+32del	100	**NC†**							
		WT1	11	32410674	ENST00000332351	Missense	p.(Arg495Gln)/c.1484G>A	45	46							
		† the *TP53* 47bp deletion generated a novel splice junction at the deletion site when tested by RNA														
THP1/LAMA84	AML/CML	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	32	25	*BCR::ABL1*	*BCR*	22	23634353	Fusion	Yes	Yes
	Cell line	*NRAS*	1	115258747	ENST00000369535	Missense	p.(Gly12Asp)/c.35G>A	18	8		*ABL1*	9	133687006			
		TP53	17	7576891	ENST00000269305	Nonsense	p.(Lys319*)/c.955A>T	85	94	*ABL1::BCR*	*ABL1*	9	133687002	Reciprocal	Yes	Yes
		TP53	17	7578384	ENST00000269305	Frameshift	p.(Arg174Leufs*3)/c.520_545del	14	**NC‡**		*BCR*	22	23634350			
		‡ the *TP53* 26bp deletion generated a novel splice junction at the deletion site when tested by RNA														

SNVs and indels										*BCR-ABL1* genomic breakpoints and Ph-associated rearrangements						
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**DNA VAF%**	**RNA VAF%**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**
BV173	CML	*CREBBP*	16	3781794	ENST00000262367	Missense	p.(Met1625Leu)/c.4873A>T	51	46	*BCR::ABL1*	*BCR*	22	23632434	Fusion	Yes	Yes
	Cell line	*KMT2D*	12	49434810	ENST00000301067	Missense	p.(Arg2248His)/c.6743G>A	43	45		*ABL1*	9	133610925			
										ABL1::BCR	*ABL1*	9	133611322	Reciprocal	Yes	Yes
											BCR	22	23632428			
Jurkat	T-ALL	*BAP1*	3	52441419	ENST00000460680	Missense	p.(Ala145Thr)/c.433G>A	49	51							
	Cell line	*CREBBP*	16	3790458	ENST00000262367	Frameshift	p.(Phe1358Leufs*18)/c.4074del	46	26							
		CREBBP	16	3779587	ENST00000262367	Nonsense	p.(Gln1821*)/c.5461C>T	NC	2.0							
		CREBBP	16	3786784	ENST00000262367	Missense	p.(Pro1476Leu)/c.4427C>T	5.8	4.1							
		CREBBP	16	3807988	ENST00000262367	Missense	p.(Thr1144Ile)/c.3431C>T	39	26							
		CUX1	7	101845476	ENST00000360264	Missense	p.(Gly978Arg)/c.2932G>A	46	44							
		DNMT3A	2	25470573	ENST00000264709	Missense	p.(Arg301Trp)/c.901C>T	50	43							
		EP300	22	41553308	ENST00000263253	Missense	p.(Arg1133Trp)/c.3397C>T	7.2	8.6							
		KMT2D	12	49431873	ENST00000301067	Frameshift	p.(Val3089Trpfs*30)/c.9265del	44	38							
		KMT2D	12	49426972	ENST00000301067	Frameshift	p.(Gln3839Serfs*42)/c.11515del	9.3	7.4							
		KMT2D	12	49415917	ENST00000301067	Missense	p.(Cys5477Tyr)/c.16430G>A	44	45							
		KMT2D	12	49447099	ENST00000301067	Missense	p.(Pro282Leu)/c.845C>T	50	54							
		NOTCH1	9	139408998	ENST00000651671	Missense	p.(Pro724Leu)/c.2171C>T	41	59							
		NOTCH1	9	139402693	ENST00000651671	Nonsense	p.(Gln1106*)/c.3316C>T	21	NC							
		NSD2	4	1980558	ENST00000508803	Frameshift	p.(Pro1343Glnfs*49)/c.4028del	51	42							
		NUP98	11	3723692	ENST00000533346	Splice donor	c.3512+1G>A	47	84							
		RUNX1	21	36252917	ENST00000300305	Missense	p.(Ala149Thr)/c.445G>A	55	47							
		SETD1B	12	122265461	ENST00000267197	Missense	p.(Arg1749Trp)/c.5245C>T	39	58							
		SETD1B	12	122252549	ENST00000267197	Nonsense	p.(Gln810*)/c.2428C>T	49	44							
		SETD1B	12	122242657	ENST00000267197	Frameshift	p.(His8Thrfs*27)/c.22del	42	30							
		SF3B1	2	198263191	ENST00000335508	Missense	p.(Ala1043Val)/c.3128C>T	46	56							
		SMARCA4	19	11143994	ENST00000344626	Missense	p.(Arg539His)/c.1616G>A	51	51							
		SMARCA4	19	11106911	ENST00000344626	Missense	p.(Arg1192His)/c.3575G>A	35	46							
		SMARCA4	19	11144150	ENST00000344626	Missense	p.(Arg1244His)/c.3731G>A	46	40							
		SMC3	10	112361730	ENST00000361804	Nonsense	p.(Arg967*)/c.2899C>T	52	11							
		TP53	17	7573943	ENST00000269305	Nonsense	p.(Arg196*)/c.586C>T	41	80							
		TP53	17	7578263	ENST00000269305	Frameshift	p.(Ser362Alafs*8)/c.1083del	46	14							
		TP53	17	7579312	ENST00000269305	Splice altering	p.(Thr125Thr)/c.375G>A	41	82							
		ZRSR2	X	15841230	ENST00000307771	Inframe insertion	p.(Ser445_Arg448dup)/c.1332_1343dup	80	57							
		NOTCH1	9	139396887	ENST00000651671	Inframe insertion	p.(Ala1740_Ala1741insGlnAlaValGluProProProProAlaGlnLeuHis	33	11							
REH	B-ALL	*KMT2D*	12	49434325	ENST00000301067	Nonsense	p.(Arg2410*)/c.7228C>T	50	43							
	Cell line	*KMT2D*	12	49443880	ENST00000301067	Missense	p.(Thr1164Ile)/c.3491C>T	7.8	9.0							
		NOTCH1	9	139390623	ENST00000651671	Missense	p.(Ser2523Leu)/c.7568C>T	17	21							
		NOTCH1	9	139412273	ENST00000651671	Missense	p.(Ser458Pro)/c.1372T>C	49	51							
		PAX5	9	36882049	ENST00000358127	Frameshift	p.(Ala322Argfs*19)/c.963dup	46	29							
		TP53	17	7578389	ENST00000269305	Missense	p.(Arg181Cys)/c.541C>T	22	25							
		TP53	17	7577121	ENST00000269305	Missense	p.(Arg273Cys)/c.817C>T	4.2	3.6							

SNVs and indels										*BCR-ABL1* genomic breakpoints and Ph-associated rearrangements						
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**DNA VAF%**	**RNA VAF%**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**
RS4;11	B-ALL	*CREBBP*	16	3801797	ENST00000262367	Missense	p.(Cys1237Arg)/c.3709T>C	30	32							
	Cell line	*EP300*	22	41566577	ENST00000263253	Splice donor	c.4452+2T>G	21	36							
		FLT3	13	28644740	ENST00000241453	Frameshift	p.(Ser18Phefs*24)/c.52dup	11	13							
		IDH2	15	90631874	ENST00000330062	Missense	p.(Leu160Pro)/c.479T>C	13	12							
		NRAS	1	115258748	ENST00000369535	Missense	p.(Gly12Cys)/c.34G>T	33	37							
		NSD2	4	1962801	ENST00000508803	Missense	p.(Glu1099Lys)/c.3295G>A	49	47							
		SETBP1	18	42532264	ENST00000282030	Missense	p.(Arg987Trp)/c.2959C>T	21	NC							
		SH2B3	12	111856427	ENST00000341259	Missense	p.(Ala160Thr)/c.478G>A	48	55							
		SMARCA4	19	11097008	ENST00000344626	Missense	p.(Arg167Trp)/c.499C>T	49	51							
		STAG2	X	123179048	ENST00000218089	Missense	p.(Pro166His)/c.497C>A	15	28							
		XPO1	2	61719472	ENST00000401558	Missense	p.(Glu571Lys)/c.1711G>A	50	46							
TOM1	B-ALL	*BCOR*	X	39933661	ENST00000378444	Inframe deletion	p.(Gln312del)/c.935_937del	92	93							
	Cell line	*CREBBP*	16	3778716	ENST00000262367	Frameshift	p.(Asn2111Argfs*34)/c.6331_6332insGAGGA	24	17							
Z119	B-ALL															
	Cell line															
OM9:22	B-ALL															
	Cell line															
K562/Kasumi-1	CML/AML	*ASXL1*	20	31022288	ENST00000375687	Nonsense	p.(Tyr591*)/c.1773C>A	17	15	*BCR::ABL1*	*BCR*	22	23632737	Fusion	Yes	Yes
	Cell line	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	12	12		*ABL1*	9	133607147			
		CREBBP	16	3831192	ENST00000262367	Splice region intronic	c.1676+3_1676+12del	22	53	*NUP214::XKR3*	*NUP214*	9	134074583	Ph-associated	Yes	Yes
		KIT	4	55599340	ENST00000288135	Missense	p.(Asn822Lys)/c.2466T>A	52	73		*XKR3*	22	17300238	inversion		
		NOTCH1	9	139413165	ENST00000651671	Missense	p.(Gly326Val)/c.977G>T	14	9.4							
		NSD2	4	1941422	ENST00000508803	Nonsense	p.(Arg600*)/c.1798C>T	18	13							
		RAD21	8	117866657	ENST00000297338	Frameshift	p.(Lys330Profs*6)/c.988delinsCCGGG	18	2.2							
		SMARCA4	19	11145745	ENST00000344626	Frameshift	p.(Gly1370Alafs*14)/c.4109del	22	5.9							
		TP53	17	7577538	ENST00000269305	Missense	p.(Arg248Gln)/c.743G>A	35	74							
		TP53	17	7578523	ENST00000269305	Frameshift	p.(Gln136Profs*13)/c.406dup	56	19							

	Genomic breakpoints of deletions and other rearrangements									Fusion transcripts and novel splicing only detectable using RNA			
Sample ID	**Deletion / Fusion / Tandem Duplication**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**	**Novel RNA splicing**	**Deletion / Fusion**	**Chr**	**Transcript type / novel splicing**	**Comment**
Pt1	*CLIC6::RUNX1*	*CLIC6*	21	36080470	34	Deletion	Yes	Yes	No	*BCR::ABL1*	22 and 9	e14e2	Fusion
		RUNX1	21	36421160						*ABL1::* 17q12	9 and 17	e1-intergenic cryptic splice site	Ph-associated inversion
	HBS1L::MYB intergenic locus	*HBS1L*	6	135366696	71	Deletion	Yes	No	No	*ABL1::* 17q12	9 and 17	e1-intergenic cryptic splice site	2nd Ph-associated inversion
		Intergenic	6	135437658						*IKZF1::IGK* locus	7 and 2	e1-intergenic *IGKV3-7* and *IGKV1-8*	Fusion
	IKZF1::IGKV3-7	*IKZF1*	7	50348584		Interchromosomal fusion	Yes	Yes	No				
		IGKV3-7	2	89277898									
	IGKV3-7::IKZF1	*IGKV3-7*	2	89277981		Reciprocal interchromosomal	Yes	No	No				
		IKZF1	7	50348584									
Pt2-S2										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion
Pt2-S3	*IKZF1*	*IKZF1*	7	50412895-50463650	51	Exon 4 to 7 deletion (predominant)	Yes	Yes	Yes	*BCR::ABL1*	22 and 9	e14e2	Fusion
	IKZF1	*IKZF1*	7	50412895-50463630	116	Exon 4 to 7 deletion (minor)	Yes	Yes	Yes	*ABL1::BCR*	9 and 22	e1e15	Reciprocal fusion
	IKZF1	*IKZF1*	7	50347391-50463629	116	Exon 2 to 7 deletion	Yes	No	Yes				
Pt3-S1										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion
Pt4										*BCR::ABL1*	22 and 9	e13e2 and e14e2	Fusion
										ABL1::TRBV5-5	9 and 7	e1-within *TRBV5-5*	Ph-associated inversion
Pt5-S2	*IKZF1*	*IKZF1*	7	50412896-50463633	51	Exon 4 to 7 deletion	Yes	Yes	Yes	*BCR::ABL1*	22 and 9	e14e2	Fusion
	HBS1L::MYB intergenic locus	*HBS1L*	6	135366699	65	Deletion	Yes	No	No				
		Intergenic	6	135437659									
Pt22										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion
										RUNX1::PRDM16	21 and 1	e2e4	Fusion
Pt23										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion

	Genomic breakpoints of deletions and other rearrangements									Fusion transcripts and novel splicing only detectable using RNA			
Sample ID	**Deletion / Fusion / Tandem Duplication**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**	**Novel RNA splicing**	**Deletion / Fusion**	**Chr**	**Transcript type / novel splicing**	**Comment**
Pt24										*BCR::ABL1*	22 and 9	e14e2	Fusion
										EXOSC2::BCR	9 and 22	e9e2	Ph-associated
Pt25										*BCR::ABL1*	22 and 9	Atypical transcript	Fusion
												Genomic break within *BCR* exon 13	
												Fusion transcript of partial *BCR* exon 13 fused to *ABL1* exon 2 with 20 nucleotides derived from an intergenic sequence upstream of *ABL1* between *BCR* and *ABL1*	
Pt26										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion
K562										*BCR::ABL1*	22 and 9	e14e2	Fusion
										NUP214::XKR3	9 and 22	e29e2	Fusion
Kasumi-1										*RUNX1::RUNX1T1*	21 and 8	e6e2	Fusion
										RUNX1T1::RUNX1	8 and 21	e8e6	Reciprocal fusion? RNA circle?
KCL22										*BCR::ABL1*	9 and 22	e13a2	Fusion
										ABL1::BCR	9 and 22	e1a14	Reciprocal fusion
ME1	*CBFB::MYH11*	*CBFB*	16	15815077		Inversion	Yes	Yes	No	*CBFB::MYH11*	16	e5e34	Fusion
		MYH11	16	67132445									
	MYH11::CBFB	*MYH11*	16	67132446		Reciprocal inversion	Yes	No	No				
		CBFB	16	15815076									
	MYC	*MYC*	8	128752858		Tandem duplication	Yes	Yes	No				
		MYC	8	128753194									

	Genomic breakpoints of deletions and other rearrangements									Fusion transcripts and novel splicing only detectable using RNA			
Sample ID	**Deletion / Fusion / Tandem Duplication**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Breakpoint detected using DNA**	**Breakpoint detected using RNA**	**Novel RNA splicing**	**Deletion / Fusion**	**Chr**	**Transcript type / novel splicing**	**Comment**
THP1/LAMA84										*BCR::ABL1*	22 and 9	e14e2	Fusion
										ABL1::BCR	9 and 22	e1e15	Reciprocal fusion
										KMT2A::MLLT3	11 and 9	e8e6	Fusion
										SNAPC3::KMT2A	9 and 11	e8e11	Reciprocal fusion inversion
BV173	*IKZF1*	*IKZF1*	7	50345516-50440831	95	Exon 2 to 3 deletion	Yes	Yes	Yes	*BCR::ABL1*	9 and 22	e13a2	Fusion
	IKZF1	*IKZF1*	7	50412881-50463667	51	Exon to 8 deletion	Yes	Yes	Yes	*ABL1::BCR*	9 and 22	e1a14	Reciprocal fusion
	IKZF1	*IKZF1*	7	50315285-50346013	31	Exon 1 deletion (non-coding exon)	Yes	No	No				
	CDKN2A/B	*CDKN2A/B*	9	21861306-22008310	147	*CDKN2A* deletion, *CDKN2B* exon 2 deletion	Yes	Yes	Yes				
	CDKN2A/B	*CDKN2A/B*	9	21973619-22008304	35	*CDKN2A* exon 1 deletion, *CDKN2B* exon 2 deletion	Yes	Yes	Yes				
Jurkat													

	Genomic breakpoints of deletions and other rearrangements									Fusion transcripts and novel splicing only detectable using RNA			
Sample ID	Deletion / Fusion / Tandem Duplication	Gene	Chr	Genomic coordinates (hg19)	Size (Kb)	Comment	Breakpoint detected using DNA	Breakpoint detected using RNA	Novel RNA splicing	Deletion / Fusion	Chr	Transcript type / novel splicing	Comment
REH	*BTG1*	*BTG1*	12	92278186-92538067	260	Exon 2 deletion	Yes	Yes	No	*ETV6::RUNX1*	12 and 21	e5e3	Fusion
										RUNX1::PRMD7	12 and 21	e2e4	Reciprocal Fusion
RS4;11	*KMT2A::AFF1*	*KMT2A*	11	118355075		Fusion	Yes	No	No	*KMT2A::AFF1*	11 and 4	e9e5	Fusion
		AFF1	4	88001854						*AFF1::KMT2A*	4 and 11	e4e10	Reciprocal Fusion
TOM1	*IKZF1*	*IKZF1*	7	50412894-50484462	72	Exon 4 to 8 deletion	Yes	Yes	Yes				
	CDKN2A	*CDKN2A*	9	21832257-21993825	162	Exon 2 to 3 deletion	Yes	Yes	Yes				
	PAX5	*PAX5*	9	36877489-37030694	153	Exon 2 to 8 deletion	Yes	Yes	Yes				
Z119	*IKZF1*	*IKZF1*	7	50346004-50463629	117	Inversion	Yes	Yes	No	*BCR::ABL1*	22 and 9	e1e2	Fusion
	IKZF1	*IKZF1*	7	50346008-50463630	117	Reciprocal inversion	Yes	Yes	No	*ABL1::BCR*	9 and 22	e1e2	Reciprocal fusion
	IKZF1	*IKZF1*	7	50362776-50484457	122	Exon 3 to 8 deletion	Yes	Yes	Yes				
	BTG1	*BTG1*	12	92205595-92538065	333	Exon 2 deletion	Yes	Yes	Yes				
	PAX5	*PAX5*	9	36929054-37030697	102	Exon 2 to 5 deletion	Yes	No	Yes				
	RB1	*RB1*	13	48984563-49074960	90	Exon 18 to 27 deletion	Yes	No	Yes				
	RB1	*RB1*	13	48984556-49176754	192	Exon 18 to 27 deletion	Yes	No	Yes				
	CDKN2A/B	*CDKN2A/B*	9	21902179-22008304	106	*CDKN2A* deletion, *CDKN2B* exon 2 deletion	Yes	Yes	No				
	CDKN2A/B	*CDKN2A/B*	9	21973636-22008306	35	*CDKN2A* exon 1 deletion, *CDKN2B* exon 2 deletion	Yes	Yes	No				
OM9:22	*IKZF1*	*IKZF1*	7	50412879-50463633	51	Exon 4 to 7 deletion	Yes	Yes	Yes	*BCR::ABL1*	22 and 9	e1e2	Fusion
	IKZF1	*IKZF1*	7	50518237-50412874	105	Exon 4 to 8 deletion	Yes	Yes	Yes	*ABL1::BCR*	9 and 22	e1e2	Reciprocal fusion
	BTG1	*BTG1*	12	92415562-92538064	122	Exon 2 deletion	Yes	Yes	No				
	CDKN2A	*CDKN2A*	9	21950793-21993815	43	Exon 2 to 3 deletion	Yes	Yes	Yes				
	RB1	*RB1*	13	48984564-49176732	192	Exon 18 to 27 deletion	Yes	No	Yes				

	Genomic breakpoints of deletions and other rearrangements									Fusion transcripts and novel splicing only detectable using RNA			
Sample ID	Deletion / Fusion / Tandem Duplication	Gene	Chr	Genomic coordinates (hg19)	Size (Kb)	Comment	Breakpoint detected using DNA	Breakpoint detected using RNA	Novel RNA splicing	Deletion / Fusion	Chr	Transcript type / novel splicing	Comment
K562/Kasumi-1										*BCR::ABL1*	22 and 9	e14e2	Fusion
										NUP214::XKR3	9 and 22	e29e2	Fusion
										RUNX1::RUNX1T1	21 and 8	e6e2	Fusion
										RUNX1T1::RUNX1	8 and 21	e8e6	Reciprocal fusion

	Summary of primary structural variant or novel splicing associated with SNVs/indels detected using DNA and/or RNA		
Sample ID	**Structural variant / Splice site variant and splicing effect**	**Structural variant / novel splicing detected using DNA**	**Structural variant / novel splicing detected using RNA**
Pt1	*BCR::ABL1* fusion	Yes	Yes
	Ph-associated rearrangement, inversion	Yes	Yes
	CLIC6::RUNX1 deletion	Yes	Yes
	HBS1L::MYB intergenic locus deletion	Yes	**No**
	IKZF1::IGK fusion	Yes	Yes
Pt2-S2	*BCR::ABL1* fusion	Yes	Yes
Pt2-S3	*BCR::ABL1* fusion	Yes	Yes
	IKZF1 exon 4-7 deletion	Yes	Yes
	IKZF1 exon 4 to 7 deletion (minor)	Yes	Yes
	IKZF1 exon 2-7 deletion	Yes	Yes
	RUNX1 c.508+2T>C splice donor variant: activation of cryptic splice donor site at 21:36252876 in exon 5 and predicted premature termination	**No**	Yes
Pt3-S1	*BCR::ABL1* fusion	Yes	Yes
	RUNX1 c.614-5860G>T: variant activated pseudo exon 21:36212688-36212743 and predicted premature termination (detected by novel splicing)	**No**	Yes
Pt4	*BCR::ABL1* fusion	Yes	Yes
	Ph-associated rearrangement, inversion	Yes	Yes
Pt5-S2	*BCR::ABL1* fusion	Yes	Yes
	IKZF1 exon 4-7 deletion	Yes	Yes
	HBS1L::MYB intergenic locus deletion	Yes	**No**
Pt22	*BCR::ABL1* fusion	Yes	Yes
	RUNX1::PRDM16 fusion	**No**	Yes
Pt23	*BCR::ABL1* fusion	Yes	Yes

Sample ID	Summary of primary structural variant or novel splicing associated with SNVs/indels detected using DNA and/or RNA Structural variant / Splice site variant and splicing effect	Structural variant / novel splicing detected using DNA	Structural variant / novel splicing detected using RNA
Pt24	*BCR::ABL1* fusion	**No**	Yes
	Ph-associated rearrangement	Yes	Yes
Pt25	*BCR::ABL1* fusion	**No**	Yes
	Ph-associated rearrangement	Yes	Yes
Pt26	*BCR::ABL1* fusion	Yes	Yes
K562	*BCR::ABL1* fusion	Yes	Yes
	Ph-associated rearrangement, inversion	Yes	Yes
Kasumi-1	*RUNX1::RUNX1T1* fusion	**No**	Yes
	CREBBP c.1676+3_1676+12del splice region intronic variant: skipping of exon 8 and predicted premature termination	**No**	Yes
KCL22	*BCR::ABL1* fusion	Yes	Yes
	KMT2D p.Cys279Cys/c.837C>T splice region synonymous variant: in-frame skipping of exon 6	**No**	Yes
ME1	*CBFB::MYH11* fusion	Yes	Yes
	MYC tandem duplication	Yes	Yes
	TP53 p.Gly302_Arg306del/c.905_919+32del splice donor + disruptive in-frame deletion: skipping of exon 8 and predicted premature termination	**No**	Yes
THP1/LAMA84	*BCR::ABL1* fusion	Yes	Yes
	KMT2A::MLLT3 fusion	**No**	Yes

	Summary of primary structural variant or novel splicing associated with SNVs/indels detected using DNA and/or RNA		
Sample ID	**Structural variant / Splice site variant and splicing effect**	**Structural variant / novel splicing detected using DNA**	**Structural variant / novel splicing detected using RNA**
BV173	*BCR::ABL1* fusion	Yes	Yes
	IKZF1 deletion	Yes	Yes
	IKZF1 deletion	Yes	Yes
	IKZF1 deletion	Yes	**No**
	CDKN2A/B deletion	Yes	Yes
	CDKN2A/B deletion	Yes	Yes
Jurkat	*TP53* p.Thr125Thr/c.375G>A splice region synonymous: activation of cryptic splice donor site at 17:7579511 in exon 4 and predicted premature termination	**No**	Yes
	NUP98 c.3512+1G>A splice donor variant: multiple effects, activation of cryptic splice donor site in exon 23 at 11:3724002, skipping of exons 23 and 24, skipping of exon 23 and predicted premature termination	**No**	Yes
REH	*ETV6::RUNX1* fusion	**No**	Yes
	BTG1 deletion	Yes	Yes

	Summary of primary structural variant or novel splicing associated with SNVs/indels detected using DNA and/or RNA		
Sample ID	**Structural variant / Splice site variant and splicing effect**	**Structural variant / novel splicing detected using DNA**	**Structural variant / novel splicing detected using RNA**
RS4;11	*KMT2A::AFF1* fusion	Yes	Yes
	EP300 c.4452+2T>G splice donor variant: skipping of exon 27 and predicted premature termination	**No**	Yes
TOM1	*IKZF1* deletion	Yes	Yes
	CDKN2A deletion	Yes	Yes
	PAX5 deletion	Yes	Yes
Z119	*BCR::ABL1* fusion	**No**	Yes
	IKZF1 inversion	Yes	Yes
	IKZF1 deletion	Yes	Yes
	BTG1 deletion	Yes	Yes
	PAX5 deletion	Yes	Yes
	RB1 deletion	Yes	Yes
	RB1 deletion	Yes	Yes
	CDKN2A/B deletion	Yes	Yes
	CDKN2A/B deletion	Yes	Yes
OM9:22	*BCR::ABL1* fusion	**No**	Yes
	IKZF1 deletion	Yes	Yes
	IKZF1 deletion	Yes	Yes
	BTG1 deletion	Yes	Yes
	CDKN2A deletion	Yes	Yes
	RB1 deletion	Yes	Yes
K562/Kasumi-1	*BCR::ABL1* fusion	Yes	Yes
	Ph-associated rearrangement, inversion	Yes	Yes
	RUNX1::RUNX1T1 fusion	**No**	Yes
	CREBBP c.1676+3_1676+12del splice region intronic variant: skipping of exon 8 predicted premature termination	**No**	Yes

Supplementary Table 11: Average read depth of 3 hot spot mutations sites across CEBPA for DNA and RNA in patient samples

***CEBPA* hot spot mutation site**	**COSMIC ID**	**DNA average reads**	**RNA average reads**
c.68dup:p.(H24Afs*84)	COSV57195669	410	521
c.247del:p.(Q83Sfs*77)	COSV57196263	379	206
c.937_939dup:p.(K313dup)	COSV57195607	1152	750

Supplementary Table 12: In silico model variant splicing prediction scores

DNA panel results shaded in red
RNA panel results shaded in green

Abbreviations
Pt = Patient
S = Sample
NC = Not called
Chr = Chromosome
VAF = Variant allele frequency

*In silico splicing prediction score changes. The score change from wild-type is indicated.
†RNA-seq only. DNA was sequenced using version 1 of the panel where ATM was not captured
[ ] indicates the scoring range for the individual prediction model, an increased score indicates a strengthened splice site, 0 indicates complete loss of splicing is predicted
Generally 3 of 4 in silico prediction models are required to derive a consensus outcome

Splicing events

											In silico splicing prediction score changes *						
Sample ID	**Gene**	**Chr**	**Genomic coordinates (GRCh37/hg19)**	**Transcript ID**	**Effect**	**SnpEff Annotation**	**Variants**	**DNA VAF%**	**RNA VAF%**	**SSF* [0-100]**	**MaxEnt* [0-12]**	**NNSPLICE* [0-1]**	**GeneSplicer* [0-24]**	**Predicted effect generated from Alamut based on in silico model assessment**	**Splicing consequences derived from RNA sequencing**	Figure demonstration
Pt2-S3	*RUNX1*	21	36252852	ENST00000300305	splice donor	HIGH	c.508+2T>C	36	47	86.16→86.97	10.2→0	0.99→0	4.29→0	The consequence of this change is not predictable, but a skip of exon 5 is very likely	Premature termination: Cryptic splice donor site utilized within exon 5	Figure 5
Pt3-S1	*RUNX1*	21	36212758	ENST00000300305	intron variant	MODIFIER	c.614-5860G>T	9	16	92.78→92.78	5.79→8.38	0.94→0.97	4.92→8.99	The consequence of this change is not predictable.	Premature termination: Creation of a variant activated pseudo-exon	Supplementary Figure 7
Pt9	*ATM*	11	108225537	ENST00000278616	splice acceptor	HIGH	c.8787-1G>A	Not evaluable	76†	87.32→0	9.74→0	0.97→0	9.19→0	Loss of splice acceptor site of exon 62. The consequence of this change is not predictable, but a skip of exon 62 is very likely.	Premature termination: Skipping of exon 61 and activation of intronic cryptic splice donor and acceptor sites resulting in pseudo-exon formation	Figure 4F
Kasumi-1	*CREBBP*	16	3831192	ENSG00000171456	splice region	LOW	c.1676+3_1676+12delAAGACCCTGT	100	100	82.23→0	6.91→0	0.99→0	3.48→0	Loss of splice donor site of exon 7. The consequence of this change is not predictable, but a skip of exon 7 is possible.	Premature termination: Skipping of exon 7 and intron 7 retention	Figure 4C
Kasumi-1/K562	*CREBBP*	16	3831192	ENSG00000171456	splice region	LOW	c.1676+3_1676+12delAAGACCCTGT	14	55	82.23→0	6.91→0	0.99→0	3.48→0	Loss of splice donor site of exon 7. The consequence of this change is not predictable, but a skip of exon 7 is possible.	Premature termination: Skipping of exon 7 and intron 7 retention	Figure 4C
KCL22	*KMT2D*	12	49447261	ENST00000301067	splice region + synonymous variant	LOW	p.Cys279Cys/c.837C>T	45	50	84.59→80.46	9.79→7.00	0.94→0.78	9.31→4.6	This variant does not alter the protein sequence although there may be a change to the splice donor site of exon 6.	Premature termination: Skipping of exon 6	Figure 4A
Jurkat	*TP53*	17	7579312	ENSG00000141510	splice region + synonymous variant	LOW	p.Thr125Thr/c.375G>A	43	83	80.90→0	8.02→0.29	0.59→0	5.90→0	The consequence of this change is not predictable, but a skip of exon 4 is very likely.	Premature termination: Intron 5 retention and activation of multiple exonic and intronic cryptic splice sites	Figure 4D
Jurkat	*NUP98*	11	3723692	ENST00000533346	splice donor	HIGH	c.3512+1G>A	49	85	73.11→0	9.40→0	0.92→0	-	The consequence of this change is not predictable, but a skip of exon 23 is very likely due to loss of the splice donor site of exon 23.	Premature termination: Single and two exon skipping, plus activation of an exonic cryptic splice site	Figure 4E
RS4;11	*EP300*	22	41566577	ENST00000263253	splice donor	HIGH	c.4452+2T>G	22	40	89.81→0	8.68→0	0.90→0	4.10→0	The consequence of this change is not predictable, but a skip of exon 27 is very likely due to loss of splice acceptor site of exon 26.	Premature termination: Skipping of exon 26 and activation of an exonic cryptic splice site variant within exon 26	Figure 4B
ME1	*TP53*	17	7576986	ENSG00000141510	splice donor variant + disruptive inframe deletion + splice region variant + intron variant	HIGH	p.Gly302_Arg306del/c.905_919+32del0	100	NC	91.17→0	9.86→0	1.00→0	10.74→0	This variation leads to the loss of 5 residues. Predicted change at donor site 0 bps upstream is -100.0% resulting in loss of splice donor site on exon 7	Premature termination: Skipping of exon 8 and intron 8 retention	Figure 6
KCL22	*SMC3*	10	112342306	ENST00000361804	splice region variant+ intron variant	LOW	c.724-6delT	49	71	86.4→86.1	6.2→4.7	1	3.8→3.3	The consequence of this change is not predictable, but a skip of exon 10 is possible.	No effect	
KCL22	*XPO1*	2	61719891	ENST00000401558	splice region variant + intron variant	LOW	c.1385-8C>T	49	50	87.3→87.2	7.8 → 7.2	1	8.5 → 7.8	The consequence of this change is not predictable.	No effect	
KCL22	*EP300*	22	41527386	ENST00000263253	splice region variant + intron variant	LOW	c.1283-6C>G	46	50	84.1→83.4	9.9→8.6	1.0→0.8	8.8→6.7	The consequence of this change is not predictable.	No effect	
KCL22	*KMT2D*	12	49439699	ENST00000301067	splice region variant + intron variant	LOW	c.4741+4A>G	50	46	95.6→85.6	10.7→8.6	1.0→0.9	10.3→3.7	The consequence of this change is not predictable.	No effect	
Kasumi	*SETD1B*	12	122243128	ENST00000267197	splice region variant + intron variant	LOW	c.273+6A>C	44	36	84.3→83.8	9.5→10.1	1	9.3→9.4	The consequence of this change is not predictable.	No effect	

Supplementary Table 13: Recall rate of variants using RNA
Additional variants detected using the gene panel are indicated in blue text
RNA panel results shaded in green

Abbreviations
Pt = Patient
S = Sample
Chr = Chromosome
ND = Not detected
NT = Not tested for that sample
VAF = Variant allele frequency
MBC = Myeloid blast crisis
LBC = Lymphoid blast crisis

† VAF derived from whole exome sequencing data using DNA
‡ Where a unique variant was detected using the targeted gene panel for a sample without prior NGS, the variant was considered verified if the same variant was detected in another sample from the same patient
§ Deletion identified by atypical splicing only
‖ Atypical splicing was not detected, breakpoints detectable

1. Not called by Vardict but probes did not cover this region of *RUNX1* in the design version. When manually examined, the mutation is present
2. Poor read coverage over this region

										***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**					
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37)/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**Pre-characterized VAF**	**Gene panel VAF**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**
Pt1	LBC	*BCORL1*	X	129147354	ENST00000218147	Frameshift	p.(Ile203Trpfs*52)/c.606_607insTGGGC	100	70	*BCR::ABL1* genomic	*BCR*	22	23634010		Yes
		ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.756G>C	29	29		*ABL1*	9	133641245		
		ABL1	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	19	17						
		ABL1	9	133738357	ENST00000318560	Missense	p.(Tyr253His)/c.757T>C	15 (Sanger)	9	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
		ABL1	9	133738363	ENST00000318560	Missense	p.(Glu255Lys)/c.763G>A	20 (Sanger)	24		*ABL1*	9	133729450		
		SMC3	10	112361923	ENST00000361804	Nonsense	p.(Leu1031*)/c.3092T>A	1.5	2						
		ABL1	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.764A>T	ND	3.3	*ABL1* ::17q12 transcript	*ABL1*	9	133589843	Ph-associated	Yes
		EZH2	7	148506461	ENST00000320356	Missense	p.(Arg684His)/c.2051G>A	ND	4.2		intergenic	17	36309399	inversion	
		ABL1	9	133738356	ENST00000318560	Missense	p.(Phe359Val)/c.10757>G	ND	1.8						
										ABL1 ::17q12 transcript	*ABL1*	9	133641030	Ph-associated	Yes
											Intergenic	17	36310216	inversion	
Pt2-S2	Diagnosis	*ASXL1*	20	31024043	ENST00000375687	Frameshift	p.(Leu1177Profs*5)/c.3529dup	9	3.5	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes
		SETD1B	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	4	5.3		*ABL1*	9	133704830		
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	ND	1.2						
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt2-S3	MBC - 1st	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	52†	40	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	51†	45		*ABL1*	9	133704830		
		PHF6	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	91†	89						
		RUNX1	21	36252852	ENST00000300305	Splice donor	c.508+2T>C	41†	47	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt2-S4	MBC - 2nd	*SETD1B*	12	122255792	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T	31	27	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes
		U2AF1	21	44514777	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G	33	32		*ABL1*	9	133704830		
		PHF6	X	133551322	ENST00000332070	Missense	p.(Gly320Arg)/c.958G>A	75	69						
		RUNX1	21	36252852	ENST00000300305	Splice donor	c.508+2T>C	6	6	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
		ABL1	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	ND	7.9		*ABL1*	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt3-S1	Diagnosis	*RUNX1*	21	36212758	ENST00000300305	Intronic splice altering	c.614-5860G>T	ND	ND[1]	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes
											ABL1	9	133597820		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		

										***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**					
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37)/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**Pre-characterized VAF**	**Gene panel VAF**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**
Pt3-S2	LBC - 2nd	*ABL1*	9	133755488	ENST00000318560	Missense	p.(Phe486Ser)/c.1514T>C	32†	82	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes
		ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>T	57†	52		*ABL1*	9	133597820		
		ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>C	10†	29						
		RUNX1	21	36212758	ENST00000300305	Intronic variant	c.614-5860G>T	44†	42	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt3-S3	LBC - 3rd	*ABL1*	9	133755488	ENST00000318560	Missense	p.(Phe486Ser)/c.1514T>C	100 (Sanger)	77	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes
		ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>C	40 (Sanger)	32		*ABL1*	9	133597820		
		ABL1	9	133738364	ENST00000318560	Missense	p.(Glu255Val)/c.821A>T	75 (Sanger)	54						
		ABL1	9	133738356	ENST00000318560	Missense	p.(Gln252His)/c.813G>C	20 (Sanger)	19	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
		RUNX1	21	36212758	ENST00000300305	Intronic splice altering	c.614-5860G>T	NT	37‡		*ABL1*	9	133729450		
		ABL1	9	133747588	ENST00000318560	Missense	p.(Val299Leu)/c.952G>T	ND (Sanger)	2						
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt4	CP	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.944C>T	21	29	*BCR::ABL1* genomic	*BCR*	22	23634569		Yes
	treatment	*ASXL1*	20	31022441	ENST00000375687	Frameshift	p.(Gly646Trpfs*12)/c.1934dup	19	ND[2]		*ABL1*	9	133615099		
	failure														
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1:: intergenic chr 7q	*ABL1*	9	133589843	Ph-associated	Yes
											intergenic chr 7q	7	142208742	reciprocal inversion	
Pt5-S2	LBC	*ABL1*	X	129155104	ENST00000318560	Missense	p.(Gly250Glu)/c.749G>A	82	86	*BCR::ABL1* genomic	*BCR*	22	23633455		Yes
		BCORL1	9	133738349	ENST00000218147	Missense	p.(Arg1196*)/c.3586C>T	95	97		*ABL1*	9	133685776		
		RUNX1	21	36231860	ENST00000300305	Missense	p.(Leu175Gln)/c.524T>A	31	25						
		RUNX1	21	36231855	ENST00000300305	Frameshift	p.(Ile177Serfs*34)/c.528del	25	22	*ABL1::BCR* genomic	*ABL1*	9	133685778		Additional
											BCR	22	23633457		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt12	Diagnosis									*BCR::NFIX* genomic	*BCR*	22	23633094	Ph-associated	Yes
											NFIX	19	13115916		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::LLGL2 transcript	*ABL1*	9	133589843	Ph-associated	Yes
											LLGL2	17	73539477		

										***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**					
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37)/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**Pre-characterized VAF**	**Gene panel VAF**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**
Pt13	Diagnosis	*ASXL1*	20	31022592	ENST00000375687	Nonsense	p.(Arg693*)/c.2077C>T	45	39	*BCR::ABL1* genomic	*BCR*	22	23631881		Yes
											ABL1	9	133730133		
										BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes
											ABL1	9	133730187		
										TNRC6B::NEK6 genomic	*TNRC6B*	22	40580783	Ph-associated	Yes
											NEK6	9	127112860		
										NEK6::TNRC6B genomic	*NEK6*	9	1237112862	Ph-associated	Yes
											TNRC6B	22	40580785		
										URM1::GGT1 genomic	*URM1*	9	131150170	Ph-associated	No
											GGT1	22	25003751	inversion	
										ST13:: 9q34.11 genomic	*ST13*	22	41247301	Ph-associated	No
											Intergenic 5' of *ASS1*	9	133313307	inversion	
										TNRC6B::NEK6 transcript	*TNRC6B*	22	40574145	Ph-associated	Yes
											NEK6	9	127113115		
Pt14	Diagnosis									*BCR::ABL1* genomic	*BCR*	22	23632144		Yes
											ABL1	9	133708168		
										ABL1::BCR genomic	*ABL1*	9	133707916	Ph-associated	Yes
											BCR	22	23636599	inversion	
										BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes
											ABL1	9	133729450		
										MYH9::BCR transcript	MYH9	22	36705326	Ph-associated	Yes
											BCR	22	23637210	inversion	
										MYH9::BCR transcript	MYH9	22	36705326	Ph-associated	Yes
											BCR	22	23632525	inversion	
										MYH9::BCR transcript	MYH9	22	36703827	Ph-associated	Yes
											BCR	22	23636274	inversion	
Pt15	Diagnosis									*BCR::ABL1* genomic	*BCR*	22	23633758		Yes
											ABL1	9	133680224		
										ABL1::BCR genomic	*ABL1*	9	133680221		Yes
											BCR	22	23633758		
														ENST00000375687:c.1934dupG	
										BCR::ABL1 transcript	*BCR*	22	236332601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		

										***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**					
Sample ID	**Disease phase**	**Gene**	**Chr**	**Genomic coordinates (GRCh37)/hg19)**	**Transcript ID**	**Effect**	**Expected variants**	**Pre-characterized VAF**	**Gene panel VAF**	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**
Pt16	Accelerated	*BCOR*	X	39933593	ENST00000378444	Frameshift	p.(Ser336Leufs*45)/c.1005dup	NT	42‡	*BCR::ABL1* genomic	*BCR*	22	23633152		Yes
	phase	*ABL1*	9	133748283	ENST00000318560	Missense	p.(Thr315Ile)/c.1001C>T	75 (Sanger)	23		*ABL1*	9	133589720		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
Pt17	Diagnosis									*BCR::ABL1* genomic	*BCR*	22	23634297		Yes
											ABL1	9	133592378		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt18	Diagnosis									*BCR::ABL1* transcript	*BCR*	22	23631809	e13e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	23634727		
Pt19	Diagnosis									*BCR::ABL1* genomic	*BCR*	22	23631991		Yes
											ABL1	9	133614844		
										BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e14	Yes
											BCR	22	23632525		
Pt20	LBC	*ABL1*	9	133750330	ENST00000318560	Missense	p.(Leu387Phe)/c.1218G>T	48	51	*BCR::ABL1* genomic	*BCR*	22	23634692		Yes
		IDH2	15	90631934	ENST00000330062	Missense	p.(Arg140Gln)/c.419G>A	41	40		*ABL1*	9	133719753		
										ABL1::BCR genomic	*ABL1*	9	133719747		Yes
											BCR	22	23634686		
										BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes
											ABL1	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	13634727		
Pt21	MBC	*GATA2*	3	128200116	ENST00000341105	Frameshift	p.(Asn397Thrfs*80)/c.1188del	77	79	*BCR::ABL1* genomic	*BCR*	22	23633027		Yes
		GATA2	3	128200766	ENST00000341105	In-frame deletion	p.(Ala341_Gly346del)/c.1021_1038del	72	70		*ABL1*	9	133652394		
		TET2	4	106180795	ENST00000540549	Missense	p.(Gly1275Arg)/c.3823G>C	15	15						
		DNMT3A	2	25469965	ENST00000264709	Nonsense	p.(Tyr359*)/c.1077C>A	ND	3.7	*BCR::ABL1* transcript	*BCR*	22	23632601	e14e2	Yes
		ABL1	9	133748414	ENST00000318560	Missense	p.(Phe359Val)/c.1075T>G	ND	1.9		*ABL1*	9	133729450		
										ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes
											BCR	22	13634727		

															Summary of pre-characterized primary structural variant detected using DNA and the gene panel	
	***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**						**Genomic breakpoints of focal deletions and other rearrangements**									
Sample ID	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Detected by gene panel**		**Pre-characterized rearrangement**	**Rearrangement detected using RNA**
Pt1	*BCR::ABL1* genomic	*BCR*	22	23634010		Yes	*CLIC6::RUNX1*[ll]	*CLIC6*	21	36080470	34	deletion	Yes		*BCR::ABL1* fusion	Yes
		ABL1	9	133641245				*RUNX1*	21	36421160					Ph-associated rearrangement, inversion	Yes
															CLIC6::RUNX1 deletion	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes	*HBS1L::MYB* intergenic locus	*HBS1L*	6	135366696	71	deletion	No		*IKZF1::IGKV3-7* fusion	Yes
		ABL1	9	133729450				Intergenic	6	135437658					*HBS1L::MYB* intergenic locus deletion	No
	ABL1 ::17q12 transcript	*ABL1*	9	133589843	Ph-associated	Yes	*IKZF1::IGKV3-7* intergenic genomic	*IKZF1*	7	50348584		interchromosomal fusion	Yes			
		intergenic	17	36309399	inversion			*IGKV3-7*	2	89277898						
	ABL1 ::17q12 transcript	*ABL1*	9	133641030	Ph-associated	Yes	*IKZF1::IGKV3-7* intergenic transcript	*IKZF1*	7	50348503		interchromosomal fusion transcript	Yes			
		Intergenic	17	36310216	inversion			*IGKV3-7* intergenic	2	89285709						
							IKZF1::IGKV3-7 intergenic alternate transcript	*IKZF1*	7	50348503		interchromosomal fusion transcript	Yes			
								IGKV3-7 intergenic	2	89285712						
Pt2-S2	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes									*BCR::ABL1* fusion	Yes
		ABL1	9	133704830												
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes										
		ABL1	9	133729450												
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes										
		BCR	22	23634727												
Pt2-S3	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes	*IKZF1*	*IKZF1*	7	50412895-50463650	51	Exon 4 to 7 deletion (predominant)	Yes		*BCR::ABL1* fusion	Yes
		ABL1	9	133704830											*IKZF1* exon 4-7 deletion	Yes
							IKZF1	*IKZF1*	7	50412895-50463630	116	Exon 4 to 7 deletion (minor)	Yes		*IKZF1* exon 4 to 7 deletion (minor)	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes										
		ABL1	9	133729450			*IKZF1*	*IKZF1*	7	50347391-50463629	116	Exon 2 to 7 deletion	Additional		Additional	
															IKZF1 exon 2-7 deletion	
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes	Atypical splicing associated with the deletions									
		BCR	22	23634727												
Pt2-S4	*BCR::ABL1* genomic	*BCR*	22	23633088		Yes	*IKZF1*	*IKZF1*	7	50347391-50463629	116	Exon 2 to 7 deletion	Yes		*BCR::ABL1* fusion	Yes
		ABL1	9	133704830			Atypical splicing associated with the deletion								*CBFB::MYH11* fusion	Yes
													Yes		*IKZF1* exon 2-7 deletion	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes	*CBFB::MYH11* fusion	*CBFB*	16	67116212						
		ABL1	9	133729450				*MYH11*	16	15814909						
													Additional		Additional	
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes	*MYH11::CBFB* reciprocal fusion	*MYH11*	16	15718625					*MYH11::CBFB* reciprocal fusion	
		BCR	22	23634727				*CBFB*	16	67063629						
Pt3-S1	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes									*BCR::ABL1* fusion	Yes
		ABL1	9	133597820												
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes										
		ABL1	9	133729450												
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes										
		BCR	22	23634727												

														Summary of pre-characterized primary structural variant detected using DNA and the gene panel	
	***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**						**Genomic breakpoints of focal deletions and other rearrangements**								
Sample ID	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Detected by gene panel**	**Pre-characterized rearrangement**	**Rearrangement detected using RNA**
Pt3-S2	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes	*IKZF1*	*IKZF1*	7	50412884-50463636	51	Exon 4 to 7 deletion	Yes	*BCR::ABL1* fusion	Yes
		ABL1	9	133597820			Atypical splicing associated with the deletion							*IKZF1* exon 4-7 deletion	Yes
														HBS1L::MYB intergenic locus deletion	No
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes	*HBS1L::MYB* intergenic locus	*HBS1L*	6	135366118	72	intergenic deletion	No		
		ABL1	9	133729450				Intergenic	6	135437668					
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											
Pt3-S3	*BCR::ABL1* genomic	*BCR*	22	23632899		Yes	*IKZF1*	*IKZF1*	7	50412884-50463636	51	Exon 4 to 7 deletion	Yes	*BCR::ABL1* fusion	Yes
		ABL1	9	133597820			Atypical splicing associated with the deletion							*IKZF1* exon 4-7 deletion	Yes
														HBS1L::MYB intergenic locus deletion	No
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes	*HBS1L::MYB* intergenic locus	*HBS1L*	6	135366118	72	intergenic deletion	No		
		ABL1	9	133729450				Intergenic	6	135437668					
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											
Pt4	*BCR::ABL1* genomic	*BCR*	22	23634569		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133615099										Ph-associated rearrangement, inversion	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1:: intergenic chr 7q	*ABL1*	9	133589843	Ph-associated reciprocal inversion	Yes									
		intergenic chr 7q	7	142208742											
Pt5-S2	*BCR::ABL1* genomic	*BCR*	22	23633455		Yes	*IKZF1*	*IKZF1*	7	50412896-50463633	51	Exon 4 to 7 deletion	Yes	*BCR::ABL1* fusion	Yes
		ABL1	9	133685776			Atypical splicing associated with the deletion							*IKZF1* exon 4-7 deletion	Yes
	ABL1::BCR genomic	*ABL1*	9	133685778		Additional									
		BCR	22	23633457											
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											

														Summary of pre-characterized primary structural variant detected using DNA and the gene panel	
	***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**						**Genomic breakpoints of focal deletions and other rearrangements**								
Sample ID	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Detected by gene panel**	**Pre-characterized rearrangement**	**Rearrangement detected using RNA**
Pt12	*BCR::NFIX* genomic	*BCR*	22	23633094	Ph-associated	Yes								*BCR::ABL1* fusion	Yes
		NFIX	19	13115916										Ph-associated rearrangement	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::LLGL2 transcript	*ABL1*	9	133589843	Ph-associated	Yes									
		LLGL2	17	73539477											
Pt13	*BCR::ABL1* genomic	*BCR*	22	23631881		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133730133										Ph-associated rearrangement, inversion	Yes
	BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes									
		ABL1	9	133730187											
	TNRC6B::NEK6 genomic	*TNRC6B*	22	40580783	Ph-associated	Yes									
		NEK6	9	127112860											
	NEK6::TNRC6B genomic	*NEK6*	9	1237112862	Ph-associated	Yes									
		TNRC6B	22	40580785											
	URM1::GGT1 genomic	*URM1*	9	131150170	Ph-associated	No									
		GGT1	22	25003751	inversion										
	*ST13::*9q34.11 genomic	*ST13*	22	41247301	Ph-associated	No									
		Intergenic 5' of *ASS1*	9	133313307	inversion										
	TNRC6B::NEK6 transcript	*TNRC6B*	22	40574145	Ph-associated	Yes									
		NEK6	9	127113115											
Pt14	*BCR::ABL1* genomic	*BCR*	22	23632144		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133708168										Ph-associated rearrangement, inversion	Yes
	ABL1::BCR genomic	*ABL1*	9	133707916	Ph-associated	Yes									
		BCR	22	23636599	inversion										
	BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes									
		ABL1	9	133729450											
	MYH9::BCR transcript	MYH9	22	36705326	Ph-associated	Yes									
		BCR	22	23637210	inversion										
	MYH9::BCR transcript	MYH9	22	36705326	Ph-associated	Yes									
		BCR	22	23632525	inversion										
	MYH9::BCR transcript	MYH9	22	36703827	Ph-associated	Yes									
		BCR	22	23636274	inversion										

															Summary of pre-characterized primary structural variant detected using DNA and the gene panel	
	***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**						**Genomic breakpoints of focal deletions and other rearrangements**									
Sample ID	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Detected by gene panel**	**Pre-characterized rearrangement**	**Rearrangement detected using RNA**
Pt15	*BCR::ABL1* genomic	*BCR*	22	23633758		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133680224										Ph-associated rearrangement	Yes
	ABL1::BCR genomic	*ABL1*	9	133680221		Yes									
		BCR	22	23633758											
	BCR::ABL1 transcript	*BCR*	22	236332601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											
Pt16	*BCR::ABL1* genomic	*BCR*	22	23633152		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133589720											
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
Pt17	*BCR::ABL1* genomic	*BCR*	22	23634297		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133592378											
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											
Pt18	*BCR::ABL1* transcript	*BCR*	22	23631809	e13e2	Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	23634727											
Pt19	*BCR::ABL1* genomic	*BCR*	22	23631991		Yes								*BCR::ABL1* fusion	Yes
		ABL1	9	133614844											
	BCR::ABL1 transcript	*BCR*	22	23631809	e13e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e14	Yes									
		BCR	22	23632525											

															Summary of pre-characterized primary structural variant detected using DNA and the gene panel	
	***BCR-ABL1* genomic breakpoints, *BCR-ABL1* transcripts and Ph-associated rearrangements**						**Genomic breakpoints of focal deletions and other rearrangements**									
Sample ID	**Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Comment and transcript type**	**Detected by gene panel**	**Deletion / Fusion**	**Gene**	**Chr**	**Genomic coordinates (hg19)**	**Size (Kb)**	**Comment**	**Detected by gene panel**	**Pre-characterized rearrangement**	**Rearrangement detected using RNA**
Pt20	*BCR::ABL1* genomic	*BCR*	22	23634692		Yes	*CDKN2A/B*[ǁ]	*CDKN2A/B*	9	21902166-22008313	106	Deletion of *CDKN2A/B*	No	*BCR::ABL1* fusion	Yes
		ABL1	9	133719753										*CDKN2A/B* deletion	Yes
	ABL1::BCR genomic	*ABL1*	9	133719747		Yes									
		BCR	22	23634686											
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	13634727											
Pt21	*BCR::ABL1* genomic	*BCR*	22	23633027		Yes	*RUNX1*	*RUNX1*	21	36392146-36201259	191	Exon 3 to 7 deletion	Yes[§]	*BCR::ABL1* fusion	Yes
		ABL1	9	133652394			Atypical splicing associated with the deletion							*RUNX1* deletion	Yes
	BCR::ABL1 transcript	*BCR*	22	23632601	e14e2	Yes									
		ABL1	9	133729450											
	ABL1::BCR transcript	*ABL1*	9	133589843	e1e15	Yes									
		BCR	22	13634727											

Supplementary Table 14: Intra- and inter-assay analysis using RNA: Structural variants

Abbreviations

Det = Detected

Chr = chromosome

Sample 1 of the intra-assay analysis was used for run 1 of the inter-assay analysis

Unlike the DNA samples, Manta was not used to detect genomic breakpoints and normalised read counts are not available either

Sample ID	Gene/s	First break	Second break	Type	Predicted RNA effect for genomic events	Intra-assay reproducibility			Inter-assay repeatability		
						Detection		Concordant detection	Detection		Concordant detection
						1	2	across the 2 runs?	1	2	across the 2 replicates?
Pt16	*BCR::ABL1* genomic break	chr22:23633094	chr9:133591769	genomic fusion	*BCR::ABL1* e14a2 transcript	Det	Det	Yes			
Pt16	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript		Det	Det	Yes			
Pt23	*BCR::ABL1* genomic break	chr22:23632880	chr9:133715120	genomic fusion	*BCR::ABL1* e14a2 transcript	Det	Det	Yes			
Pt23	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript		Det	Det	Yes			
Pt23	*ABL1::BCR* transcript	chr9:133589843	chr22:23634727	e1e15 reciprocal transcript		Det	Det	Yes			
Pt24	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript		Det	Det	Yes			
Pt24	*EXOSC2::BCR* transcript	chr9:133579736	chr22:23634727	3'UTRe15 reciprocal transcript		Det	Det	Yes			
Pt2-S2	*BCR::ABL1* genomic break	chr22:23633088	chr9:133704830	genomic fusion	*BCR::ABL1* e14a2 transcript				Det	Det	Yes
Pt2-S2	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript					Det	Det	Yes
Pt2-S2	*ABL1::BCR* transcript	chr9:133589843	chr22:23634727	e1e15 reciprocal transcript					Det	Det	Yes
Pt5-S2	*BCR::ABL1* genomic break	chr22:23633455	chr9:133685776	genomic fusion	*BCR::ABL1* e14a2 transcript				Det	Det	Yes
Pt5-S2	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript					Det	Det	Yes
Pt5-S2	*ABL1::BCR* transcript	chr9:133589843	chr22:23634727	e1e15 reciprocal transcript					Det	Det	Yes
Pt5-S2	*IKZF1* deletion	chr7:50412896	chr7:50463633	exon 4-7 deletion	*IKZF1* e3e8 transcript				Det	Det	Yes
Pt22	*BCR::ABL1* genomic break	chr22:23632880	chr9:133629142	genomic fusion	*BCR::ABL1* e14a2 transcript				Det	Det	Yes
Pt22	*BCR::ABL1* transcript	chr22:23632601	chr9:133729450	e14a2 transcript					Det	Det	Yes
Pt22	*ABL1::BCR* transcript	chr9:133589843	chr22:23634727	e1e15 reciprocal transcript					Det	Det	Yes
Pt22	*BCR::ABL1* alternate transcript	chr22:23631809	chr9:133729450	e13a2 alternate transcript					Det	Det	Yes
Pt22	*RUNX1::PRDM16* fusion	chr21:36421138	chr1:3301715	e2e4 transcript					Det	Det	Yes

Supplementary Table 15: Intra- and inter-assay analysis using RNA: SNVs and indels

Abbreviations

ND = Not detected
Chr = Chromosome
CV = coefficient of variation
SD = standard deviation

Variants in blue met criteria for clinical significance
The other variants are likely germline

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt16	X	39933593	*BCOR*	ENST00000378444	Frameshift	p.(Ser336Leufs*45)/c.1005dup	41.77	40.8	41.3	0.7	1.6					
Pt16	9	133748283	*ABL1*	ENST00000372348	Missense	p.(Thr315Ile)/c.1001C>T	22.54	27.5	25.0	3.5	14.1					
Pt16	11	108180917	*ATM*	ENST00000278616	Synonymous	p.(Ala1931Ala)/c.5793T>C	99.38	99.5	99.4	0.1	0.1					
Pt16	11	108119695	*ATM*	ENST00000278616	Synonymous	p.(Ser367Ser)/c.1101T>A	46.36	48.1	47.2	1.2	2.6					
Pt16	X	100608191	*BTK*	ENST00000308731	Synonymous	p.(Cys633Cys)/c.1899C>T	99.39	99.5	99.4	0.1	0.1					
Pt16	11	119168129	*CBL*	ENST00000264033	Missense	p.(Thr730Met)/c.2189C>T	49.64	53.7	51.7	2.9	5.5					
Pt16	16	3778427	*CREBBP*	ENST00000262367	Synonymous	p.(Gln2207Gln)/c.6621A>G	99.44	98.7	99.1	0.5	0.5					
Pt16	16	3900336	*CREBBP*	ENST00000262367	Missense	p.(Ala254Thr)/c.760G>A	49.77	51.1	50.4	1.0	1.9					
Pt16	16	3820723	*CREBBP*	ENST00000262367	Missense	p.(Thr910Ser)/c.2728A>T	48.9	48.3	48.6	0.4	0.9					
Pt16	16	3860640	*CREBBP*	ENST00000262367	Synonymous	p.(Asp313Asp)/c.939T>C	47.87	48.9	48.4	0.7	1.5					
Pt16	16	3795363	*CREBBP*	ENST00000262367	Missense	c.3837-8C>T	44.37	38.4	41.4	4.2	10.2					
Pt16	7	101755060	*CUX1*	ENST00000360264	Missense	c.640+6A>G	99.57	100.0	99.8	0.3	0.3					
Pt16	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A	99.8	99.8	99.8	0.0	0.0					
Pt16	7	101844851	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G	99.88	99.7	99.8	0.1	0.1					
Pt16	11	85968623	*EED*	ENST00000263360	Synonymous	p.(Leu207Leu)/c.619C>T	48.64	48.3	48.5	0.2	0.5					
Pt16	22	41513263	*EP300*	ENST00000263253	Missense	p.(Thr56Ser)/c.167C>G	52.63	53.1	52.9	0.4	0.7					
Pt16	7	50467767	*IKZF1*	ENST00000439701	Synonymous	p.(Pro292Pro)/c.876C>A	46.89	47.7	47.3	0.6	1.2					
Pt16	7	50435777	*IKZF1*	ENST00000439701	Synonymous	p.(Pro78Pro)/c.234T>G	44.21	45.6	44.9	1.0	2.2					
Pt16	1	65310489	*JAK1*	ENST00000342505	Synonymous	p.(Pro733Pro)/c.2199A>G	46.94	46.9	46.9	0.1	0.1					
Pt16	1	65321388	*JAK1*	ENST00000342505	Missense	c.1459-7C>T	44.43	44.4	44.4	0.0	0.0					
Pt16	12	402167	*KDM5A*	ENST00000399788	Missense	p.(Ala1542Ser)/c.4624G>T	44.24	46.3	45.3	1.5	3.2					
Pt16	X	44929077	*KDM6A*	ENST00000377967	Missense	p.(Thr726Lys)/c.2177C>A	43.17	43.6	43.4	0.3	0.7					
Pt16	4	55602765	*KIT*	ENST00000288135	Synonymous	p.(Leu862Leu)/c.2586G>C	45.41	48.0	46.7	1.8	3.9					
Pt16	4	55593464	*KIT*	ENST00000288135	Missense	p.(Met541Leu)/c.1621A>C	41.52	43.5	42.5	1.4	3.3					
Pt16	4	55598096	*KIT*	ENST00000288135	Missense	p.(Asp765Tyr)/c.2293G>T	45.93	43.2	44.6	1.9	4.4					
Pt16	12	49424534	*KMT2D*	ENST00000301067	Synonymous	p.(Pro4563Pro)/c.13689C>T	99.73	99.5	99.6	0.1	0.1					
Pt16	6	135513637	*MYB*	ENST00000341911	Missense	p.(Thr235Pro)/c.703A>C	46.62	46.9	46.8	0.2	0.5					
Pt16	17	29553485	*NF1*	ENST00000358273	Synonymous	p.(Pro678Pro)/c.2034G>A	48.35	48.9	48.6	0.4	0.9					
Pt16	18	42643270	*SETBP1*	ENST00000282030	Missense	p.(Glu1466Asp)/c.4398G>T	51.72	45.3	48.5	4.5	9.3					
Pt16	18	42532693	*SETBP1*	ENST00000282030	Missense	p.(Pro1130Thr)/c.3388C>A	48.59	43.0	45.8	4.0	8.7					
Pt16	3	47162886	*SETD2*	ENST00000409792	Missense	p.(Met1080Ile)/c.3240G>A	99.84	99.8	99.8	0.0	0.0					
Pt16	19	11145691	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1351Asp)/c.4053C>T	50.07	50.2	50.2	0.1	0.2					
Pt16	19	11105608	*SMARCA4*	ENST00000429416	Synonymous	p.(His508His)/c.1524T>C	46.62	47.7	47.2	0.8	1.7					
Pt16	4	106196951	*TET2*	ENST00000540549	Missense	p.(Ile1762Val)/c.5284A>G	99.68	99.9	99.8	0.1	0.1					
Pt16	11	32417945	*WT1*	ENST00000332351	Synonymous	p.(Arg369Arg)/c.1107A>G	100	98.9	99.5	0.7	0.8					

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt23	1	36937065	*CSF3R*	ENST00000373103	Synonymous	p.(Arg418Arg)/c.1254T>C	99.36	99.1	99.2	0.2	0.2					
Pt23	X	44929077	*KDM6A*	ENST00000377967	Missense	p.(Thr726Lys)/c.2177C>A	99.17	99.3	99.2	0.1	0.1					
Pt23	X	39932907	*BCOR*	ENST00000378444	Synonymous	p.(Ala564Ala)/c.1692A>G	98.67	99.7	99.2	0.7	0.7					
Pt23	4	106196829	*TET2*	ENST00000540549	Missense	p.(Leu1721Trp)/c.5162T>G	97.97	100.0	99.0	1.4	1.5					
Pt23	X	129147373	*BCORL1*	ENST00000540052	Missense	p.(Gly209Ser)/c.625G>A	91.72	96.1	93.9	3.1	3.3					
Pt23	2	25469502	*DNMT3A*	ENST00000264709	Synonymous	p.(Leu422Leu)/c.1266G>A	97.12	99.4	98.3	1.6	1.7					
Pt23	7	101844851	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G	49.14	62.2	55.6	9.2	16.5					
Pt23	12	25398295	*KRAS*	ENST00000256078	Synonymous	p.(Val8Val)/c.24A>G	51.21	42.9	47.0	5.9	12.6					
Pt23	10	112349422	*SMC3*	ENST00000361804	Synonymous	p.(Tyr455Tyr)/c.1365T>C	51.02	48.0	49.5	2.2	4.4					
Pt23	2	209108317	*IDH1*	ENST00000415913	Missense	p.(Val178Ile)/c.532G>A	48.08	48.2	48.1	0.1	0.1					
Pt23	19	11105608	*SMARCA4*	ENST00000429416	Synonymous	p.(His508His)/c.1524T>C	48.92	48.2	48.6	0.5	1.0					
Pt23	12	406292	*KDM5A*	ENST00000399788	Synonymous	p.(Ser1383Ser)/c.4149C>T	46.72	37.1	41.9	6.8	16.2					
Pt23	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A	49.17	54.1	51.7	3.5	6.8					
Pt23	19	11170839	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1629Asp)/c.4887T>C	48.66	48.2	48.4	0.3	0.7					
Pt23	17	29553485	*NF1*	ENST00000358273	Synonymous	p.(Pro678Pro)/c.2034G>A	49.67	44.6	47.1	3.6	7.6					
Pt23	4	106156187	*TET2*	ENST00000540549	Missense	p.(Pro363Leu)/c.1088C>T	47.06	49.9	48.5	2.0	4.1					
Pt23	22	23523630	*BCR*	ENST00000305877	Synonymous	p.(Ile161Ile)/c.483C>A	47.43	50.0	48.7	1.8	3.7					
Pt23	2	25536827	*DNMT3A*	ENST00000264709	Synonymous	p.(Pro9Pro)/c.27C>T	46.83	55.8	51.3	6.3	12.3					
Pt23	12	12037444	*ETV6*	ENST00000396373	Synonymous	p.(Arg359Arg)/c.1075C>A	45.74	48.6	47.2	2.0	4.3					
Pt23	12	49427919	*KMT2D*	ENST00000301067	Synonymous	p.(Pro3557Pro)/c.10671A>G	45.79	44.8	45.3	0.7	1.6					
Pt23	1	65311262	*JAK1*	ENST00000342505	Synonymous	p.(Ser683Ser)/c.2049C>T	44.51	45.8	45.2	0.9	2.0					
Pt23	12	427575	*KDM5A*	ENST00000399788	Missense	p.(Met865Thr)/c.2594T>C	43.89	46.7	45.3	2.0	4.4					
Pt23	2	209113192	*IDH1*	ENST00000415913	Synonymous	p.(Gly105Gly)/c.315C>T	38.46	39.6	39.0	0.8	2.0					
Pt23	22	23655084	*BCR*	ENST00000305877	Synonymous	p.(Asp1111Asp)/c.3333C>T	99.4	98.5	98.9	0.7	0.7					
Pt23	11	85968623	*EED*	ENST00000263360	Synonymous	p.(Leu207Leu)/c.619C>T	49.14	56.1	52.6	4.9	9.3					
Pt23	18	42532606	*SETBP1*	ENST00000282030	Missense	p.(Val1101Ile)/c.3301G>A	45.38	54.4	49.9	6.4	12.8					

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt24	20	31022297	*ASXL1*	ENST00000375687	Missense	p.(Cys594*)/c.1782C>A	42.78	35.7	39.2	5.0	12.8					
Pt24	9	133760617	*ABL1*	ENST00000372348	Synonymous	p.(Pro999Pro)/c.2997C>T	29.52	25.9	27.7	2.6	9.4					
Pt24	11	108115587	*ATM*	ENST00000278616	Synonymous	p.(Val245Val)/c.735C>T	37.68	42.8	40.2	3.6	9.0					
Pt24	X	39932907	*BCOR*	ENST00000378444	Synonymous	p.(Ala564Ala)/c.1692A>G	97.27	95.7	96.5	1.1	1.1					
Pt24	22	23523630	*BCR*	ENST00000305877	Synonymous	p.(Ile161Ile)/c.483C>A	47.02	42.7	44.9	3.1	6.8					
Pt24	19	33792631	*CEBPA*	ENST00000498907	Synonymous	p.(Thr230Thr)/c.690G>T	41.98	49.3	45.7	5.2	11.4					
Pt24	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A	50.4	49.2	49.8	0.8	1.7					
Pt24	7	101844851	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G	50.37	48.3	49.3	1.5	3.0					
Pt24	7	101892229	*CUX1*	ENST00000360264	Synonymous	p.(Pro1486Pro)/c.4458C>A	38	40.7	39.4	1.9	4.9					
Pt24	11	85968623	*EED*	ENST00000263360	Synonymous	p.(Leu207Leu)/c.619C>T	100	99.3	99.6	0.5	0.5					
Pt24	22	41548008	*EP300*	ENST00000263253	Missense	p.(Ile997Val)/c.2989A>G	52.28	49.8	51.0	1.8	3.5					
Pt24	22	41574383	*EP300*	ENST00000263253	Missense	p.(Gln2223Pro)/c.6668A>C	45.86	44.5	45.2	1.0	2.2					
Pt24	7	50435777	*IKZF1*	ENST00000439701	Synonymous	p.(Pro78Pro)/c.234T>G	99.2	98.6	98.9	0.4	0.4					
Pt24	1	65311214	*JAK1*	ENST00000342505	Synonymous	p.(Ala699Ala)/c.2097C>G	45.68	46.9	46.3	0.9	1.9					
Pt24	1	65321250	*JAK1*	ENST00000342505	Synonymous	p.(Ile530Ile)/c.1590C>T	51.58	47.8	49.7	2.7	5.3					
Pt24	1	65321388	*JAK1*	ENST00000342505	Splice region	c.1459-7C>T	43.65	45.8	44.7	1.5	3.5					
Pt24	1	65335095	*JAK1*	ENST00000342505	Synonymous	p.(Gly182Gly)/c.546A>G	45.59	48.5	47.1	2.1	4.4					
Pt24	9	5050706	*JAK2*	ENST00000381652	Synonymous	p.(His163His)/c.489C>T	49.47	50.9	50.2	1.0	2.0					
Pt24	19	17940941	*JAK3*	ENST00000458235	Synonymous	p.(Pro1061Pro)/c.3183G>T	55.83	45.5	50.7	7.3	14.4					
Pt24	12	406292	*KDM5A*	ENST00000399788	Synonymous	p.(Ser1383Ser)/c.4149C>T	46.57	47.2	46.9	0.5	1.0					
Pt24	12	427575	*KDM5A*	ENST00000399788	Missense	p.(Met865Thr)/c.2594T>C	46.9	47.7	47.3	0.6	1.2					
Pt24	4	55602765	*KIT*	ENST00000288135	Synonymous	p.(Leu862Leu)/c.2586G>C	52.54	45.9	49.2	4.7	9.6					
Pt24	17	29553485	*NF1*	ENST00000358273	Synonymous	p.(Pro678Pro)/c.2034G>A	99.27	99.5	99.4	0.2	0.2					
Pt24	10	104934709	*NT5C2*	ENST00000343289	Missense	p.(Thr3Ala)/c.7A>G	43.51	46.8	45.2	2.3	5.2					
Pt24	8	117864217	*RAD21*	ENST00000297338	Synonymous	p.(Ala480Ala)/c.1440T>C	58.99	51.4	55.2	5.4	9.7					
Pt24	2	198288565	*SF3B1*	ENST00000335508	Synonymous	p.(Tyr54Tyr)/c.162C>T	45.68	48.1	46.9	1.7	3.6					
Pt24	19	11105608	*SMARCA4*	ENST00000429416	Synonymous	p.(His508His)/c.1524T>C	47.56	48.3	47.9	0.5	1.0					
Pt24	19	11170839	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1629Asp)/c.4887T>C	47.45	48.0	47.7	0.4	0.8					
Pt24	17	74733168	*SRSF2*	ENST00000392485	Synonymous	p.(Thr25Thr)/c.75C>T	54.55	53.3	53.9	0.9	1.7					
Pt24	4	106156163	*TET2*	ENST00000540549	Missense	p.(Gly355Asp)/c.1064G>A	46.63	46.2	46.4	0.3	0.7					
Pt24	4	106196951	*TET2*	ENST00000540549	Missense	p.(Ile1762Val)/c.5284A>G	99.72	99.3	99.5	0.3	0.3					
Pt24	4	55593464	*KIT*	ENST00000288135	Missense	p.(Met541Leu)/c.1621A>C	47.09	55.3	51.2	5.8	11.3					
Pt24	12	25362777	*KRAS*	ENST00000256078	Synonymous	p.(Asp173Asp)/c.519T>C	55.13	57.7	56.4	1.8	3.3					

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt2-S2	12	122255792	*SETD1B*	ENST00000267197	Nonsense	p.(Glu1091*)/c.3271G>T						5.3	4.9	5.1	0.3	5.5
Pt2-S2	20	31024043	*ASXL1*	ENST00000375687	Frameshift	p.(Leu1177Profs*5)/c.3529dup						3.5	3.3	3.4	0.1	4.2
Pt2-S2	21	44514777	*U2AF1*	ENST00000291552	Missense	p.(Gln157Arg)/c.470A>G						1.2	0.7	1.0	0.4	37.2
Pt2-S2	5	170818427	*NPM1*	ENST00000296930	Missense	p.(Thr86Met)/c.257C>T						48.5	39.44	44.0	6.4	14.6
Pt2-S2	19	11098596	*SMARCA4*	ENST00000429416	Missense	p.(Tyr372His)/c.1114T>C						41.68	49.48	45.6	5.5	12.1
Pt2-S2	1	36939403	*CSF3R*	ENST00000373103	Missense	p.(Glu149Asp)/c.447G>C						42.56	41.19	41.9	1.0	2.3
Pt2-S2	2	209108317	*IDH1*	ENST00000415913	Missense	p.(Val178Ile)/c.532G>A						49.43	50.75	50.1	0.9	1.9
Pt2-S2	13	28623587	*FLT3*	ENST00000241453	Missense	p.(Asp324Asn)/c.970G>A						45.39	46.8	46.1	1.0	2.2
Pt2-S2	4	106156187	*TET2*	ENST00000540549	Missense	p.(Pro363Leu)/c.1088C>T						47.12	45.68	46.4	1.0	2.2
Pt2-S2	18	42532693	*SETBP1*	ENST00000282030	Missense	p.(Pro1130Thr)/c.3388C>A						45.05	51.51	48.3	4.6	9.5
Pt2-S2	4	106196829	*TET2*	ENST00000540549	Missense	p.(Leu1721Trp)/c.5162T>G						44.18	51.2	47.7	5.0	10.4
Pt2-S2	4	106196951	*TET2*	ENST00000540549	Missense	p.(Ile1762Val)/c.5284A>G						53.95	53.9	53.9	0.0	0.1
Pt2-S2	9	133760029	*ABL1*	ENST00000372348	Synonymous	p.(Pro803Pro)/c.2409C>G						48.35	39.21	43.8	6.5	14.8
Pt2-S2	9	133755528	*ABL1*	ENST00000372348	Synonymous	p.(Glu518Glu)/c.1554A>G						43.07	42.72	42.9	0.2	0.6
Pt2-S2	1	36937065	*CSF3R*	ENST00000373103	Synonymous	p.(Arg418Arg)/c.1254T>C						99.45	99.62	99.5	0.1	0.1
Pt2-S2	12	25362777	*KRAS*	ENST00000256078	Synonymous	p.(Asp173Asp)/c.519T>C						46.45	60.73	53.6	10.1	18.8
Pt2-S2	11	85968623	*EED*	ENST00000263360	Synonymous	p.(Leu207Leu)/c.619C>T						46.65	55.83	51.2	6.5	12.7
Pt2-S2	7	50435777	*IKZF1*	ENST00000439701	Synonymous	p.(Pro78Pro)/c.234T>G						44.24	51.9	48.1	5.4	11.3
Pt2-S2	7	101844851	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G						41.7	46.05	43.9	3.1	7.0
Pt2-S2	7	50467767	*IKZF1*	ENST00000439701	Synonymous	p.(Pro292Pro)/c.876C>A						46.88	49.62	48.3	1.9	4.0
Pt2-S2	22	23631801	*BCR*	ENST00000305877	Synonymous	p.(Asn900Asn)/c.2700T>C						52.3	49.95	51.1	1.7	3.2
Pt2-S2	19	11105608	*SMARCA4*	ENST00000429416	Synonymous	p.(His508His)/c.1524T>C						49.27	46.75	48.0	1.8	3.7
Pt2-S2	22	23655084	*BCR*	ENST00000305877	Synonymous	p.(Asp1111Asp)/c.3333C>T						45.47	48.2	46.8	1.9	4.1
Pt2-S2	2	25469502	*DNMT3A*	ENST00000264709	Synonymous	p.(Leu422Leu)/c.1266G>A						49.56	45.06	47.3	3.2	6.7
Pt2-S2	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A						45.68	46.57	46.1	0.6	1.4
Pt2-S2	19	11145691	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1351Asp)/c.4053C>T						47.62	45.85	46.7	1.3	2.7
Pt2-S2	9	5050706	*JAK2*	ENST00000381652	Synonymous	p.(His163His)/c.489C>T						45.69	43.82	44.8	1.3	3.0
Pt2-S2	2	209113192	*IDH1*	ENST00000415913	Synonymous	p.(Gly105Gly)/c.315C>T						42.86	38.3	40.6	3.2	7.9

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt5-S2	X	129155104	*BCORL1*	ENST00000540052	Nonsense	p.(Arg1196*)/c.3586C>T						97.1	100	98.6	2.1	2.1
Pt5-S2	9	133738349	*ABL1*	ENST00000372348	Missense	p.(Gly250Glu)/c.749G>A						86.4	90.7	88.6	3.0	3.4
Pt5-S2	21	36231860	*RUNX1*	ENST00000300305	Missense	p.(Leu175Gln)/c.524T>A						24.9	25.3	25.1	0.3	1.1
Pt5-S2	21	36231855	*RUNX1*	ENST00000300305	Frameshift	p.(Ile177fs)/c.528delC						21.9	22.9	22.4	0.7	3.2
Pt5-S2	9	133760617	*ABL1*	ENST00000372348	Synonymous	p.(Pro999Pro)/c.2997C>T						80.02	81.09	80.6	0.8	0.9
Pt5-S2	1	36937065	*CSF3R*	ENST00000373103	Synonymous	p.(Arg418Arg)/c.1254T>C						52.48	56.65	54.6	2.9	5.4
Pt5-S2	7	101844851	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G						47	42.59	44.8	3.1	7.0
Pt5-S2	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A						49.53	46.15	47.8	2.4	5.0
Pt5-S2	22	41548008	*EP300*	ENST00000263253	Missense	p.(Ile997Val)/c.2989A>G						48.92	55.8	52.4	4.9	9.3
Pt5-S2	22	41542780	*EP300*	ENST00000263253	Missense	p.(Ser697Arg)/c.2091T>G						41.97	49.58	45.8	5.4	11.8
Pt5-S2	2	209108317	*IDH1*	ENST00000415913	Missense	p.(Val178Ile)/c.532G>A						48.95	47.55	48.3	1.0	2.1
Pt5-S2	2	209113192	*IDH1*	ENST00000415913	Synonymous	p.(Gly105Gly)/c.315C>T						40.98	40.97	41.0	0.0	0.0
Pt5-S2	7	50435777	*IKZF1*	ENST00000439701	Synonymous	p.(Pro78Pro)/c.234T>G						54.17	60.51	57.3	4.5	7.8
Pt5-S2	7	50467767	*IKZF1*	ENST00000439701	Synonymous	p.(Pro292Pro)/c.876C>A						45.4	40.44	42.9	3.5	8.2
Pt5-S2	9	5050706	*JAK2*	ENST00000381652	Synonymous	p.(His163His)/c.489C>T						46.85	53.47	50.2	4.7	9.3
Pt5-S2	19	17945471	*JAK3*	ENST00000458235	Synonymous	p.(Ala753Ala)/c.2259C>T						47.66	46.74	47.2	0.7	1.4
Pt5-S2	12	49444545	*KMT2D*	ENST00000301067	Synonymous	p.(Ile942Ile)/c.2826C>T						48.45	50.73	49.6	1.6	3.3
Pt5-S2	12	49424534	*KMT2D*	ENST00000301067	Synonymous	p.(Pro4563Pro)/c.13689C>T						50.81	45.64	48.2	3.7	7.6
Pt5-S2	9	139410467	*NOTCH1*	ENST00000277541	Synonymous	p.(Asp545Asp)/c.1635C>T						46.96	48	47.5	0.7	1.5
Pt5-S2	9	139396933	*NOTCH1*	ENST00000277541	Synonymous	p.(Thr1725Thr)/c.5175C>T						47.42	44.6	46.0	2.0	4.3
Pt5-S2	10	104934709	*NT5C2*	ENST00000343289	Missense	p.(Thr3Ala)/c.7A>G						45.09	48.19	46.6	2.2	4.7
Pt5-S2	8	117864217	*RAD21*	ENST00000297338	Synonymous	p.(Ala480Ala)/c.1440T>C						51.73	53.2	52.5	1.0	2.0
Pt5-S2	19	11170839	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1629Asp)/c.4887T>C						50.33	50.4	50.4	0.0	0.1
Pt5-S2	9	133755528	*ABL1*	ENST00000372348	Synonymous	p.(Glu518Glu)/c.1554A>G						21.01	14.9	18.0	4.3	24.1
Pt5-S2	9	133760029	*ABL1*	ENST00000372348	Synonymous	p.(Pro803Pro)/c.2409C>G						18.69	16.3	17.5	1.7	9.9
Pt5-S2	22	23655084	*BCR*	ENST00000305877	Synonymous	p.(Asp1111Asp)/c.3333C>T						10.64	11.74	11.2	0.8	7.0

Sample ID	Chr	Genomic coordinates (GRCh37/hg19)	Gene	Transcript ID	Effect	Variant	Intra-assay reproducibility					Inter-assay repeatability				
							Allele frequency %		Mean	SD	CV%	Allele frequency %		Mean	SD	CV%
							1	2				1	2			
Pt22	X	133559247	*PHF6*	ENST00000332070	Missense	p.(His329Tyr)/c.985C>T						89.6	89.6	89.6	0.0	0.0
Pt22	7	101843371	*CUX1*	ENST00000360264	Nonsense	p.(Arg672*)/c.2014C>T						6.4	7.2	6.8	0.6	8.3
Pt22	7	101847749	*CUX1*	ENST00000360264	Nonsense	p.(Arg1007*)/c.3019C>T						3.5	3.8	3.7	0.2	5.8
Pt22	20	31024466	*ASXL1*	ENST00000375687	Frameshift	p.(Arg1318fs)/c.3952dup						1.7	1.4	1.6	0.2	13.7
Pt22	X	39932808	*BCOR*	ENST00000378444	Synonymous	p.(His597His)/c.1791C>T						100	99.43	99.7	0.4	0.4
Pt22	22	23631801	*BCR*	ENST00000305877	Synonymous	p.(Asn900Asn)/c.2700T>C						99.35	99.24	99.3	0.1	0.1
Pt22	22	23523630	*BCR*	ENST00000305877	Synonymous	p.(Ile161Ile)/c.483C>A						49.27	53.5	51.4	3.0	5.8
Pt22	16	3900384	*CREBBP*	ENST00000262367	Missense	p.(Val238Leu)/c.712G>C						51.36	53.0	52.2	1.1	2.2
Pt22	16	3779115	*CREBBP*	ENST00000262367	Missense	p.(Asn1978Ser)/c.5933A>G						33	47.66	40.3	10.4	25.7
Pt22	1	36937065	*CSF3R*	ENST00000373103	Synonymous	p.(Arg418Arg)/c.1254T>C						40.31	44.67	42.5	3.1	7.3
Pt22	7	128200072	*CUX1*	ENST00000360264	Synonymous	p.(Pro769Pro)/c.2307A>G						46.34	49.48	47.9	2.2	4.6
Pt22	11	85968623	*EED*	ENST00000263360	Synonymous	p.(Leu207Leu)/c.619C>T						47.09	48.21	47.7	0.8	1.7
Pt22	3	128204877	*GATA2*	ENST00000341105	Synonymous	p.(Thr188Thr)/c.564G>C						53.23	54.95	54.1	1.2	2.2
Pt22	3	128000000	*GATA2*	ENST00000341105	Synonymous	p.(Ala411Ala)/c.1233G>A						41.79	47.38	44.6	4.0	8.9
Pt22	7	50467767	*IKZF1*	ENST00000439701	Synonymous	p.(Pro292Pro)/c.876C>A						40.5	44.18	42.4	2.6	6.1
Pt22	1	65303659	*JAK1*	ENST00000342505	Synonymous	p.(Lys1032Lys)/c.3096G>A						55.67	48.19	51.9	5.3	10.2
Pt22	1	65310489	*JAK1*	ENST00000342505	Synonymous	p.(Pro733Pro)/c.2199A>G						47.45	46.05	46.8	1.0	2.1
Pt22	1	65335062	*JAK1*	ENST00000342505	Synonymous	p.(Ala193Ala)/c.579T>C						45.28	48.65	47.0	2.4	5.1
Pt22	9	5050706	*JAK2*	ENST00000381652	Synonymous	p.(His163His)/c.489C>T						48.98	48.88	48.9	0.1	0.1
Pt22	12	49444545	*KMT2D*	ENST00000301067	Synonymous	p.(Ile942Ile)/c.2826C>T						99.49	98.54	99.0	0.7	0.7
Pt22	12	49425978	*KMT2D*	ENST00000301067	Synonymous	p.(Pro4170Pro)/c.12510A>G						98.68	98.97	98.8	0.2	0.2
Pt22	12	49445028	*KMT2D*	ENST00000301067	Missense	p.(Pro813Leu)/c.2438C>T						45.03	30.6	37.8	10.2	27.0
Pt22	12	49427652	*KMT2D*	ENST00000301067	Synonymous	p.(Gln3612Gln)/c.10836G>A						100	97.35	98.7	1.9	1.9
Pt22	9	139413908	*NOTCH1*	ENST00000277541	Synonymous	p.(Pro284Pro)/c.852G>A						52.99	46.91	50.0	4.3	8.6
Pt22	4	1902579	*NSD2*	ENST00000382895	Synonymous	p.(Asn66Asn)/c.198C>T						35.59	46.64	41.1	7.8	19.0
Pt22	10	117864217	*NT5C2*	ENST00000343289	Missense	p.(Thr3Ala)/c.7A>G						41.72	47.86	44.8	4.3	9.7
Pt22	8	118000000	*RAD21*	ENST00000297338	Synonymous	p.(Ala480Ala)/c.1440T>C						54.29	49.62	52.0	3.3	6.4
Pt22	19	11105608	*SMARCA4*	ENST00000429416	Synonymous	p.(His508His)/c.1524T>C						99.71	99.59	99.7	0.1	0.1
Pt22	19	11101993	*SMARCA4*	ENST00000429416	Synonymous	p.(Lys471Lys)/c.1413G>A						53.85	48.78	51.3	3.6	7.0
Pt22	19	11170839	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1629Asp)/c.4887T>C						48.48	44.49	46.5	2.8	6.1
Pt22	19	11105593	*SMARCA4*	ENST00000429416	Synonymous	p.(Ala503Ala)/c.1509A>G						44.77	49.76	47.3	3.5	7.5
Pt22	19	11169514	*SMARCA4*	ENST00000429416	Synonymous	p.(Asp1528Asp)/c.4584C>T						43.15	42.13	42.6	0.7	1.7
Pt22	7	101837149	*CUX1*	ENST00000360264	Synonymous	p.(Pro379Pro)/c.1137G>A						48.5	47.85	48.2	0.5	1.0
									Average CV		3.8			Average CV		6.4

6 Correlation of additional genetic abnormalities identified at diagnosis with survival and molecular outcomes in chronic phase CML treated with frontline imatinib

Preface

Genomic interrogation in chronic myeloid leukaemia (CML) has lagged behind other haematological diseases and the incidence at diagnosis of CML of potentially pathogenic variants and their clinical relevance is unknown. In 2016, Mitani (Mitani et al, Blood, 2016) reported whole exome sequencing (WES) in 15 newly diagnosed chronic phase (CP)-CML patients who were selected based on achievement of complete cytogenetic response following treatment with a tyrosine kinase inhibitor (TKI). Additional somatic mutations were identified in BCR::ABL1 positive cells prior to TKI therapy but only a few involved known cancer genes. Similarly, Togasaki (Togasaki et al, Blood Cancer Journal, 2017) published whole exome sequencing of 24 newly diagnosed CP-CML patients. Multiple somatic mutations in addition to BCR::ABL1 were identified in each patient, the number of mutations correlating with achievement of major molecular response. Likewise, Mologni (Mologni et al. *American Journal of Hematology*, 2017) performed whole exome sequencing in 19 consecutive newly diagnosed CP-CML patients, treated with upfront imatinib. Patients with additional somatic mutations at diagnosis with high oncogenic potential were more likely to be associated with imatinib resistance. The deep sequencing results of 100 CP-CML patients, treated with a range of TKIs, was published by Kim and colleagues (Kim et al, Blood, 2017). This study identified the presence of additional somatic variants at the time of diagnosis and in some instances, appeared to influence patient outcomes. The exploratory integrative genomic analysis performed in 2018 by Branford and colleagues investigated 47 CML patients who were highly selected based on their known outcome: optimal versus poor. Patients who progressed to

blast phase CML had a higher incidence of cancer-associated gene variants at diagnosis. Moreover, a novel genetic abnormality associated with the formation of the Philadelphia (Ph)-chromosome, termed Ph-associated rearrangements, was also identified at a higher incidence in patients who progressed to blast phase CML.

These published works were either limited by sample size or were subject to substantial selection bias as patients chosen for sequencing were selected upon eventual disease progression or molecular outcomes. Therefore currently, evidence-based treatment decisions based on the genetic profile at diagnosis cannot be made. The true incidence of potentially pathogenic variants at diagnosis of CP-CML is not known as their effect on treatment failure and molecular response has not been clarified in a consecutive, uniformly treated cohort of patients. Furthermore, while all studies identified somatic variants in addition to BCR::ABL1, although predominantly focusing on single nucleotide variants and small insertions/deletions, the impact of novel additional rearrangements associated with the formation of the Ph chromosome has only been explored in the 2018 Branford et al. paper and the true effect and association with outcome remains unclear.

This study involved the genomic investigation of diagnostic samples of a large consecutive cohort of newly diagnosed CP-CML patients who were uniformly treated with first-line imatinib 600mg daily. Variants of potential clinical relevance in key genes linked to blood cancer were detected at diagnosis using whole exome sequencing, RNA-seq and a novel RNA-based hybridisation capture technique that targeted specific genes. The sequencing strategy allowed the detection of various mutation types, including single nucleotide variants, small insertions and deletions, larger deletions and gene fusions. The capture technique proved capable of detecting all of these variant types in a single assay in addition to aberrant RNA splicing secondary to splicing variants. The strategy also detected novel rearrangements associated with the formation of the Ph chromosome, including deletions, inversions and unique gene fusions. This diversity of genetic abnormalities has never been reported in a consecutive cohort of CML patients at diagnosis.

The genomic investigation demonstrated that one third of patients with CP-CML had additional genetic abnormalities (AGAs) at diagnosis. These patients had a higher incidence of treatment failure and BCR::ABL1 kinase domain mutations, and inferior major and deep molecular responses. Importantly, switching to nilotinib in the event of suboptimal response failed to rescue deep molecular response in patients with AGAs. These findings establish the foundation for clinical trials where the genomic profile will determine the therapeutic approach. Specifically, these results will lead to future studies to determine whether more potent first-line TKI therapy will overcome the negative impact of AGAs or whether a TKI plus drugs targeting other aberrant pathways will be more effective in this setting. Our data provide critical evidence to support revised clinical guidelines to incorporate next generation sequencing for the diagnostic work up of CP-CML patients for genome-based risk stratification.

The results of the clinical significance of the AGAs in an imatinib treated population has been submitted as a revised manuscript for publication consideration in Haematologica as "*Impact of additional genetic abnormalities at diagnosis of chronic myeloid leukemia for first-line imatinib-treated patients receiving proactive treatment intervention*". Supplementary data follows the main manuscript. The included manuscript has been modified following initial review by the journal and has been accepted for publication by Haematologica.

Impact of additional genetic abnormalities at diagnosis of chronic myeloid leukemia for first-line imatinib-treated patients receiving proactive treatment intervention

Running Head: Additional genetic abnormalities in CP-CML

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6.1 Abstract

The BCR::ABL1 gene fusion initiates chronic myeloid leukemia (CML), however evidence has accumulated from studies of highly selected cohorts that variants in other cancer-related genes are associated with treatment failure. Nevertheless, the true incidence and impact of additional genetic abnormalities (AGAs) at diagnosis of chronic phase (CP)-CML is unknown. We sought to determine whether AGAs at diagnosis in a consecutive imatinib-treated cohort of 210 patients enrolled in the TIDEL-II trial influenced outcome despite a highly proactive treatment intervention strategy. Survival outcomes including overall survival, progression-free survival, failure-free survival and BCR::ABL1 kinase domain mutation acquisition were evaluated. Molecular outcomes were measured at a central laboratory and included major molecular response (MMR, BCR::ABL1 ≤0.1%IS), MR4 (BCR::ABL1 ≤0.01%IS) and MR4.5 (BCR::ABL1 ≤0.0032%IS). AGAs included variants in known cancer genes and novel rearrangements involving the formation of the Philadelphia chromosome. Clinical outcomes and molecular response were assessed based on the genetic profile and other baseline factors. AGAs were identified in 31% of patients. Potentially pathogenic variants in cancer-related genes were detected in 16% of patients at diagnosis (including gene fusions and deletions) and structural rearrangements involving the Philadelphia chromosome (Ph-associated rearrangements), detected in 18%. Multivariable analysis demonstrated that the combined genetic abnormalities plus the ELTS clinical risk score were independent predictors of lower molecular response rates and higher treatment failure. Despite a highly proactive treatment intervention strategy, first-line imatinib-treated patients with AGAs had poorer response rates. This data provides evidence for the incorporation of genomically-based risk assessment for CML.

6.2 Introduction

Chronic myeloid leukemia (CML) is characterized by the translocation that forms the Philadelphia (Ph) chromosome and the *BCR::ABL1* gene fusion. Targeted therapy with tyrosine kinase inhibitors (TKIs)[1-5] inhibits the activated BCR::ABL1 enzyme and while generally very effective,[6] treatment outcomes remain heterogenous. Up to 15% of patients are resistant to one or more TKIs and progression to blast phase (BP) still occurs in

approximately 5% of patients.[7] While *BCR*::*ABL1* kinase domain point mutations remain the primary known mechanism of acquired resistance and direct sequential therapy selection,[7-9] these mutations are identified in only approximately 50% of cases of TKI resistance.[10,11] Kinase domain mutations are not detected at diagnosis in chronic phase (CP) CML and are not the cause of primary resistance, which remains poorly understood. This suggests the existence of alternative resistance pathways.

Unrestrained BCR::ABL1 activity promotes genetic instability and can trigger the development of single nucleotide variants, insertions and deletions.[12-16] We previously performed an integrative genomic analysis in a discovery cohort of CP-CML patients at diagnosis who were specifically selected based on optimal or very poor outcomes, and found that patients who subsequently progressed to BP had a significantly higher frequency of cancer-related gene variants at diagnosis compared with patients who achieved an optimal molecular response (54% versus 16%).[17] Furthermore, all patients tested at BP had one or more cancer gene variants in addition to *BCR::ABL1*, which included gene fusions and gene deletions.[17,18]

Genomic profiling also uncovered novel genomic rearrangements associated with the formation of the Ph chromosome, termed Ph-associated rearrangements.[17] These were structural variants defined as aberrant fusions formed at the time of the Ph translocation, involving rearrangement of genes or sequences on the translocated chromosomes. It is likely that many such variants constitute near-simultaneous genomic rearrangements due to repair of additional double strand breaks related to the formation of the Ph chromosome. They were characterized by sequence fragmentation, non-contiguous deletion, inversion, and imperfect reassembly, likely resulting from genomic 'shattering' and attempted realignment into a mosaic patchwork of genomic fragments.[17,19] These chromoplexy-like rearrangements[20] were highly complex in some patients and have been described in other malignancies to correlate with accelerated cancer evolution.[21] In our discovery cohort, Ph-associated rearrangements were more frequently observed in patients with poor outcomes (33%) compared with those achieving optimal molecular targets (11%).[17] These genomic findings notwithstanding, the true clinical implications of cancer gene variants and Ph-associated rearrangements at the time of CP-CML diagnosis is unknown. Multiple studies have linked cancer gene variants at diagnosis and poor outcome for imatinib-treated patients[17,22,23] but there has been no

systematic evaluation of unselected cohorts to establish the true incidence and clinical risk conferred by genetic abnormalities at diagnosis.

In this study, we investigated the clinical relevance of variants in cancer-related genes that were classified as pathogenic or potentially pathogenic (including genes fusions and deletions), and Ph-associated rearrangements detected at the time of diagnosis in a consecutive cohort of imatinib-treated patients who received rapid treatment intervention that was largely based on time-dependent molecular milestone response values. We show that these genetic abnormalities when detected at the time of diagnosis have a significant impact on treatment response and outcome. The prognostic effect of the genetic abnormalities was independent of the EUTOS long term survival (ELTS) score, which has already been demonstrated to be discriminative of survival in the TKI era.[24] Our data validates and expands the findings from previous discovery cohort studies where the results suffered from selection bias since patients were selected for sequencing based on their known outcome.[17,22,23]

6.3 Methods

6.3.1 Patients

The total cohort of 210 adult CP-CML patients enrolled in the Australasian Leukemia and Lymphoma Group CML9 study (TIDEL-II, ACTRN12607000325404) were investigated.[25] Patients were treated with first-line imatinib 600 mg daily with active intervention: either dose escalation or nilotinib switch for lack of achievement of time-dependent molecular milestones. Imatinib was also dose escalated for subtherapeutic trough levels (<1000ng/mL) at day 22. The molecular milestones were *BCR::ABL1* transcript ratios of ≤10%, ≤1%, and ≤0.1%^{IS} at 3, 6 and 12 months, respectively, similar to those subsequently adopted by the European LeukemiaNet (ELN) as optimal time-dependent responses to TKI therapy.[26] Patients were also switched to nilotinib for imatinib intolerance or loss of response, defined as at least one of: loss of confirmed complete hematological response or major cytogenetic response; cytogenetic clonal evolution; a confirmed more than five-fold increase in *BCR*::*ABL1* ratio from nadir to a level >0.1% resulting in loss of MMR; a greater than two-fold increase from nadir in *BCR*::*ABL1* ratio to a level of >10%; detection of >50% mutant *BCR*::*ABL1*; or disease

transformation to accelerated phase (AP) or BP.[25] The method of TKI compliance assessment is included in the Supplemental Patient compliance assessment

6.3.2 Next-generation sequencing

Diagnostic blood samples were sequenced using a customized RNA-based capture panel of 126 genes[27] (Supplemental Table 1), or whole exome and/or whole transcriptome sequencing, as previously described[17] following appropriate approval from the local institutional review board. We previously demonstrated that total RNA is suitable for detecting multiple different types of variants, including single nucleotide variants (SNVs), small insertions and deletions (indels), fusions (genomic and transcripts), and gene deletions and their corresponding genomic breakpoints from pre-spliced RNA.[17,27] Identified variants were reviewed using strict criteria, as detailed previously.[17] Of the variants that met our criteria for damaging, we only included those considered as likely pathogenic or pathogenic using the criteria set out in joint consensus standards for the classification of somatic variants.[28,29] Further detail on sequencing methods are provided in the Supplemental methods.

6.3.3 Statistical analysis

We assessed the association between specific baseline variables and molecular response and outcome by 4 years, including additional genetic abnormalities (AGAs), ELTS clinical risk scores, age, sex, and *BCR::ABL1* transcript type. AGAs were assessed both in aggregate and in separate categories (cancer gene variants and Ph-associated rearrangements). Variables with a P value <0.05 using univariate analysis were assessed in bi/multivariable analysis for the relevant outcome. Confounding variables that were significant using univariate testing required separate assessment on bi/multivariable analysis, such as age and ELTS. The Bayesian information criterion indicated the best multivariable model for selection. Variables that emerged as significant on univariate analysis were assessed for independence utilizing the variance inflation factor to test for multicollinearity. Multiple comparison correction was performed using the Benjamini-Hochberg Method.[30] Overall survival (OS) and failure-free survival (FFS) were calculated through Kaplan-Meier estimates and log-rank tests. Patients were censored at study withdrawal or completion of follow up visits. Independent predictors

of OS and FFS were tested using Cox regression analysis. Failure events were as currently defined by the ELN,[7] which included failure to achieve time-dependent molecular milestones, acquisition of *BCR::ABL1* kinase domain mutations, AP/BP and death by any cause. The cumulative incidence of *BCR*::*ABL1* kinase domain mutation acquisition and disease progression to AP or BP were assessed through Fine-Gray modelling, as were the molecular outcomes of major molecular response (MMR, *BCR::ABL1* ≤0.1%^{IS}), MR4 (*BCR::ABL1* ≤0.01%^{IS}) and MR4.5 (*BCR::ABL1* ≤0.0032%^{IS}). Schoenfeld residuals were used to test the assumption of proportional hazards. Death not related to AP/BP and progression to AP/BP were considered competing risks. Statistical analysis was performed using GraphPad Prism 8.0.0 and R version 4.1.2.

6.4 Results

Diagnostic samples were successfully sequenced for 200 of the 210 patients (95%) with characteristics of these patients summarized in Table 1. The median follow-up of patients was 37 months (2.5-48 months). RNA capture panel sequencing was available for 188 patients and 12 patient samples were sequenced using whole-exome sequencing and/or whole transcriptome RNA-Seq.[17] Data for 17 samples were previously reported.[17] Six samples were sequenced using RNA-seq and repeated using the RNA capture panel to demonstrate concordance and reported previously.[27] Results were not available for nine patients due to RNA degradation or inadequate sample. *BCR::ABL1* fusion transcripts were detected in all samples and the genomic breakpoints that generated the *BCR*::*ABL1* transcript were detected in 85% of patients (Supplemental Table 2).

Table 1: Patient characteristics

Patient characteristics	**All patients with sequencing data n=200**	**Sub-group breakdown**			
	Number (%) or median [range]	**No AGAs (n=139)**	**Cancer-gene variants alone (n=25)**	**Ph-associated rearrangements alone (n=28)**	**Both cancer-gene variants and Ph-associated rearrangements (n=8)**
Male	113 (57)	77 (55)	17 (68)	15 (54)	4 (50)
Age at diagnosis, years	50 [17-81]	50 [17-81]	51 [23-71]	45 [17-79]	47 [24-55]
Transcript					
- e13a2	82 (41)	59 (42)	5 (20)	16 (57)	2 (25)
- e14a2	80 (40)	56 (40)	11 (44)	9 (32)	4 (50)
- Both e13a2 and e14a2	36 (18)	24 (17)	8 (32)	3 (11)	1 (13)
- Other*	2 (1)	0	1 (4)	0	1 (13)

ELTS - Low - Intermediate - High - N/A	 126 (63) 46 (23) 19 (9) 9 (4)	 92 (66) 27 (19) 13 (9) 7 (5)	 15 (60) 8 (32) 1 (4) 1 (4)	 15 (54) 9 (32) 3 (11) 1 (4)	 4 (50) 2 (25) 2 (25) 0
Progressed to BP	8 (4)	4 (3)	2 (8)	1 (3)	1 (13)
BCR::ABL1 kinase domain mutation acquisition	12 (6)	3 (2)	2 (8)	5 (17)	2 (25)
Nilotinib switch for time-dependent suboptimal molecular response	53 (26)	28 (20)	9 (36)	12 (41)	4 (50)

BP: blast phase (no patient progressed to accelerated phase); N/A: not available

* Included one patient with e1a2 and one patient with e13a3 *BCR::ABL1* transcripts

6.4.1 Genomic findings

At the time of diagnosis, 40 SNVs and indels in cancer genes were detected in 33 patients (16%) across 10 genes (Figure 1A). One of these patients also had a gene fusion involving *IKZF1* and a deletion involving *RUNX1*, and rapidly progressed to BP (shown in Figure 2). Ph-associated rearrangements were identified in 36 patients (18%). The complexity of these rearrangements is detailed in Figure 1B, Supplemental Figure 1 and in the Supplemental Results. There was no significant difference in the age of patients without AGAs (median age 50, range 17-81 years) compared to those with cancer gene variants (median age 51, range 23-71 years) or Ph-associated rearrangements (median age 45, range 17-79 years). Overall, 121/200 patients (61%) expressed the reciprocal *ABL1::BCR* transcript. Ph-rearrangements were strongly associated with the absence of *ABL1::BCR.* Of 36 patients with Ph-associated rearrangements, only one expressed *ABL1::BCR.* The rearrangement for this patient comprised a genomic inversion between *BCR* and *ABL1* that was immediately adjacent to the standard *BCR::ABL1* genomic breakpoint. Lack of a reciprocal transcript in 97% of patients with a Ph-associated rearrangement is consistent with deletion or rearrangement of sequence adjacent to the *ABL1* and/or *BCR* breakpoints in many cases. Supplemental Table 2 details the predicted size of deletions based on the genomic location of the fusion partners of patients with Ph-associated rearrangements.

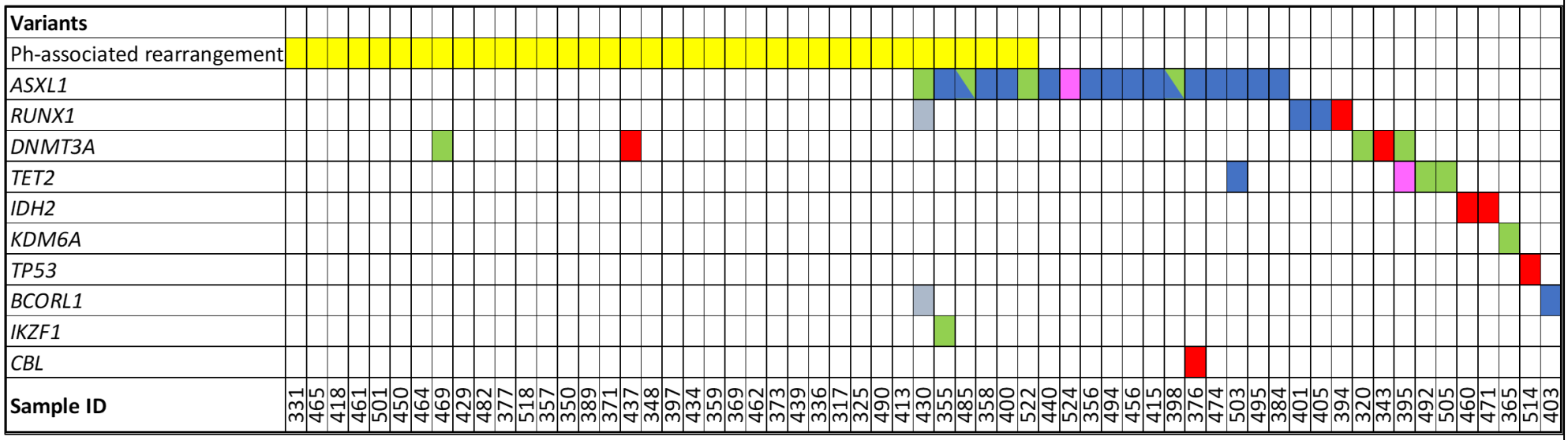
Eight patients had both cancer gene variants and Ph-associated rearrangements, accounting for 4.5% of the sequenced population. Collectively, 61 patients (31%) harbored additional genetic abnormalities, which we have termed AGAs. The data for individual patients are detailed in Supplemental Table 2.

ASXL1 was the most frequently mutated gene at diagnosis (Figure 1A) with 20 variants observed in 18 of 200 patients (9.0%). Further detail of patients with mutated *ASXL1* is provided in section 'Outcome for patients with mutated *ASXL1*'. Other genes mutated in multiple patients were *RUNX1, BCORL1, IDH2*, *DNMT3A* and *TET2*. Two patients had *BCORL1* variants at diagnosis and both progressed to BP, one of whom is depicted in Figure 2. Four patients had evidence of clonal hematopoiesis of indeterminate potential with somatic variants affecting *DNMT3A*, *ASXL1* or *TET2* that were detected in both the diagnosis and

remission samples, and therefore likely pre-dated the acquisition of the Ph chromosome. Three of these 4 patients achieved an optimal response, and one failed therapy and acquired a *BCR::ABL1* kinase domain mutation. This patient also had a distinct somatic *ASXL1* variant exclusively within the leukaemic clone at diagnosis. Variants that affected RNA splicing were also identified by this methodology (Figure 1C-D).

Figure 1: The genomic findings of the TIDEL-II cohort at diagnosis
(A) Oncoplot of AGAs identified at diagnosis. (B) The predominant Ph-associated rearrangements for individual patients based on the fusion read counts are shown. The diversity of the partner genes (outer circle) involved in the Ph-associated rearrangements is illustrated. The color of the inner circle corresponds to the chromosome location of the fusion partner. The width of the ribbon correlates with the frequency of co-occurrence of the fusion partner. All events were in addition to the primary BCR::ABL1 transcript. The complete list of Ph-associated rearrangements is documented in Supplemental Table 2. Fusions between BCR and ABL1 in the Circos plot represent inversions. (C) One of the added benefits of utilizing RNA as the genetic material for sequencing is the ability to identify aberrant splicing. Aberrant splicing associated with a TET2 splice acceptor variant demonstrated by an Integrative Genomics Viewer screenshot showing the wild-type sequence (top panel) and mutant (bottom panel) detectable using targeted gene panel sequencing. The mutant, detected in patient 395, disrupted the canonical splice acceptor site (AG at the exon 8 junction) and led to activation of a cryptic acceptor splice site within intron 7 (AG at the sequence read junction) and exon extension. The activated cryptic splice acceptor site was 48 nucleotides upstream of the border of exon 8. Inclusion of this sequence within the extended exon was predicted to result in premature termination. Alamut, an in-silico splice prediction tool, predicted loss of the original splice acceptor site but not activation of the cryptic splice site. (D) Sashimi plots demonstrated the atypical splicing of TET2 between exons 7 and 8 due to the splice acceptor variant. The arcs represent splice junctions that connect exons and the number of splice junction reads are indicated. Normal splicing between exons 7 and 8 is demonstrated in the top panel. The bottom panel demonstrates normal splicing plus aberrant splicing resulting from activation of the cryptic splice site (red asterisk) within intron 7. This example demonstrates the relevance of RNA sequencing for identifying and investigating the effect of splicing variants. This somatic variant existed before the acquisition of the BCR::ABL1 fusion.

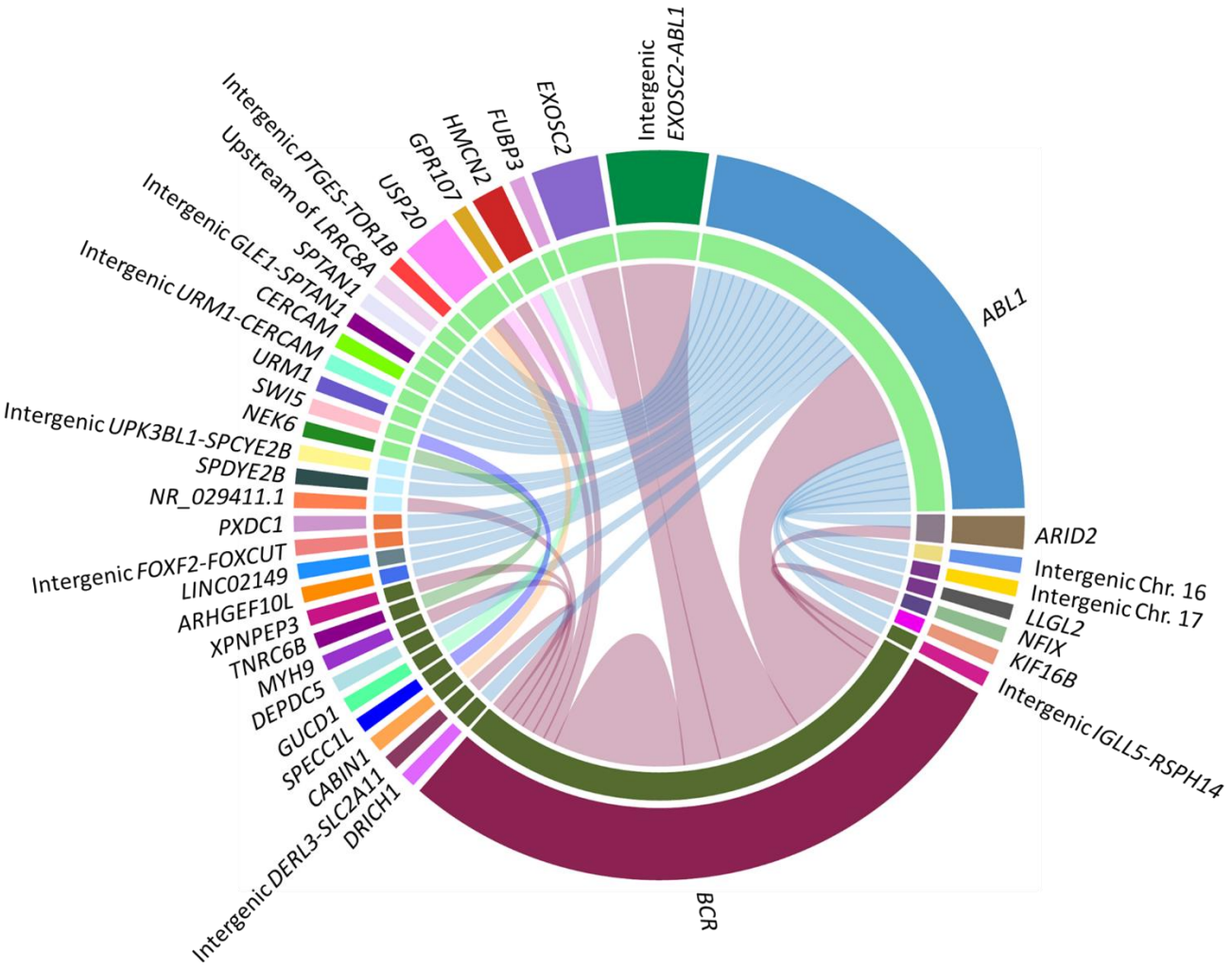
A. Oncoplot of additional genetic abnormalities identified



Variant subtype

- Missense
- Frameshift
- Nonsense
- Splice variant
- Structural variant
- Ph-associated rearrangement

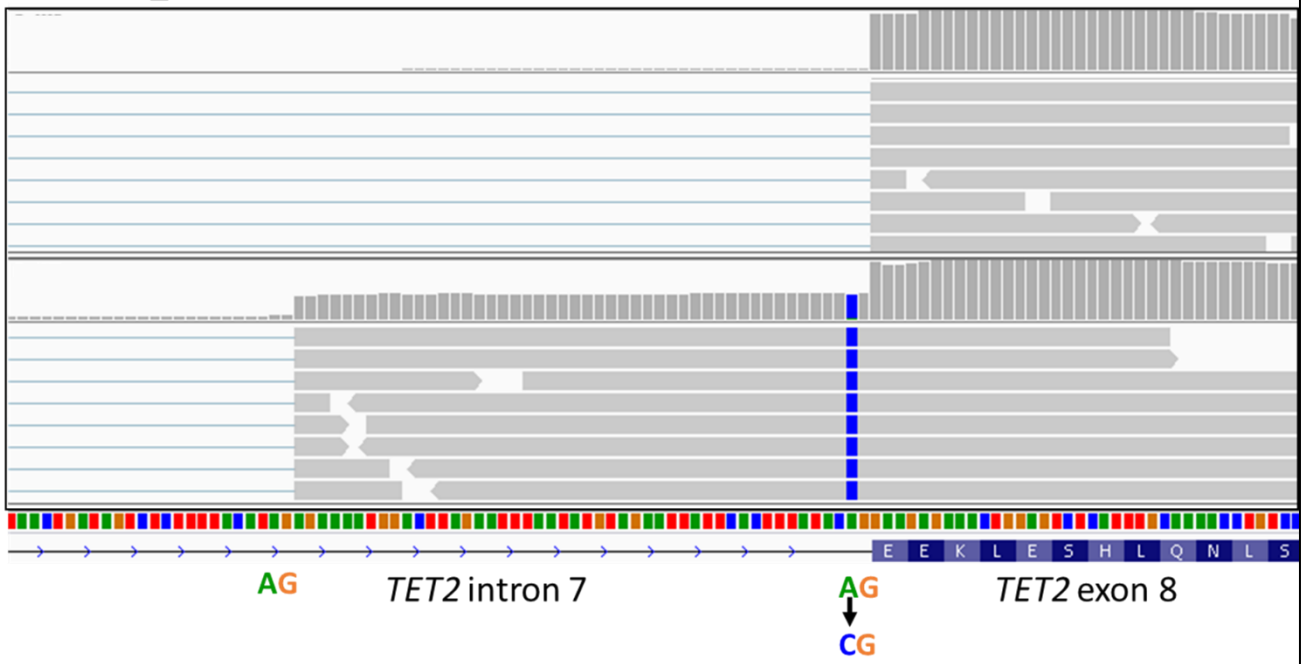
B. Circos plot of Ph-associated rearrangements



C. *TET2* splice acceptor variant NM_001127208.2:c.3955-2A>C

Representative wild-type sequence:
Sequencing reads at the exon junction

Mutant sequences:
sequencing reads at an intronic cryptic splice acceptor site and exon extension



D. Aberrant RNA splicing associated with the splice acceptor variant

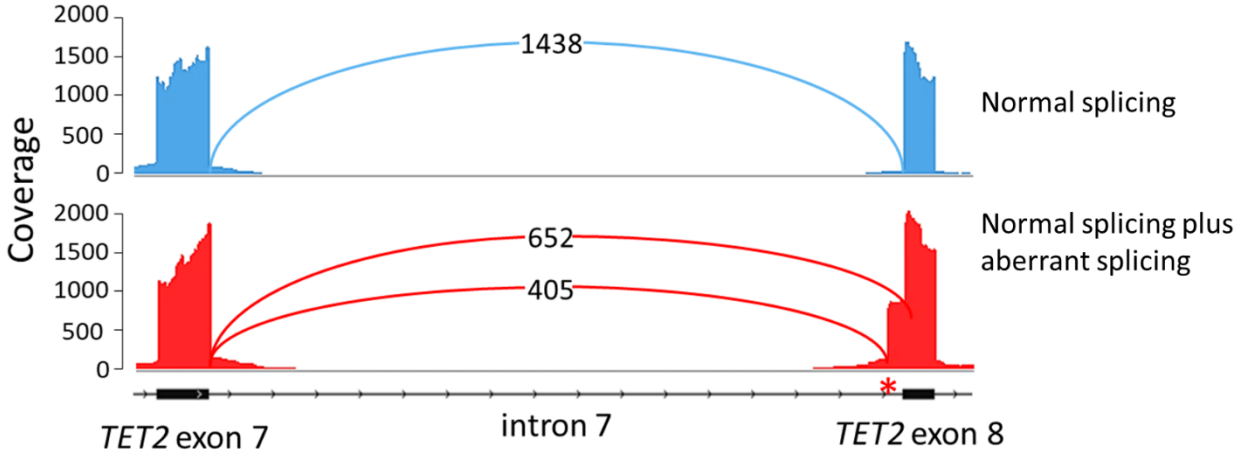
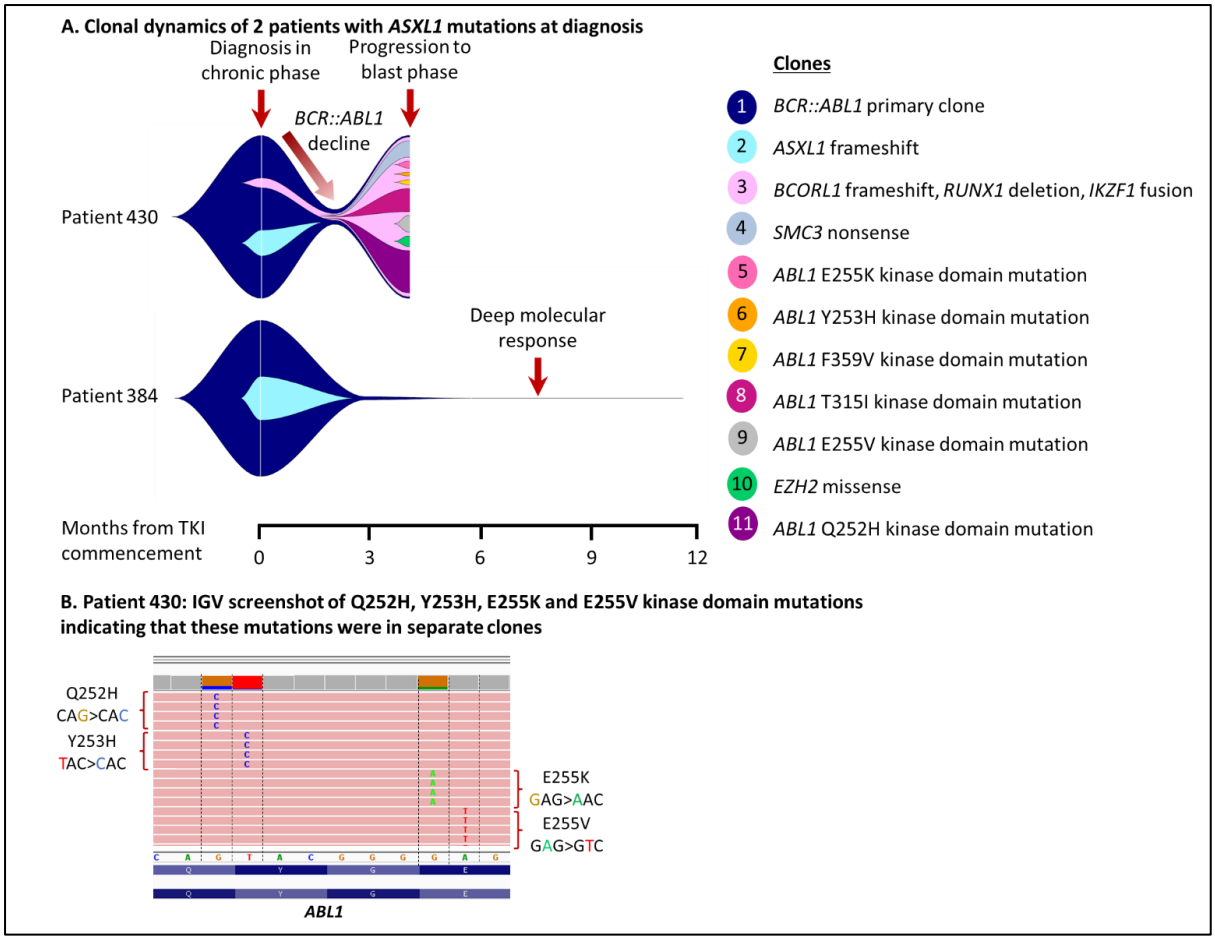


Figure 2: The clonal dynamics of two patients with ASXL1 variants at diagnosis illustrated by fishplots

(A). Patient 430 had a Ph-associated rearrangement in addition to the BCR::ABL1 clone (dark blue) at diagnosis. The Ph-associated rearrangement involved an inversion between ABL1 exon 1 and an intergenic region on chromosome 17q12. In addition, two mutant subclones were detected at diagnosis. One clone contained an ASXL1 frameshift variant (light blue) and the second subclone harbored a BCORL1 frameshift variant, a 34 Kb RUNX1 deletion and an IKZF1::IGKV3-7 fusion (represented in pink). With the decline of BCR::ABL1 in response to imatinib, the subclones also reduced and the ASXL1 clone became undetectable. The second clone expanded and evolved in lymphoid blast phase at 4 months of imatinib. Several independent subclones arose from the original BCORL1/RUNX1/IKZF1 subclone, including an SMC3 nonsense variant, an EZH2 missense variant and six ABL1 kinase domain mutations. The second patient (384) harbored a single ASXL1 nonsense variant at diagnosis. A rapid deep molecular response was achieved and the ASXL1 subclone became undetectable. The optimal response was maintained. (B). IGV screenshot of amino acids 252-255 of ABL1 of patient 430, demonstrating that the Q252H, Y253H, E255K and E255V kinase domain mutations were on separate reads, which indicates that these mutations were in separate clones and were not compound mutations. Only a proportion of each of the mutant reads are shown.



We investigated the clinical impact of the AGAs on response and outcome. The presence of any genetic abnormality (cancer gene variant or Ph-associated rearrangement) predicted inferior FFS and molecular responses.

6.4.2 Overall and progression-free survival

There were eleven deaths by the end of the 4-year follow up period with an overall survival of 94% (95% CI 87.9-97.9%), Supplemental Figure 2A. Six patients succumbed to non-CML related causes of death (four cardiac related deaths, one cerebrovascular accident, one secondary to infection and one unknown cause of death). Eight patients progressed to BP (five myeloid and three lymphoid BP) and no patient progressed to AP with a cumulative incidence of progression of 6% (95% CI 2.3-12.0%), Supplemental Figure 2B. Of the eight patients that progressed to BP, five died. No variable predicted for progression to BP or overall survival by Cox regression analysis.

6.4.3 Failure-free survival

The 4-year estimate of FFS, as defined by the ELN, was 77% (95% CI 70.2-82.3%) (Supplemental Figure 2C). Univariate analysis confirmed that AGAs at diagnosis predicted for inferior FFS, 69% versus 80% (*P*=.03), Figure 3A. The ELTS score was also a predictor of FFS, Supplemental Figure 3A. No other baseline variable predicted FFS. Multivariable analysis using Cox regression analysis confirmed that the only independent predictors of FFS were the presence of AGAs at diagnosis (p=0.04) and the ELTS score (p=0.001), Table 2.

6.4.3.1 Acquisition of *BCR*::*ABL1* kinase domain mutations

Twelve patients acquired *BCR*::*ABL1* kinase domain mutations with a cumulative incidence of 6% by 4 years (95% CI 3.3-10.2%) (Supplemental Figure 2D and Supplemental Table 2). The kinase domain mutations were acquired at a median of 323 days (range 85-722 days) from TKI commencement. By univariate analysis, having an AGA at diagnosis predicted for the acquisition of *BCR*::*ABL1* kinase domain mutations by 4 years, 15% versus 2% (p<0.001), Figure 3B. Notably, Ph-associated rearrangements were strongly associated with the acquisition of kinase domain mutations, (p<0.001, Supplemental Table 3). While the ELTS score was a significant predictor of the acquisition of kinase domain mutations on univariate analysis (Table 2), the false discovery rate corrected for this. AGAs at diagnosis remained the only predictor for the acquisition of *BCR*::*ABL1* kinase domain mutations.

6.4.4 Molecular responses by 4 years

Inferior achievement of MMR, MR4 and MR4.5 was associated with the presence of AGAs at diagnosis. MMR at 12 months of TKI therapy is an optimal response[7] and lack of MMR at the 12 month milestone was a trigger for treatment intervention for the study cohort. By 12 months, the cumulative rate of MMR was 61% (95% CI 54%-67%) for the study population (Supplemental Figure 2E). The rate of MMR by 12 months in patients with AGAs was significantly lower than those without these events, 46% versus 67%, p=0.005 (Figure 3C). The overall cumulative rate of MMR by 4 years was 82% (95% CI 76%-87%). Univariate analysis (Table 2) demonstrated that AGAs at diagnosis predicted for an inferior cumulative incidence of MMR by 4 years compared with patients without these events, 72% versus 86%, p=0.007 (Figure 3C). ELTS was a predictor for MMR by 4 years: 87%, 73% and 68% for low, intermediate and high-risk groups, respectively (p<0.001), Supplemental Figure 3B. To avoid collinearity, age and ELTS were assessed separately with AGAs at diagnosis using bivariable analysis. The strongest model contained both AGAs (p=0.025) and the ELTS score (p<0.001), Table 2.

By 4 years, 59% (95% CI 51%-65%) and 43% (95% CI 35%-50%) of evaluated patients achieved MR4 and MR4.5 respectively by Fine-Gray modelling (Supplemental Figures 2F-G). AGAs were associated with a significantly lower incidence of MR4: 37% versus 67%, p=0.001; and MR4.5: 27% versus 49%, p=0.03, (Figures 3D-E). The strongest multivariable analysis demonstrated that AGAs and the ELTS score were the only independent predictors of MR4 (AGAs p=0.004 and ELTS p<0.001) and MR4.5 (AGAs p=0.03 and ELTS p=0.02), Table 2.

Table 2: Cox regression analysis demonstrating the univariate and multivariate analysis for each 4-year outcome.
Variables with p value of ≤0.05 in univariate analysis following false-discovery correction were included in the multivariable analysis models.

Outcome measure	Variable	Cumulative incidence of event (%)	Univariate analysis			Multivariable analysis		
			Sub-distribution /Hazard ratio (95% CI)*	*p* value	False discovery rate	Sub-distribution /Hazard ratio (95% CI)*	*p* value	
Failure-free survival (number of events = 41/200)	No AGA vs AGA	80% vs 69%	1.76 (1.04-3.14)	0.02	0.038	1.82 (1.57-3.43)	0.04	
	ELTS - Low - Intermediate - High	86% 65% 58%	- 2.60 (1.23-4.73) 2.78 (1.43-7.92)	- 0.01 0.005	0.01	- 2.50 (1.24-5.04) 3.51 (1.51-8.14)	- 0.01 0.003	Wald test: *p*=0.001
	Age (<63 years vs ≥63 years)	74% vs 87%	0.45 (0.17-1.21)	0.14	0.28			
	Sex (female vs male)	82% vs 78%	1.20 (0.44-1.55)	0.56	0.70			
	Transcript type - e14a2	80%	- 1.01 (0.50-2.03)	- 0.97	0.96			

	- e13a2 - Both e14a2 & e13a2	80% 78%	1.12 (0.48-2.63)	0.78				
BCR::*ABL1* KD mutation development (number of events = 12/200)	No AGA vs AGA	2% vs 15%	7.69 (2.04-25)	<0.001	0.015	7.69 (2.04-25)	<0.001	
	ELTS - Low - Intermediate - High	3% 16% 5%	- 1.65 (1.53-1.77) 1.13 (0.98-1.91)	- 0.008 0.06	0.073			
	Age (<63 years vs ≥63 years)	10% vs 5%	1.01 (0.97-1.04)	0.71	0.70			
	Sex (female vs male)	5% vs 7%	1.55 (0.19-2.14)	0.47	0.59			
	Transcript type - e14a2 - e13a2 - Both e14a2 & e13a2	10% 4% 3%	- 0.35 0.27	- 0.12 0.22	0.20			
MMR (number of events = 163/200)	No AGA vs AGA	86% vs 72%	0.62 (0.43-0.87)	0.007	0.016	0.70 (0.47-0.94)	0.025	
	ELTS - Low - Intermediate	87%	-	-	0.005	-	-	Wald test:

	- High	73% 68%	0.53 (0.37-0.76) 0.40 (0.26-0.63)	<0.001 <0.001		0.54 (0.38-0.78) 0.41 (0.25-0.66)	0.003 0.001	*p*<0.001
	Age (<63 years vs ≥63 years)	76% vs 95%	2.26 (1.52-3.35)	0.01	0.017			
	Sex (female vs male)	87% vs 86%	1.16 (0.85-1.60)	0.36	0.45			
	Transcript type - e14a2 - e13a2 - Both e14a2 & e13a2	 81% 79% 83%	 - 0.78 (0.55-1.09) 0.92 (0.58-1.44)	 - 0.14 0.7	0.75			
MR4 (number of events = 117/200)	No AGA vs AGA	67% vs 37%	0.48 (0.31-0.76)	0.001	0.005	0.51 (0.32-0.82)	0.004	
	ELTS - Low - Intermediate - High	66% 42% 41%	- 0.47 (0.30-0.74) 0.45 (0.26-0.79)	- <0.001 0.002	0.020	- 0.51 (0.33-0.81) 0.43 (0.25-0.75)	- 0.004 0.003	Wald test: *p*<0.001

	Age (<63 years vs ≥63 years)	53% vs 78%	1.86 (1.17-2.94)	0.032	0.53		
	Sex (female vs male)	67% vs 52%	1.54 (1.08-2.2)	0.05	0.062		
	Transcript type - e14a2 - e13a2 - Both e14a2 & e13a2	 59% 55% 67%	 - 0.64 (0.43-1.44) 0.87 (0.53-1.45)	 - 0.34 0.60	0.6		
MR4.5 (number of events = 76/200)	No AGA vs AGA	47% vs 27%	0.53 (0.31-0.94)	0.015	0.037	0.54 (0.32-0.82)	0.004
	ELTS - Low - Intermediate - High	 48% 21% 18%	 - 0.29 (0.14-0.60) 0.32 (0.13-0.81)	 - <0.001 0.016	0.005	 - 0.51 (0.32-0.80) 0.43 (0.24-0.75)	 - 0.004 0.003	 Wald test: *p*=0.019
	Age (<63 years vs ≥63 years)	35% vs 52%	1.54 (0.87-2.72)	0.13	0.163			

	Sex (female vs male)	44% vs 33%	0.64 (0.41-1.09)	0.055	0.091			
	Transcript type - e14a2 - e13a2 - Both e14a2 & e13a2	 43% 33% 42%	- 0.68 (0.41-1.13) 0.90 (0.64-1.64)	- 0.13 0.74	0.46			

AGA: Additional genetic abnormalities; CI: confidence interval, KD: kinase domain, MMR: major molecular response.

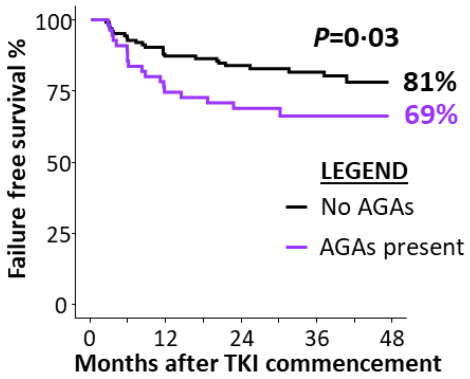
For the ELTS clinical risk groups, the *P* values are in relation to the low-risk group

* Univariate and multivariable analysis for kinase domain mutation acquisition, MMR, MR4 and MR4.5 were assessed with Fine and Gray modelling. This utilizes sub-distribution hazard ratios, which are reported here

Figure 3: AGAs at diagnosis were an independent predictor of specific outcomes and molecular responses

The graphs show the outcomes and responses according to AGA status at diagnosis. (A) Kaplan-Meier estimates of failure-free survival; (B) Cumulative incidence of the acquisition of BCR::ABL1 kinase domain mutations; (C) Cumulative incidence of MMR; (D) Cumulative incidence of MR4; (E) Cumulative incidence of MR4.5. The number at risk table is included below the relevant graphs.

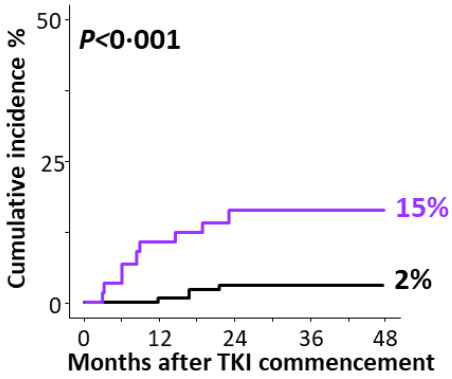
A. Failure-free survival



Number at risk

No AGAs	140	126	112	70	31
AGAs	60	48	39	20	8

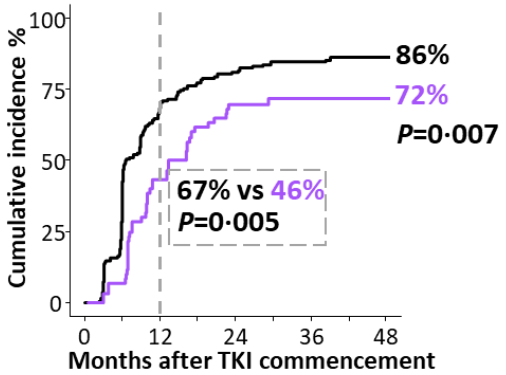
B. Acquisition of kinase domain mutations



Number at risk

No AGAs	140	132	118	70	27
AGAs	60	52	45	29	10

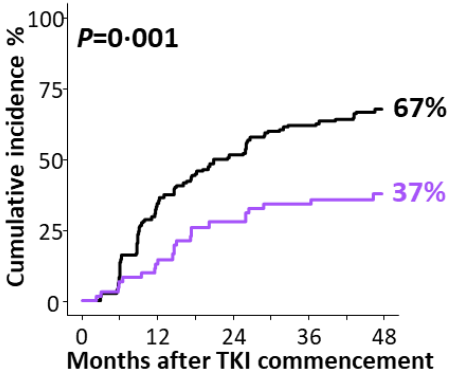
C. Major molecular response achievement



Number at risk

No AGAs	140	43	18	13	9
AGAs	60	28	9	3	3

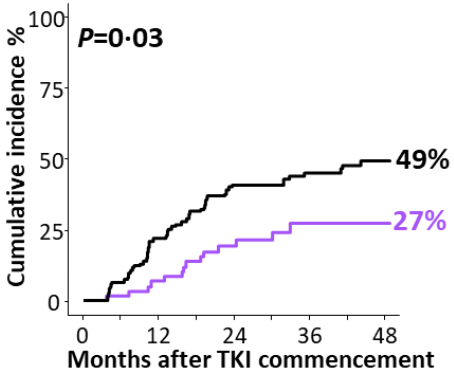
D. MR4 achievement



Number at risk

No AGAs	140	89	55	30	12
AGAs	60	46	31	13	8

E. MR4·5 achievement



Number at risk

No AGAs	140	110	74	50	26
AGAs	60	47	35	18	11

6.4.5 Combining AGAs and ELTS to predict molecular outcomes

As AGAs and ELTS were the only independent predictors of FFS, MMR, MR4 and MR4.5, the impact of combining the two variables for the prediction of these outcomes was assessed. The intermediate-risk and high-risk groups tracked together for each of the assessed outcomes and were not statistically different from each other and so the two groups were combined to increase sample size.

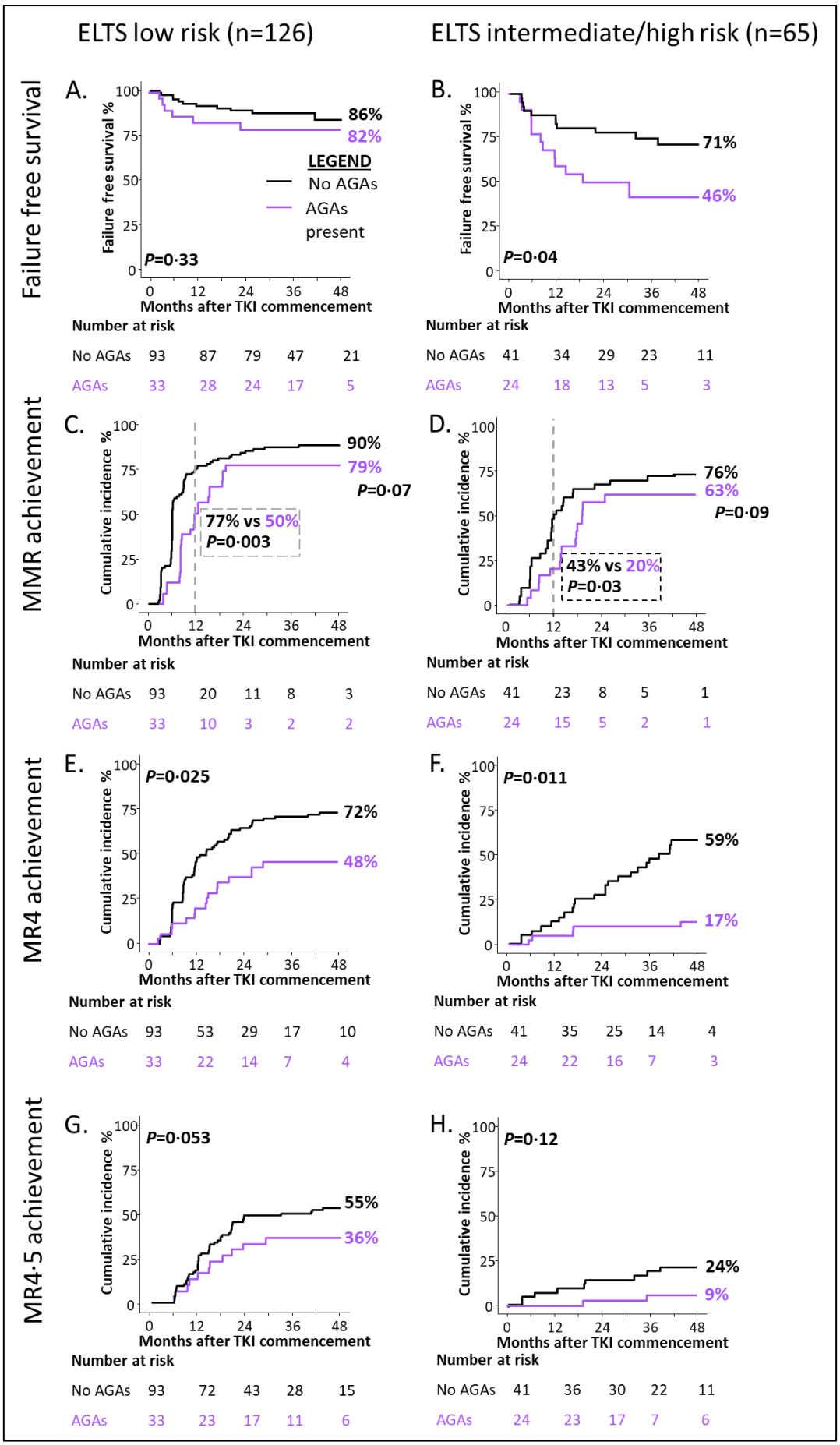
AGAs were detected in 34 of 126 patients (27%) with low ELTS score but did not predict FFS, Figure 4A. For the combined intermediate (n=46) / high-risk (n=19) patients, 25 of 65 (38%) had AGAs. FFS was significantly inferior for intermediate/high risk patients with AGAs compared with those without; 46% vs 71%, respectively p=0.04 (Figure 4B). With respect to MMR achievement, the impact of combining AGAs and ELTS was evident across the low-risk and intermediate/high-risk groups (Figures 4C-D). The cumulative incidence of MMR by 12 months for low-risk ELTS patients with AGAs was 50% (95% CI 32%-66%) compared with 77% (95% CI 67%-85%) for low-risk patients without AGAs, p=0.003 (Figure 4C). For intermediate/high-risk ELTS patients, the respective values were 20% (95% CI 7%-31%) compared with 43% (95% CI 32%-58%) for those without, p=0.03 (Figure 4D). Notably, none of the 19 patients in the high-risk ELTS group with an AGAs achieved MMR by 12 months. The statistical difference observed at 12 months was not observed at 48 months and was likely due to patients with AGAs having slower achievement of MMR but eventually reaching the target milestone. However, beyond 24 months, there were few patients available for evaluation.

Inferior MR4 and MR4.5 were predicted by the presence of AGAs among the ELTS risk groups. MR4 in low-risk ELTS patients with AGAs was lower compared with those without AGAs, 48% versus 72% by 4 years, p=0.025 (Figure 4E). The respective values for intermediate/high-risk ELTS patients were 17% versus 59%, p=0.011 (Figure 4F). No patient with high-risk ELTS with AGAs at diagnosis achieved MR4 by 4 years. The cumulative incidence of MR4.5 by 4 years in the low-risk ELTS group with AGAs was 36% versus 55% in those without AGAs, p=0.053 (Figure 4G). The respective values for intermediate/high-risk ELTS patients were 9% versus 24%, p=0.12 (Figure 4H). Again, among the high-risk ELTS patients, none with AGAs achieved

MR4.5 by 4 years.

Figure 4: Failure-free survival and molecular response among the ELTS risk groups according to AGAs at diagnosis.

FFS according to (A) Low and (B) Intermediate/high-risk ELTS; the 48-month cumulative incidence of MMR according to (C) Low and (D) Intermediate/high-risk ELTS with the 12-month values indicated; the 4-year cumulative incidence of MR4 according to (E) Low and (F) Intermediate/high-risk ELTS; and the 4-year cumulative incidence of MR4.5 according to (G) Low and (H) Intermediate/high-risk ELTS.


ELTS low risk (n=126)
ELTS intermediate/high risk (n=65)
Failure free survival
MMR achievement
MR4 achievement
MR4·5 achievement
A.
B.
C.
D.
E.
F.
G.
H.
Failure free survival %
Cumulative incidence %
Months after TKI commencement
LEGEND
No AGAs
AGAs present
86%
82%
71%
46%
P=0·33
P=0·04
90%
79%
P=0·07
77% vs 50%
P=0·003
76%
63%
P=0·09
43% vs 20%
P=0·03
72%
48%
P=0·025
59%
17%
P=0·011
55%
36%
P=0·053
24%
9%
P=0·12
Number at risk
No AGAs 93 87 79 47 21
AGAs 33 28 24 17 5
No AGAs 41 34 29 23 11
AGAs 24 18 13 5 3
No AGAs 93 20 11 8 3
AGAs 33 10 3 2 2
No AGAs 41 23 8 5 1
AGAs 24 15 5 2 1
No AGAs 93 53 29 17 10
AGAs 33 22 14 7 4
No AGAs 41 35 25 14 4
AGAs 24 22 16 7 3
No AGAs 93 72 43 28 15
AGAs 33 23 17 11 6
No AGAs 41 36 30 22 11
AGAs 24 23 17 7 6

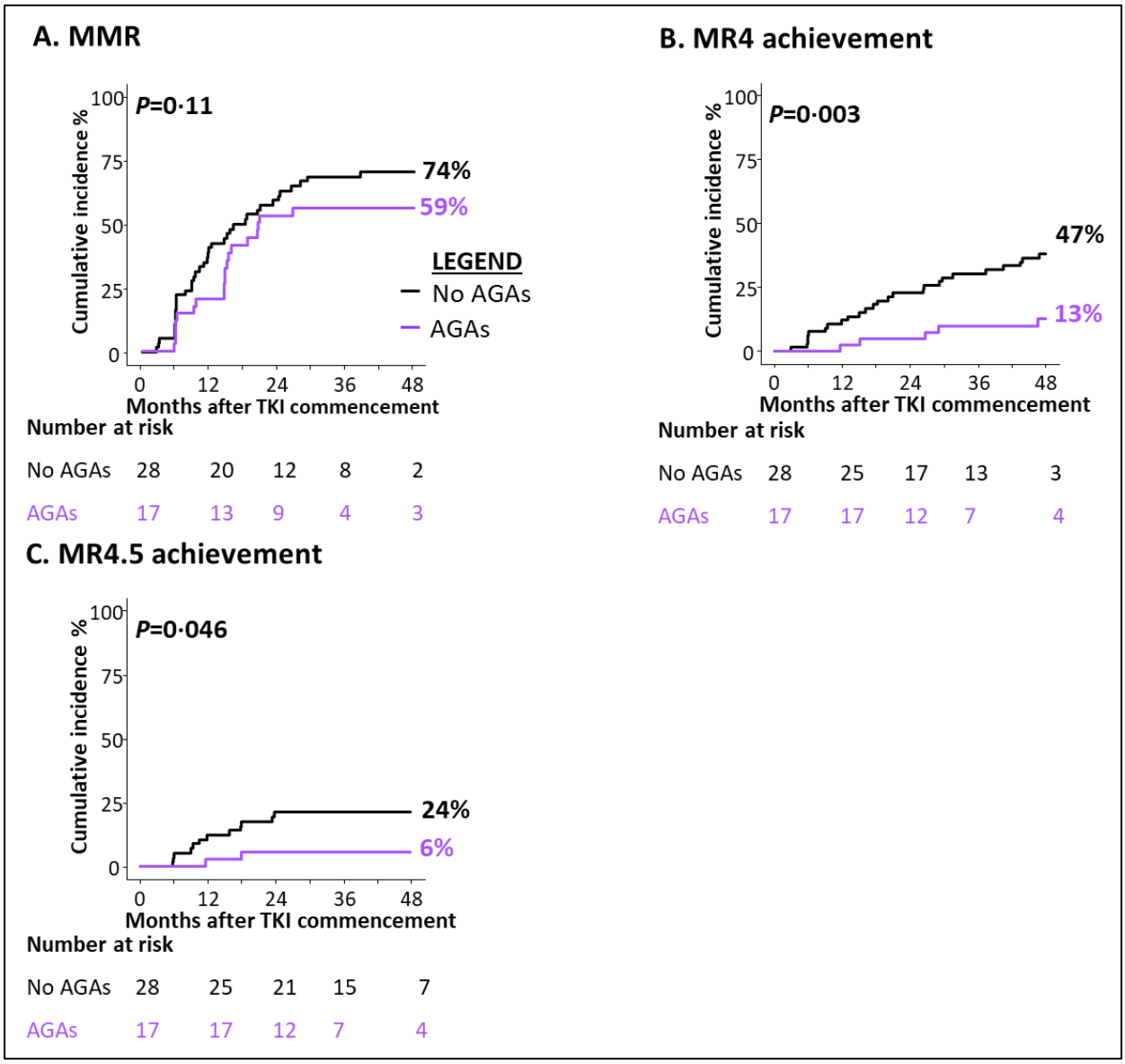
6.4.6 Impact of AGAs on molecular response for patients who switched to nilotinib

Trial-specific criteria for imatinib dose escalation or switch to nilotinib occurred for time-dependent suboptimal molecular response,[25] which was more pro-active than in current treatment guidelines. Patients also switched to nilotinib for loss of response or imatinib intolerance. Seventeen patients dose escalated imatinib to 800mg daily due to failure to meet trial-defined targets, which included 6 patients with AGAs. TKI switch to nilotinib due to failure to reach a subsequent milestone occurred in 14/16 evaluable patients. One patient switched to nilotinib due to intolerance of the higher imatinib dose, whereas 2 patients with AGAs achieved the subsequent molecular milestone. All that can be concluded from this limited data is that dose escalation of imatinib based on failure criteria had limited response, irrespective of the presence of AGAs or not. We assessed whether switch to nilotinib for any reason other than imatinib intolerance could rescue the inferior responses associated with AGAs at diagnosis. Fifty-three patients switched to nilotinib for reasons other than imatinib intolerance at a median of 189 days (85-678 days, Supplemental Table 4) and the switch occurred more often in patients with AGAs than patients without, 40% versus 20%, respectively, p=0.004.

The cumulative incidence of MMR by 4 years for the 53 patients who switched to nilotinib for reasons other than intolerance was numerically lower if AGAs were present at diagnosis, although this did not reach significance, 59% versus 74%, p=0.11 (Figure 5A). Fourteen of the 53 patients switched to nilotinib at 12 months for lack of MMR and 11 of these 14 achieved MMR following nilotinib switch. The cumulative incidence of MR4 by 4 years was significantly lower if AGAs were present at diagnosis, 13% versus 47%, p=0.003 (Figure 5B). Likewise, the 4-year cumulative incidence of MR4.5 was only 6% if AGAs were present compared with 24% for patients without AGAs, p=0.046 (Figure 5C). There was no significant difference in the cumulative incidence of 12-month MMR, 4-year MR4 or MR4.5 for the 24 patients who switched to nilotinib for imatinib intolerance, irrespective of the presence of AGAs (data not shown).

Figure 5: Impact of AGAs on molecular response for patients who switched to nilotinib for trial defined failure criteria

The graphs show the outcomes and responses according to AGA status at diagnosis in patients who switched to nilotinib for trial-defined nilotinib switch criteria, excluding imatinib intolerance. (A) Cumulative incidence of MMR, (B) Cumulative incidence of MR4 by 4 years, (C) Cumulative incidence of MR4.5 by 4 years.



6.4.7 Outcome for patients with mutated ASXL1

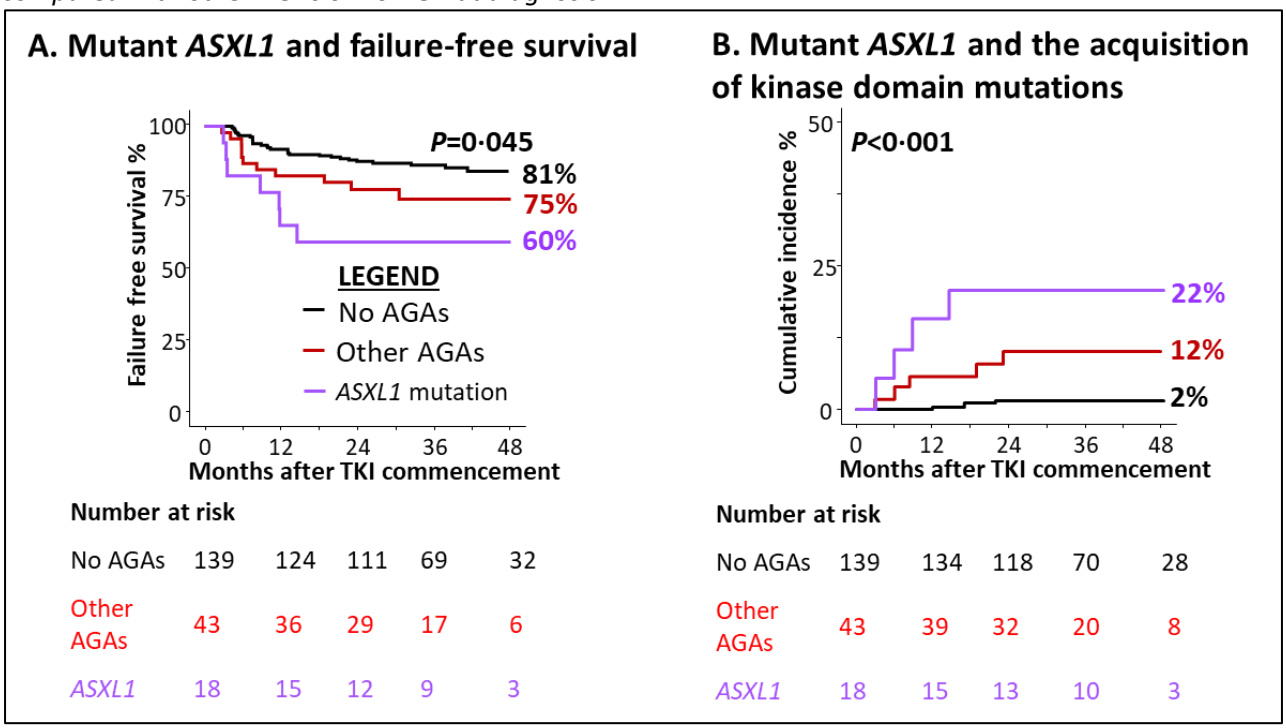
ASXL1 was the most frequently mutated cancer-associated gene at diagnosis. Eighteen patients had frameshift, nonsense or splice site *ASXL1* variants. These 18 patients had an inferior 4-year FFS compared with patients with other AGAs or without any AGAs: 60% versus 75% versus 81% respectively, *p*=0.045 (Figure 6A). Evolution to BP was only observed for 1 patient with mutated *ASXL1* at diagnosis, although the *ASXL1* mutant was noticeably absent

at progression (Figure 2). Notably, 4 of the 18 patients (22%) acquired *BCR*::*ABL1* kinase domain mutations, compared with 12% of patients with other AGAs and 2% of patients without any AGAs, $p<0.001$ (Figure 6B). None of the other molecular outcomes were significantly different between patients with mutant *ASXL1*, other AGAs or those with no AGAs. The most frequently detected *ASXL1* variant in our cohort was a nonsense variant at R693, which was identified in 6 of the 20 patients with *ASXL1* variants (Supplemental Table 2). This is among the most frequently reported *ASXL1* variants. Only 2 patients had the *ASXL1* mutant G646Wfs*12 at the time of diagnosis in our cohort, which is the most frequently reported *ASXL1* variant.

Interestingly, not all patients with *ASXL1* mutants at diagnosis had an adverse outcome. Figure 2 contrasts the dynamics of clonal expansion and disappearance for two patients with responses on either end of the therapeutic spectrum. At diagnosis, patient 430 had a Ph-associated rearrangement as part of the *BCR::ABL1* clone, plus 2 mutant subclones. One harbored an *ASXL1* frameshift variant and the other harbored a *BCORL1* frameshift variant, an *IKZF1* fusion and a *RUNX1* deletion. The patient rapidly progressed to lymphoid BP at 4 months after an initial response to imatinib and additional sequencing was performed.[17,27] The *ASXL1* subclone became undetectable, whereas the second 3-mutant subclone expanded and evolved at BP. Eight additional variants were detected at BP, including 6 *ABL1* kinase domain mutations. In contrast, patient 384 had a single *ASXL1* nonsense variant at diagnosis and an excellent response to imatinib therapy. A deep molecular response was achieved at 6 months and the *ASXL1* variant became undetectable by 4 months. The epigenetic modifier *ASXL1* may not harbor intrinsic transformation potential and additional genomic events may be necessary for progression.

Figure 6: ASXL1 variants were associated with treatment failure, including the acquisition of BCR::ABL1 kinase domain mutations.

ASXL1 variants predicted for (A) inferior FFS and the (B) acquisition of BCR::ABL1 mutations compared with other AGAs or no AGA at diagnosis.



6.5 Discussion

This study involving a large unselected cohort of consecutively treated CP-CML patients initiated with first-line imatinib,[27] expands the results of our prior genomic analysis of a highly selected patient cohort.[17] A higher frequency of AGAs at diagnosis was an independent predictor of treatment failure and inferior molecular responses. The data also expand the findings of other genomic studies where patients were retrospectively selected for sequencing based on their known clinical outcome.[22,23] Whether more potent TKIs when administered as first-line therapy can abrogate the inferior responses associated with AGAs[22] needs to be further investigated. However, the results presented in this study suggest a highly proactive approach with TKI switch for failure and sub-optimal molecular response may not completely negate the adverse impact of AGAs. In our unselected CML cohort, AGAs were detected in 31% of patients at diagnosis and were associated with inferior FFS and molecular responses, even when patients were treated with a highly proactive intervention strategy involving imatinib dose escalation and/or switch to nilotinib for suboptimal molecular responses. For example, the 4-year rate of MR4 in ENESTnd was 32% in the imatinib-treated

cohort where patients were randomized to receive 400mg daily of imatinib.[3] In comparison, the 4-year rate of MR4 in the TIDEL-II study population was 59%, which is similar to the MR4 rate achieved by the matched timepoint in the nilotinib 300mg twice daily arm of the ENESTnd study (56%).[3] Therefore conventional dosing of imatinib, i.e. 400mg daily of imatinib, will likely produce more marked differences than observed in this study.

The independent predictors of patient outcome (inferior FFS, acquisition of *BCR::ABL1* kinase domain mutations, lower rates of MMR, MR4 and MR4.5) were AGAs at diagnosis and the ELTS clinical risk score. No variable predicted overall survival or progression to AP/BP, but progression was uncommon in the study population. Combining AGAs and the ELTS risk category further differentiated subgroups with inferior molecular responses. In particular, patients with intermediate/high-risk ELTS who had AGAs at diagnosis had inferior FFS and molecular responses. Notably, none of the 8 patients in the high-risk ELTS group with AGAs achieved MMR by 12 months, or MR4 and MR4.5 by 4 years.

ASXL1 variants have been associated with an inferior outcome in many myeloid malignancies, including CML.[31] Furthermore, it is among the most frequently mutated genes at BP of CML.[17,23,32,33] Mutated *ASXL1* was associated with lower response rates to the third-generation TKI, olverembatinib, and higher risk of progression and death.[31] Consistent with other studies,[33] *ASXL1* was the most frequently mutated cancer gene at diagnosis in our cohort (9%) and *ASXL1* variants were associated with inferior FFS and higher risk of *BCR::ABL1* kinase domain mutation development compared with other AGAs. The most frequently reported *ASXL1* variant in myeloid neoplasms is G646Wfs*12.[34] The reliable detection of this mutant above background reads using next-generation sequencing requires a simple set of metrics.[35] Only 2 patients in our cohort (1%) had the *ASXL1* variant G646Wfs*12 at the time of diagnosis. In contrast, this variant has recently been reported at a high frequency in CP- or AP-CML patients with resistance to imatinib and/or second-generation TKIs.[31] The frequency of specific *ASXL1* variants may vary depending on the disease phase and characterization of much larger cohorts will be needed to confirm the prognostic effect of variants in individual genes. Ochi et al. assessed clonal evolution of genetic abnormalities in blastic transformation of CML.[32] Almost all patients with mutant *ASXL1* gained additional genetic abnormalities during progression to BP, suggesting that mutant *ASXL1* clones may gain a more transforming

phenotype through clonal evolution. Targeted therapeutic approaches for mutant *ASXL1* could be possible. Mutant *ASXL1* was reported to drive response to the *BCL2* inhibitor venetoclax in vitro[36] and a small molecule inhibitor of *BAP1* inhibited mutant *ASXL1*-driven leukaemic gene expression and impaired tumour growth in vivo.[37]

Ph-associated rearrangements are a novel genomic level event associated with the formation of the Ph chromosome that has only recently been described in CML.[17] We found a strong correlation between the presence of Ph-associated rearrangements and the acquisition of *BCR*::*ABL1* kinase domain mutations and significantly inferior molecular responses. Every rearrangement was novel and unique to individual patients. Whether these structural rearrangements directly influence response and outcome or if they are a marker of genomic instability is unknown. Ph-associated rearrangements may be associated with defective DNA double-strand break repair. When DNA repair mechanisms are not properly regulated, genome integrity may not be maintained. Major repair mechanisms of double strand breaks are homologous recombination or the error prone non-homologous end joining. It is not yet known whether homologous recombination repair deficiency plays a role in the generation of Ph-associated rearrangements, which could trigger use of error prone non-homologous end joining repair.[38-41] Complexity associated with the formation of the Ph chromosome for some patients was revealed by using higher resolution next-generation sequencing techniques, demonstrating added layers of genomic intricacies beyond the previously reported derivative 9 deletions. Furthermore, Ph-associated rearrangements were strongly associated with the absence of the *ABL1*::*BCR* reciprocal transcript, which is consistent with disruption of sequences adjacent to breakpoints on the derivative chromosomes. It is also consistent with the absence of *ABL1*::*BCR* transcripts previously reported for patients with large deletions adjacent to the translocation breakpoints identified by FISH.[42,43] Some rearrangements involved genomic inversions, fragmentation, circularization and random reassembly, potentially from genomic 'shattering'. These have been described in other malignancies and associated with therapy resistance.[44] The Ph-associated rearrangements emphasize that the formation of the *BCR::ABL1* gene fusion in some patients may result from a multi-step process.[45,46] Similar rearrangements have been identified in other fusion-based hematological malignancies, such as acute promyelocytic leukemia,[47,48] characterized by the *PML::RARA* fusion, and *RUNX1::RUNXT1* mutated acute myeloid leukemia,[49,50] a sub-type

generally associated with a favorable prognosis. In both contexts, these genomic findings have been associated with poor outcomes.[47,49] The promiscuous *KMT2A* gene defines a category of aggressive high-risk leukemia with over 100 fusion partners.[51,52] A subset of these patients had complex chromosomal rearrangements, internal tandem duplications, focal gene deletions, and 11q chromosomal inversions and insertions.[51] While further investigation is required to clarify the clinical and biological significance of these rearrangements, they may be a hallmark of genomic instability. A customized RNA-capture panel, designed specifically to identify novel genomic rearrangements associated with the expected fusions characterizing the specific leukemias, could illuminate the complexity of these events.

Risk stratification in CML in the TKI era has relied upon clinical and laboratory parameters within the Sokal risk score and more recently has included the ELTS score. The rate of early *BCR::ABL1* decline in response to TKI therapy has also been utilized to differentiate patient outcomes, with TKI switch recommended for patients failing to adequately meet the critical molecular milestones recommended within the first 12 months of TKI initiation. However, there is increasing awareness of the influence of cancer-gene variants for risk stratification in other hematologic malignancies, and genetic profile has been incorporated into diagnostics, prognostication and treatment algorithms.[53-56] Our work supports and expands the landscape of AGAs and reveals their effect on outcomes for patients with CML. Exploration of the complexities of Ph-associated rearrangements will also be useful as a novel predictor of adverse outcomes in CML and may pave the way for similar findings in other fusion-based cancers. While the TIDEL-II therapeutic approach does not reflect current treatment practices, where patients are treated with first-line second-generation TKIs or imatinib at a standard dose of 400mg, it highlights that despite higher dose first-line imatinib, aggressive monitoring and proactive TKI switching, the negative effect of AGAs could not be overcome. Whether upfront treatment with more potent second-generation TKIs can nullify the inferior outcomes we observed for patients with AGAs at diagnosis remains to be established. If this is proven, optimal TKI selection in CML could integrate the genomic landscape into risk stratification, in addition to other factors that influence selection of therapy, such as toxicity profile and patient comorbidity. Early consideration of allogeneic stem cell transplantation or novel therapeutic approaches in patients stratified as very high risk of treatment failure and inferior molecular responses based on their individual genetic and clinical profile may be justified.

Very high-risk features may include the detection of multiple AGAs at diagnosis, as was observed for patient 430 who had rapid progression at 4 months of imatinib (Figure 2). Expanded data are required to establish the genomic criteria that define very high risk.

6.6 Data Availability

De-identified participant data collected for our study can be made available to researchers once appropriate ethical approval and a signed data access agreement is obtained.

6.7 Acknowledgements

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6.8 Authorship Contributions

NS collected and analyzed the data and wrote the paper. CW designed the probes for the RNA capture panel, prepared samples and analyzed the data. NHS, VS, RRK, ML, JB and HA prepared samples and completed the required laboratory work. AWS, JF, PW, DT and CHK performed the bioinformatic analysis and pipeline development. IP, ASY, RMK, DMR, AKM, APS, APG, DTY and HS reviewed the manuscript. DTY, APG and TPH designed and conducted the TIDEL II trial and coordinated the correlative studies. TPH contributed key concepts and assisted in writing the paper. SB designed the research, analyzed the data, contributed key concepts and methodology, and assisted in writing the manuscript.

6.9 Disclosure of Conflicts of Interest

NS received honoraria from Novartis and meeting sponsorship from Novartis, Amgen, and Janssen. SB is a member of the advisory boards of Qiagen, Novartis and Cepheid, received honoraria from Qiagen, Novartis, Bristol-Myers Squibb, Incyte and Cepheid, and research funding from Novartis and Cepheid. ASY is a member of advisory board for Novartis, received research funding from Novartis, Bristol-Myers Squibb and Celgene and honoraria from Novartis and Bristol-Myers Squibb. AKM is a member of advisory boards of Sobi and Novartis, received speaker fees from Abbvie and meeting sponsorship from MSD and Amgen. DMR has received research funding and honoraria from Novartis and Bristol-Myers Squibb and honoraria from Takeda. APG received honoraria from Roche, MSD, Janssen, Novartis and Amgen while having advisory roles for MSD Oncology, Janssen and Novartis. TPH is a member of advisory boards and has received research funding and honoraria from Novartis and Bristol-Myers Squibb. The other authors declare no conflict of interest.

6.10 References

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Supplement

Supplemental Methods, Results, Tables and Figures to Shanmuganathan N, Impact of additional genetic abnormalities at diagnosis of chronic myeloid leukemia for first-line imatinib-treated patients receiving proactive treatment intervention (chapter 6)

Supplementary methods

Medication compliance assessment

Patient compliance assessments were included in the CML9 (TIDEL-II) study protocol and are as follows.

- Imatinib trough levels were performed on day 22 of imatinib therapy and patients with trough levels <1,000ng/mL who were confirmed to be compliant with imatinib administration underwent dose escalation to a total daily dose of imatinib of 800mg.
- Records of dosing diaries, dosages administered and returned as well as the interval between study visits were recorded by study personnel.
- Imatinib and/or nilotinib drug accountability was logged by the pharmacy and study staff of each study centre.
- Patients with questionable study compliance were reported to the study management committee and patients deemed to be non-compliant were withdrawn from the study following discussion with the treating physician.

Next-generation sequencing

The genes selected for inclusion in the customized panel were either recurrently mutated in CML[1] or commonly reported in acute leukemias. Specific intronic regions of *BCR* were targeted by probes to detect the genomic breakpoints of the common e13a2 and e14a2 *BCR::ABL1* fusions. To maximise the detection of Ph-associated rearrangements, we included additional genes on chromosomes 9 and 22. Furthermore, probes targeted intronic cryptic *RAG* recombination signal sequences of specific genes for the detection of *RAG*-

mediated deletions, including *IKZF1* (Supplementary Table 1). The STAR algorithm aligned the raw sequencing data to the hg19 reference genome.[2] Genomic breaks were detected using total RNA due to the presence of intron-retaining precursor mRNA, usually absent from standard poly(A) enriched RNA-seq.[3] The detection of Ph-associated rearrangements was possible, even if only one of the fusion partner regions was captured, through spanning reads that partially mapped to the partner gene. Some events were the product of deletion-bridges,[4] which were identified by copy number variation analysis or aberrant splicing.[5] Other Ph-associated rearrangements were derived from variant translocations, as evidenced by chromosome analysis.

Additional detail regarding sequencing and validation methods has been published elsewhere.[3,5] The variant callers were VarDict[6] and Mutect2.[7] Strict criteria were used to assess the pathogenicity of each SNV and indel and only variants of potential or known clinical relevance were reported, as described previously.[5] The classification standards only categorize somatic variants as likely pathogenic or pathogenic based on their potential to confer growth and survival advantages in tumor cells.[8] All variants were validated through orthogonal testing using Sanger sequencing or repeat testing using next-generation sequencing. The somatic status of variants was established by sequencing remission samples, mesenchymal stromal cells[5] or inferred by a rise in variant allele frequency (VAF) in samples at progression. SNVs and indels with VAF ≥5%[3] were assessed for their association with outcome. Gene fusions were detected and filtered as previously described.[5] Candidate Ph-associated rearrangements were supported by at least 5 unique chimeric reads. Unique fusions supported by fewer chimeric reads were considered valid if they were additionally supported by evidence of corresponding genomic breaks and/or reciprocal fusions or alternative transcripts. The reliability of this strategy for the classification of high confidence somatic fusions was validated by testing diagnosis, remission or mesenchymal stromal cells using PCR and Sanger sequencing or by repeat analysis using next-generation sequencing.[3,5] Patients where a SNV or indel of VAF <5% was the only cancer gene variant identified, were not classed as having a clinically relevant variant. Aberrant splicing was investigated through customized pipelines and sashimi plots. All variants were reviewed using the Integrative Genomics Viewer.[3,9]

We have previously demonstrated that RNA-based capture sequencing improved the diagnostic yield of mutant detection in CML and was capable of detecting a range of variant types.[3] These include gene fusions, RNA splice alterations and focal gene deletions in specifically targeted genes. Furthermore, intronic *BCR*::*ABL1* genomic breakpoints are detectable from pre-spliced RNA. Targeting intronic cryptic *RAG*-mediated recombination signal sequences in specific genes aids the detection of *RAG*-mediated deletions, which are common in lymphoid BP.[10] These deletions are also detectable through the generation of atypical RNA splicing.[3] Furthermore, deletions adjacent to *BCR* and/or *ABL1* can be inferred based on the presence of novel fusions involving these genes, including an estimation of the deletion size (Supplemental Table 2). Copy gain detection using this RNA-based methodology is not possible. DNA-based sequencing with the appropriate bioinformatic tools can detect copy changes but is unable to detect fusion transcripts or assess the effect of splice site variants on RNA splicing.

Supplementary Results

Complexity of Ph-associated rearrangements

Most Ph-associated rearrangements involved *BCR* and/or *ABL1* and a novel fusion partner, Figure 1. However, a small number of rearrangements involved other genes on chromosomes 9 or 22, with many of the rearrangements predicted to occur on the derivative chromosome 9. Genes such as *EXOSC2*, directly upstream of *ABL1* on chromosome 9, or the intergenic region between *EXOSC2* and *ABL1* were recurrently involved in Ph-associated rearrangements. Other recurrent fusion partner genes were *USP20* and *HMCN2*, also on chromosome 9 and upstream of *ABL1*. Interestingly, *HMCN2* is not normally expressed in blood[11] but was expressed when fused to another gene in a Ph-associated rearrangement. This highlights the potential for novel fusions to alter function. The reciprocal *ABL1::BCR* transcript was detected in 61% of all patients. Only one of 36 patients with a Ph-associated rearrangement had an *ABL1::BCR* reciprocal transcript detected. The rearrangement for this patient comprised a genomic inversion between *BCR* and *ABL1* that was immediately adjacent to the standard *BCR::ABL1* genomic breakpoint. Lack of a reciprocal transcript in 97% of patients with a Ph-associated rearrangement is consistent with loss or rearrangement of sequence adjacent to the *ABL1* and/or *BCR* breakpoints in many cases.

Additional layers of genomic complexity of the Ph-associated rearrangements were sequence inversions (Supplemental Figure 1A) and/or fusions involving additional chromosomes. The Ph-associated rearrangements were a combination of genomic events and transcripts (Supplemental Figure 1B-C). When multiple Ph-associated rearrangements were identified for an individual patient, they were all linked to the primary Ph chromosome. The simplest example to illustrate Ph-associated rearrangements is shown in Supplemental Figure 4, where patient 439 had a t(9;22;12) variant Ph chromosome. Targeted RNA sequencing characterized the precise genomic breakpoints at nucleotide level resolution on each chromosome, which occurred within the *ARID2* gene on chromosome 12 and generated novel gene fusions. The *ARID2::BCR* and *ABL1::ARID2* fusion transcripts were reciprocal products of the three-way chromosomal translocation. This patient also had a 17Kb genomic inversion within *BCR* intron 14, upstream of the *BCR::ABL1* genomic break. Targeting exons of *BCR* and *ABL1* in the capture panel, plus the common intronic breakpoint regions of *BCR* was sufficient to identify the novel *ARID2* partner gene, even though this gene was not specifically targeted by probes. This case highlights how next-generation sequencing can reveal further complexity of a rearrangement. Additional chromosome involvement was also identified such as observed in patient 434 who had a variant translocation, t(9;22;19). RNA sequencing and the detection of novel gene fusions involving *BCR* and *ABL1* revealed the additional involvement of chromosome 17, as detailed in Supplemental Table 2. Overall, 11 patients had a variant Ph chromosome reported and novel fusions involving genes/sequences on the involved chromosomes were identified in 7 of these. One additional patient expressed a fusion between *ABL1* and a gene on chromosome 20 but a variant translocation was not reported.

Almost half of the Ph-associated rearrangements involved sequence inversions (Supplemental Figure 1A). These included inversions within the *BCR* gene adjacent to the *BCR*::*ABL1* genomic breakpoints (Supplemental Figure 3) and even *BCR*::*ABL1* fusions where segments of either *BCR* or *ABL1* were inverted. Moreover, some Ph-associated rearrangements may have involved the formation of possible circular DNA (Supplemental

Figure 4). We did not observe a worse outcome for patients with a greater complexity of Ph-associated rearrangement.

Two patients had rearrangements involving genes upstream of *ABL1*. One was a rearrangement between *DOLK* and its adjacent gene *NUP188* that was identified using whole transcriptome sequencing and RNA panel sequencing. These genes lie approximately 2 Mb upstream of *ABL1* on chromosome 9. Cryptic deletion of *NUP188* has been reported in acute lymphoblastic leukemia.[12] However, PCR and Sanger sequencing of the diagnosis, remission and mesenchymal stromal cell samples confirmed the rearrangement was a rare germline variant and was not associated with the formation of the Ph chromosome. The second rearrangement was an inversion between *EXOSC2*, which is immediately upstream of *ABL1*, and the *FUBP3* gene approximately 70 Kb upstream of *EXOSC2*. In this case, the inversion was confirmed as somatic and likely a Ph-associated rearrangement.

Cryptic splicing of BCR::ABL1

Aberrant low level transcription of *BCR*::*ABL1* was evident in most patients. This frequently involved fusion between *BCR* exon 14 and *ABL1* intron 1 sequence at cryptic splice acceptor sites. Additionally, recurrent fusions between sequences within *BCR* intron 14 and *ABL1* intron 1 were frequently observed. The *BCR* fusion junctions occurred in the region between 22:23633075 and 22:23634757 (hg19 genome build) and the most frequent junction site was at 22:23633094. The fusion junctions within *ABL1* occurred over a wide region: 9:133591769 to 9:133729453. There was no association between the detection of these *BCR*::*ABL1* transcripts and outcome. The high coverage achieved with an RNA-based targeted sequencing strategy using total RNA leads to the ubiquitous detection of rare transcripts. The deep complexity of the transcriptome and the detection of rare transcripts was previously reported using a similar high depth sequencing strategy.[13] In that study, low level non-coding transcription was reported in intergenic regions and novel isoforms of well studied genes, such as *TP53*. Novel splice junctions occurred at regions that were enriched for canonical splice motifs, which is consistent with our observations involving *BCR*::*ABL1*.

Supplementary Tables

Supplementary Table 1: Captured genes with additional captured intronic regions

- Genes included to detect fusion transcripts are indicated, including Ph-associated rearrangements on chromosomes 9 and 22
- Genes included to detect *RAG*-mediated deletions or to aid their detection are indicated (Deletion)
- Some genes are mutated by various mechanisms (including fusions, deletions and indels), such as *IKZF1*, *PAX5* and *RUNX1*

Gene captured	Chromosome	Comment
ABL1	9	Fusion
AFF1	4	Fusion
AFF4	7	Fusion
AIF1L	9	Fusion
AKT1	14	
ASS1	9	Fusion
ASXL1	20	
ATM	11	
BAP1	3	
BCOR	X	
BCORL1	X	
BCR	22	Fusion
BTG1	12	Deletion
BTK	X	
CABIN1	22	Fusion
CACNA1B	9	Fusion
CALR	5	
CBFB	16	Fusion
CBL	11	
CDKN2A	9	Deletion
CDKN2B	9	Deletion
CDKN2B-AS1	9	Deletion
CEBPA	19	
CREBBP	16	
CRLF2	X	Fusion
CSF3R	1	
CUX1	7	
DNMT3A	2	
EED	11	
ELL	19	Fusion
EP300	22	
ERG	21	Fusion
ETV6	12	Fusion
EXOSC2	9	Fusion
EZH2	7	

FAT1	4	
FAT3	5	
FBXW7	4	
FIGNL1	7	Deletion
FLT3	13	
FOCAD	9	Deletion
GATA2	3	
GGT1	22	Fusion
HBS1L	6	Deletion
HMCN2	9	Fusion
IDH1	2	
IDH2	15	
IKZF1	7	Deletion/Fusion
IL7R	5	
JAK1	1	
JAK2	9	
JAK3	19	
KAT6A	8	Fusion
KDM5A	12	Fusion
KDM6A	X	
KIT	4	
KMT2A	11	Fusion
KMT2D	12	
KRAS	12	
MBNL1	3	Fusion
MECOM	3	Fusion
MEF2D	1	Fusion
MICAL3	22	Fusion
MLLT1	19	Fusion
MLLT10	10	Fusion
MLLT3	9	Fusion
MLLT4	6	Fusion
MLLT6	17	Fusion
MPL	1	
MSI2	17	Fusion
MTAP	9	Deletion
MYB	6	Fusion
MYC	8	Fusion
MYH11	16	Fusion
MYH9	22	Fusion
NCOR1	17	
NEK6	9	Fusion
NF1	17	
NOTCH1	9	
NPDC1	9	Fusion
NPM1	19	
NRAS	1	

NSD2	4	Fusion
NT5C2	10	
NUP188	9	Fusion
NUP214	9	Fusion
NUP98	11	Fusion
PAX5	9	Deletion/Fusion
PDGFRB	5	Fusion
PHF6	X	
PPM1D	17	
PPM1F	22	Fusion
PTEN	10	
PTPN11	12	
RAD21	8	
RAD51	15	Fusion
RARA	17	Fusion
RB1	13	Deletion
RCBTB2	13	Deletion
RUNX1	21	Fusion
RUNX1T1	8	Fusion
SETBP1	18	
SETD1B	12	
SETD2	3	
SF3B1	2	
SH2B3	12	
SHANK3	22	Fusion
SMARCA4	19	
SMARCB1	22	Fusion
SMC1A	X	
SMC3	10	
SPECC1L	22	Fusion
SRSF2	17	
STAG2	X	
STAT5B	17	
SUZ12	17	Fusion
TCF3	19	Fusion
TET2	4	
TNRC6B	22	Fusion
TP53	17	
TXNRD2	22	Fusion
U2AF1	21	
UBE2A	X	
USP20	9	Fusion
VPREB1	22	Fusion
WT1	11	
XPO1	2	
ZCCHC7	9	Fusion
ZNF384	12	Fusion

ZRSR2	X	

- Additional intergenic/intronic captured regions to detect specific focal deletions and the p210 *BCR-ABL1* genomic breakpoints

Chromosome	Start	End	Associated gene
22	23631623	23633120	*BCR* p210 breakpoint cluster region, intron 13, 14 and 15
13	48856820	48856971	RB1
13	48858675	48858990	RB1
13	48860525	48860748	RB1
13	48867360	48867576	RB1
13	48868330	48868511	RB1
13	48875705	48875934	RB1
13	48881095	48881272	RB1
13	48886125	48886222	RB1
13	48896420	48896614	RB1
13	48896685	48896876	RB1
13	48918535	48918754	RB1
13	48922545	48922716	RB1
13	48932310	48932530	RB1
13	48935915	48936052	RB1
13	48950005	48950129	RB1
13	48955670	48955842	RB1
13	48966980	48967229	RB1
13	48968650	48968728	RB1
13	48968760	48968835	RB1
13	48973245	48973461	RB1
13	48984480	48984709	RB1
13	48986460	48986668	RB1
13	48997285	48997403	RB1
13	49000080	49000301	RB1
13	49004490	49004606	RB1
13	49008150	49008251	RB1
13	49009995	49010172	RB1
13	49013975	49014106	RB1
13	49018115	49018301	RB1
13	49020730	49020954	RB1
13	49028130	49028314	RB1
13	49033545	49033721	RB1
13	49056460	49056659	RB1
13	49061545	49061761	RB1
13	49074900	49075083	RB1
21	36212278	36214100	RUNX1
6	135365878	135366126	*HBS1L-MYB* intergenic region
6	135366538	135366683	*HBS1L-MYB* intergenic region

6	135423673	135423800	*HBS1L-MYB* intergenic region
6	135423813	135423913	*HBS1L-MYB* intergenic region
6	135437513	135437758	*HBS1L-MYB* intergenic region
7	50168194	50168365	IKZF1
7	50345069	50345619	IKZF1
7	50345869	50346147	IKZF1
7	50346419	50347403	IKZF1
7	50347404	50347584	IKZF1
7	50350309	50350704	IKZF1
7	50351569	50351850	IKZF1
7	50352074	50352258	IKZF1
7	50352339	50352485	IKZF1
7	50352639	50352858	IKZF1
7	50354139	50354398	IKZF1
7	50359029	50359205	IKZF1
7	50363499	50363703	IKZF1
7	50412736	50413150	IKZF1
7	50416336	50416614	IKZF1
7	50418576	50418960	IKZF1
7	50419816	50420171	IKZF1
7	50423391	50423820	IKZF1
7	50424386	50424626	IKZF1
7	50427026	50427653	IKZF1
7	50429151	50429298	IKZF1
7	50429581	50429746	IKZF1
7	50431561	50431745	IKZF1
7	50432141	50432679	IKZF1
7	50433961	50434179	IKZF1
7	50434831	50435001	IKZF1
7	50435026	50435353	IKZF1
7	50438286	50438558	IKZF1
7	50440361	50441112	IKZF1
7	50441786	50442077	IKZF1
7	50442701	50442846	IKZF1
7	50445936	50446250	IKZF1
7	50446851	50447143	IKZF1
7	50457031	50457321	IKZF1
7	50458861	50459143	IKZF1
7	50461346	50461632	IKZF1
7	50461651	50461896	IKZF1
7	50463446	50463792	IKZF1
7	50465816	50466062	IKZF1
7	50471416	50471665	IKZF1
7	50471696	50471885	IKZF1
7	50472961	50473175	IKZF1
7	50474036	50474245	IKZF1
7	50477941	50478212	IKZF1

7	50481221	50481503	IKZF1
7	50483291	50483526	IKZF1
7	50483761	50484190	IKZF1
7	50484201	50484293	IKZF1
7	50484316	50484678	IKZF1
7	50486726	50486998	IKZF1
7	50489341	50489599	IKZF1
7	50496801	50497012	IKZF1
7	50517171	50517353	IKZF1
9	21827569	21827777	CDKN2A/B
9	21902059	21902240	CDKN2A/B
9	21967164	21967369	CDKN2A/B
9	21967864	21968006	CDKN2A/B
9	21970099	21970432	CDKN2A/B
9	21971944	21972040	CDKN2A/B
9	21975709	21975910	CDKN2A/B
9	21981199	21981481	CDKN2A/B
9	21982954	21983141	CDKN2A/B
9	21984224	21984437	CDKN2A/B
9	21985899	21986085	CDKN2A/B
9	21988794	21988930	CDKN2A/B
9	21990329	21990543	CDKN2A/B
9	21990909	21991257	CDKN2A/B
9	21991774	21991977	CDKN2A/B
9	21992704	21992866	CDKN2A/B
9	22008189	22008436	CDKN2A/B
9	36802954	36803129	PAX5
9	36806444	36806663	PAX5
9	36807204	36807457	PAX5
9	36808164	36808383	PAX5
9	36825269	36825476	PAX5
9	36828324	36828529	PAX5
9	36830839	36831052	PAX5
9	36831849	36832058	PAX5
9	36832159	36832387	PAX5
9	36833714	36833853	PAX5
9	36835399	36835581	PAX5
9	36839144	36839326	PAX5
9	36840879	36841009	PAX5
9	36841019	36841117	PAX5
9	36849184	36849360	PAX5
9	36854969	36855142	PAX5
9	36857259	36857485	PAX5
9	36857784	36857978	PAX5
9	36858969	36859189	PAX5
9	36859504	36859683	PAX5
9	36861964	36862234	PAX5

9	36863419	36863531	PAX5
9	36867249	36867458	PAX5
9	36868344	36868548	PAX5
9	36875204	36875425	PAX5
9	36876944	36877057	PAX5
9	36877384	36877594	PAX5
9	36880869	36880974	PAX5
9	36882489	36882701	PAX5
9	36885144	36885373	PAX5
9	36885659	36886042	PAX5
9	36892769	36892970	PAX5
9	36894534	36894743	PAX5
9	36895884	36896065	PAX5
9	36896184	36896398	PAX5
9	36911864	36912079	PAX5
9	36912169	36912361	PAX5
9	36913384	36913612	PAX5
9	36914019	36914232	PAX5
9	36914334	36914554	PAX5
9	36914559	36914777	PAX5
9	36921529	36921735	PAX5
9	36922184	36922408	PAX5
9	36925694	36925902	PAX5
9	36926149	36926353	PAX5
9	36928944	36929114	PAX5
9	36930509	36930715	PAX5
9	36931889	36932016	PAX5
9	36933854	36934064	PAX5
9	36942344	36942516	PAX5
9	36943559	36943675	PAX5
9	36945524	36945625	PAX5
9	36946769	36946970	PAX5
9	36947159	36947366	PAX5
9	36963449	36963659	PAX5
9	36967114	36967325	PAX5
9	36970584	36970967	PAX5
9	36970989	36971401	PAX5
9	36975164	36975378	PAX5
9	36977089	36977247	PAX5
9	36980524	36980744	PAX5
9	36981539	36981745	PAX5
9	36981779	36981997	PAX5
9	37007129	37007342	PAX5
9	37009124	37009305	PAX5
9	37011789	37012015	PAX5
9	37014584	37014752	PAX5
9	37018584	37018795	PAX5

9	37022529	37022711	PAX5
9	37022919	37023274	PAX5
9	37024539	37024751	PAX5
9	37024764	37024941	PAX5
9	37026559	37026787	PAX5
9	37027609	37027740	PAX5
9	37030544	37030792	PAX5
9	37032619	37032842	PAX5
9	37032844	37033002	PAX5
9	37033009	37033107	PAX5
9	37034614	37034798	PAX5
9	37042229	37042439	PAX5
9	37048214	37048379	PAX5
9	37052179	37052449	PAX5

Supplementary Table 2: All additional genetic abnormalities (AGAs) identified in sequenced patients

Cancer gene variants: The median variant allele frequency of the 40 mutations was 29% (range 4.1-91.3%)

*Failure criteria are defined by the ELN, Hochhaus et al, Leukemia 2020:34(4);966-984.

**Failure criterion = death without transformation or transplant

i = intron, e = exon. ABL1 exons are indicated by the symbol 'a' for consistency with the commonly used nomenclature

VAF = variant allele frequency

Cancer gene variant = clinically relevant variant with VAF >5% in a cancer-related gene

Blank cells = not detected

8 patients have both cancer gene mutations and Ph-associated rearrangements

Other abbreviations: H = High; I = Intermediate; L = Low; inv = inversion; NA = Not available

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
317	71	M	H	e14a2		Yes	No	Yes	No	No	Yes	23634582	133637182	i14i1	21
320	38	M	NA	e14a2	a1e15	No	Yes	No	No	No	Yes	23634055	133604365	i14i1	69
389	48	M	I	e14a2		Yes	No	Yes	Yes	Yes	No				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
469	32	F	L	e13a2, e14a2		Yes	Yes	Yes	Yes	No	Yes	23634518	133639230	i14i1	81
325	76	M	I	e13a2		Yes	No	No	No	No	No				
505	63	F	L	e14a2		No	Yes	No	No	No	Yes	23633896	133714077	i14i1	39
494	33	M	L	e13a2, e14a2		No	Yes	No	No	No	Yes	23633032	133681722	i14i1	58
460	25	M	L	e14a2	a1e15	No	Yes	No	Yes	No	No	23634540	133592160	i14i1	19
348	53	F	L	e14a2		Yes	No	No	No	No	Yes				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
495	68	F	I	e1a2	a1e2	No	Yes	Yes	Yes	No	No				
465	20	F	I	e13a2		Yes	No	Yes	Yes	No	No	23632106	133644789	i13i1	50
429	36	M	L	e13a2		Yes	No	No	No	No	Yes				
439	63	F	L	e14a2		Yes	No	No	No	No	Yes	23634627	133636390	i14i1	7
474	66	F	I	e14a2	a1e15	No	Yes	Yes	Yes	Yes	No	23632699	133606508	i14i1	9

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
418	23	M	L	e13a2		Yes	No	Yes	No	No	No				
430	37	M	I	e14a2		Yes	Yes	No	Yes	Yes	No	23634010	133641245	i14i1	199
331	17	M	I	e13a2		Yes	No	No	Yes	No	Yes				
471	32	F	L	e13a2		No	Yes	No	No	No	Yes	23632276	133607586	i13i1	24

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
462	57	F	L	e13a2		Yes	No	No	No	No	No	23632158	133646194	i13i1	33
461	24	F	L	e13a2		Yes	No	No	No	No	Yes				
524	68	M	I	e13a2	a1e14	No	Yes	No	No	No	Yes	23632070	133650307	i13i1	105
350	45	M	I	e14a2		Yes	No	No	Yes	Yes	No				
440	23	F	L	e13a2, e14a2	a1e15	No	Yes	No	No	No	Yes	23634152	133683213	i14i2	6
403	50	M	I	e13a2, e14a2	a1e15	No	Yes	Yes	Yes	No	No	23633626	133632153	i14i1	57
501	30	F	L	e13a2		Yes	No	No	No	No	No				
376	65	M	L	e14a2		No	Yes	No	No	No	Yes	23633618	133675348	i14i1	16

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
377	39	M	L	e13a2		Yes	No	No	No	No	No	23632440	133674454	i13i1	51
434	55	F	L	e13a2, e14a2		Yes	No	No	No	No	Yes				
373	62	F	I	e13a2		Yes	No	No	No	No	Yes				
336	70	M	I	e13a2, e14a2		Yes	No	No	No	No	Yes				
490	79	F	H	e14a2		Yes	No	Yes	No	No	No	23634205	133667504	i14i1	99
398	52	F	I	e13a2, e14a2	a1e15	No	Yes	No	No	No	No	23632768	133636254	i14i1	76
394	55	M	L	e13a2	a1e14	No	Yes	No	No	No	No	23632304	133619807	e13i1	20

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
384	47	M	L	e13a2	a1e14	No	Yes	No	No	No	Yes	23632318	133627041	i13i1	5
437	49	M	L	e13a2		Yes	Yes	No	No	No	No	23633598	133610360	i14i1	10
514	40	M	L	e13a2	a1e14	No	Yes	No	Yes	No	No	23632119	133708410	i13i1	2
395	71	M	I	e13a2, e14a2		No	Yes	Yes	No	No	Yes				
456	34	M	L	e14a2	a1e15	No	Yes	Yes	No	No	No	23634601	133709994	i14i1	32
369	57	F	L	e13a2		Yes	No	No	Yes	Yes	No	23632144	133708168	i13i1	40

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
400	55	F	L	e13a3		Yes	Yes	No	No	No	No	23631881	133730113	i13i1	13
405	52	F	L	e13a2, e14a2	a1e16	No	Yes	No	No	No	Yes	23634755	133616494	e15i1	18
503	67	M	L	e14a2	a1e15	No	Yes	Yes	Yes	Yes	No	23633566	133590455	i14i1	129
365	51	M	I	e14a2	a1e15	No	Yes	Yes	No	No	No	23633221	133591248	i14i1	3
401	32	M	L	e14a2	a1e15	No	Yes	No	No	No	No	23634426	133629095	i14i1	21

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
355	45	M	H	e14a2	a1e16	Yes	Yes	Yes	No	No	No	23634777	133608203	e15i1	10
450	32	M	L	e13a2		Yes	No	No	No	No	Yes	23632327	133630022	i13i1	69
356	29	M	I	e14a2	a1e15	No	Yes	No	No	No	No	23634134	133714442	i14i1	46
485	24	M	I	e13a2		Yes	Yes	Yes	Yes	No	No	23632225	133602047	i13i1	11
492	61	M	L	e14a2	a1e15	No	Yes	Yes	No	No	No	23634576	133681281	i14i1	100
518	43	F	L	e14a2		Yes	No	Yes	No	No	No	23634382	133646443	i14i1	8
357	44	M	L	e14a2		Yes	No	No	No	No	No	23633873	133629417	i14i1	21
358	53	F	H	e14a2		Yes	Yes	Yes	Yes	Yes	No				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
413	40	M	NA	e13a2		Yes	Yes	Yes	No	No	No				
359	57	M	I	e14a2		Yes	No	Yes	Yes	Yes	No	23634567	133711605	i14i1	50
343	58	F	L	e13a2/e14a2	a1e15	No	Yes	No	No	No	Yes	23634488	133660079	i14i1	61
482	36	F	L	e13a2		Yes	No	Yes	No	No	No				
522	55	F	L	e13a2		Yes	No	No	No	No	Yes				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
415	46	M	H	e13a2	a1e15	No	Yes	Yes	Yes	No	No	23632580	133607821	e14i1	3
397	53	M	L	e13a2		Yes	No	Yes	Yes	Yes	No				
371	49	M	H	e13a2,e14a2		Yes	No	Yes	No	No	No	23634043	133684352	i14i1	68
464	32	M	I	e13a2		Yes	No	Yes	No	No	No				
318	47	M	L	e13a2	a1e15	No	No	No	No	No	Yes	23632332	133591489	i13i1	9
319	32	M	L	e13a2		No	No	No	No	No	Yes	23632370	133690379	i13i1	
321	60	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23633226	133684933	i14i1	
322	68	F	H	e13a2		No	No	No	No	No	Yes	23631950	133600690	i13i1	31
323	47	F	L	e13a2	a1e14	No	No	No	No	No	Yes	23633556	133669987	i14i1	57
324	40	M	I	e14a2	a1e15	No	No	No	No	No	Yes	23634659	133644751	i14i1	2
326	48	M	L	e14a2	a1e15	No	No	No	Yes**	No	Yes	23633514	133700414	i14i1	2
327	53	F	L	e13a2/e14a2		No	No	No	No	No	Yes	23634423	133616821	i14i1	
328	45	M	I	e13a2	a1e14	No	No	No	No	No	No				
329	46	M	I	e13a2	a1e14	No	No	No	No	No	Yes	23632247	133601045	i13i1	20
330	18	F	L	e13a2/e14a2		No	No	No	Yes	No	No	23632942	133608369	i14i1	2
333	80	M	H	e13a2/e14a2		No	No	No	Yes**	No	Yes	23634136	133608065	i14i1	25
334	19	M	H	e13a2	a1e14	No	No	No	No	No	Yes	23632350	133708491	i13i1	82
335	25	F	L	e14e2		No	No	No	Yes	No	No	23634573	133599071	i14i1	14
337	32	M	L	e14a2		No	No	No	No	No	Yes	23633466	133590172	i14i1	11
338	78	M	I	e14a2	a1e16	No	No	No	No	No	Yes	23634805	133608786	e15i1	70
339	69	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23633189	133695712	i14i1	8
340			L	e13a2/e14a2	a1e16	No	No	No	No	No	Yes				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
341	47	F	L	e13a2		No	No	No	No	No	Yes	23631852	133701480	i13i1	14
342	39	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23633146	133664478	i14i1	16
344	72	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23634361	133680157	i14i1	27
345	29	M	L	e14a2	a1e15	No	No	Yes	No	No	No	23634297	133592378	i14i1	132
346	28	M	L	e13e2	a1e15	No	No	Yes	No	No	No				
347	58	M	I	e13a2	a1e14	No	No	No	No	No	Yes	23632206	133602429	i13i1	24
349	50	M	L	e13a2	a1e15	No	No	No	Yes	No	No	23632582	133608128	e14i1	6
351	58	F	I	e14a2	a1e15	No	No	No	No	No	Yes	23634455	133718495	i14i1	8
352	57	M	I	e14a2	a1e15	No	No	No	Yes	No	No	23634355	133704019	i14i1	2
353	24	M	L	e14a2		No	No	No	No	No	Yes	23634339	133638712	i14i1	3
354	34	M	I	e13e2	a1e15	No	No	No	No	No	No	23632256	133685168	i13i1	5
528	63	F	H	e13a2/e14a2	a1e15	No	No	No	Yes**	No	No	23632892	133623193	i14i1	49
360	48	F	L	e14a2	a1e15	No	No	No	No	No	Yes				
361	59	M	I	e13a2/e14a2	a1e15	No	No	Yes	Yes	Yes	Yes	23632635	133592170	i14i1	20
363	57	F	L	e13a2	a1e14	No	No	Yes	No	No	Yes	23632257	133685929	i13i1	30
364	63	F	L	e14a2		No	No	No	No	No	Yes	23633758	133610358	i14i1	21
366	46	F	L	e13a2		No	No	No	No	No	No	23631926	133696860	i13i1	6
523	78	M	NA	e14a2	a1e15	No	No	No	No	No	Yes	23634023	133697800	i14i1	4
367	37	M	L	e14a2		No	No	Yes	No	No	Yes	23633049	133714929	i14i1	65
368	63	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23634147	133690262	i14i1	25
370	51	M	L	e13a2/e14a2	a1e14	No	No	No	Yes**	No	No				
372	39	M	I	e14a2		No	No	No	No	No	No	23633166	133648235	i14i1	17
374	46	F	H	e13a2	a1e14	No	No	Yes	Yes	No	No				
378	60	M	H	e13a2		No	No	Yes	Yes	No	No	23632352	133701640	i13i1	16
379	47	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23634118	133617568	i14i1	19
380	67	M	L	e13a2	a1e15	No	No	No	No	No	Yes	23632505	133709318	i13i1	62
383	63	M	L	e14a2	a1e15	No	No	No	No	No	No	23634728	133656178	e15i1	
385	67	M	L	e13a2	a1e14	No	No	No	No	No	Yes	23631953	133597913	i13i1	17
386	75	F	L	e14a2		No	No	No	No	No	Yes	23633944	133709304	i14i1	2
387	67	M	L	e14e2		No	No	No	No	No	Yes	23632924	133716169	i14i1	2
388	21	F	NA	e14a2	a1e15	No	No	No	No	No	Yes	23634484	133684078	i14i1	5
390	56	M	L	e14a2	a1e15	No	No	No	No	No	Yes				
391	67	F	L	e14e2		No	No	No	No	No	Yes	23634718	133600589	i14i1	128
392	67	F	I	e13a2	a1e14	No	No	No	No	No	Yes	23632404	133608642	i13i1	3
393	21	F	L	e14a2	a1e17	No	No	No	No	No	Yes	23632776	133716696	i14i1	27
396	34	M	L	e13a2/e14a2	a1e15	No	No	No	No	No	Yes	23634331	133668607	i14i1	172
399	47	F	L	e14e2	a1e15	No	No	No	No	No	Yes	23633883	133658561	i14i1	26
402	38	F	L	e13a2	a1e14	No	No	No	No	No	Yes	23632063	133687563	i13i1	4
404	51	F	L	e14a2	a1e15	No	No	No	No	No	Yes				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
406	40	F	L	e14e2		No	No	No	No	No	Yes	23634752	133709764	e15i1	47
407	50	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23633636	133723128	i14i1	4
408	67	F	L	e14a2	a1e15	No	No	No	No	No	Yes				
410	48	F	I	e13a2		No	No	Yes	Yes	No	No	23632125	133593762	i13i1	5
411	67	M	NA	e13a2	a1e14	No	No	No	No	No	Yes	23631847	133597480	i13i1	2
412	27	F	NA	e14e2	a1e15	No	No	No	No	No	No				
414	63	M	NA	e13a2/e14a2	a1e15	No	No	Yes	Yes	No	No	23633941	133692744	i14i1	30
416	51	M	L	e13a2		No	No	No	No	No	Yes	23631909	133621894	i13i1	19
417	44	F	L	e14a2		No	No	No	No	No	Yes				
419	66	M	L	e14a2	a1e15	No	No	No	Yes**	No	Yes	23633725	133620081	i14i1	7
420	73	F	NA	e14a2	a1e15	No	No	No	No	No	Yes	23633821	133618546	i14i1	4
421	57	F	L	e14a2	a1e15	No	No	Yes	No	No	No	23633114	133720858	i14i1	8
422	69	M	L	e13a2/e14a2	a1e15	No	No	No	No	No	Yes	23634234	133640673	i14i1	79
423	60	M	L	e13a2	a1e14	No	No	No	No	No	Yes	23632461	133645564	i13i1	77
425	61	M	H	e13a2	a1e14	No	No	Yes	Yes	No	No	23631991	133614844	i13i1	37
426	74	F	I	e14a2	a1e16	No	No	No	No	No	Yes	23634719	133692481	i14i1	6
427	52	F	L	e13e2		No	No	No	No	No	Yes	23631921	133663759	i13i1	26
428	40	F	H	e13a2		No	No	No	No	No	Yes	23632588	133662845	e14i1	18
432	33	M	L	e14a2	a1e15	No	No	Yes	No	No	No	23633026	133591292	i14i1	44
433	36	M	L	e13a2/e14a2	a1e15	No	No	Yes	No	No	No				
435	53	M	H	e13a2		No	No	Yes	No	No	No				
436	63	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23633879	133698166	i14i1	
438	42	M	L	e14a2	a1e16	No	No	No	No	No	Yes	23634723	133601765	i14i1	37
441	17	M	L	e13a2	a1e15	No	No	No	No	No	Yes	23632108	133643037	i13i1	16
442	44	M	I	e13a2	a1e15	No	No	No	No	No	Yes	23632547	133637604	e14i1	95
443	46	M	L	e13a2/e14a2		No	No	No	No	No	Yes	23634126	133623382	i14i1	
444	50	M	L	e13a2/e14a2	a1e16	No	No	No	No	No	No	23633574	133631139	i14i1	53
445	74	M	I	e13a2/e14a2	a1e15	No	No	No	No	No	No	23634361	133622199	i14i1	36
446	62	F	L	e13a2/e14a2		No	No	No	No	No	Yes	23633632	133589561	i14i1	
447	46	M	L	e14a2	a1e15	No	No	Yes	No	No	No	23634067	133663964	i14i1	10
448	52	F	L	e13a2	a1e14	No	No	Yes	Yes	No	No	23632243	133683360	i13i1	8
449	68	M	I	e14a2	a1e15	No	No	No	No	No	No	23634468	133636150	i14i1	8

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
451	39	F	I	e13e2	a1e15	No	No	No	No	No	No	23634574	133651150	i14i1	153
452	27	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23632718	133632410	i14i1	21
453	45	M	I	e13a2	a1e14	No	No	Yes	No	No	No	23632217	133608106	i13i1	
454	65	M	L	e13a2	a1e14	No	No	Yes	No	No	Yes	23631978	133622493	i13i1	12
455	68	M	I	e13a2	a1e14	No	No	No	No	No	Yes	23632126	133602839	i13i1	8
457	32	M	L	e14a2	a1e15	No	No	No	No	No	No	23632874	133592456	i14i1	3
458	61	M	L	e14e2	a1e15	No	No	No	No	No	Yes	23633094	133683371	i14i1	5
461	42	M	L	e14e2	a1e15	No	No	No	No	No	No	23633091	133727511	i14i1	3
463	18	M	L	e13a2/e14a2	a1e15	No	No	Yes	No	No	No	23634675	133714142	i14i1	13
466	51	M	L	e13a2		No	No	No	No	No	Yes	23631950	133602842	i13i1	7
467	23	M	H	e14e2	a1e15	No	No	Yes	Yes	No	Yes				
468	66	M	I	e13a2	a1e14	No	No	No	No	No	Yes	23632121	133717939	i13i1	67
470	51	M	L	e13a2		No	No	No	No	No	Yes				
472	61	F	I	e14a2	a1e15	No	No	Yes	Yes	No	No	23633758	133680224	i14i1	
473	31	F	L	e13a2/e14a2	a1e16	No	No	Yes	No	No	Yes	23633739	133650718	i14i1	108
475	54	F	I	e13a2	a1e14	No	No	Yes	No	No	Yes	23632180	133622484	i13i1	1
476	49	M	L	e13a2	a1e14	No	No	No	No	No	No	23632341	133646153	i13i1	70
477	51	F	L	e13a2/e14a2	a1e16	No	No	No	No	No	Yes	23634291	133647265	i14i1	24
478	62	M	I	e13e1		No	No	No	No	No	Yes				
479	64	F	L	e13a2/e14a2		No	No	No	No	No	Yes	23634398	133651378	i14i1	44
480	60	M	L	e13a2		No	No	No	No	No	No	23632009	133672774	i13i1	15
481	67	F	L	e13e2	a1e15	No	No	No	No	No	Yes	23632505	133638938	i13i1	58
483	66	F	L	e14e2	a1e15	No	No	No	No	No	Yes	23633884	133601203	i14i1	3
484	53	M	L	e13a2	a1e14	No	No	No	No	No	Yes				
486	47	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23632889	133637646	i14i1	35
487	52	F	L	e13a2	a1e14	No	No	No	No	No	No	23632114	133641238	i13i1	79
488	34	F	L	e14a2	a1e15	No	No	No	No	No	Yes				
525	81	F	H	e13a2	a1e14	No	No	No	No	No	Yes	23632410	133692344	i13i1	65
489	81	F	L	e13a2	a1e14	No	No	Yes	No	No	No	23632359	133649581	i13i1	13
491	47	M	L	e14a2	a1e15	No	No	No	No	No	Yes	23634089	133640933	i14i1	
493	19	F	I	e13e2		No	No	No	No	No	Yes	23632075	133627422	i13i1	28
496	63	M	H	e13a2	a1e14	No	No	No	No	No	Yes	23631991	133723083	i13i1	3
497	61	M	NA	e13e2		No	No	No	No	No	Yes	23632004	133634630	i13i1	7
498	54	F	L	e13a2		No	No	No	No	No	Yes				

Demographics				Transcript type		Ph-associated rearrangement?	Cancer gene variant	Nilotinib switch for suboptimal molecular response	ELN Failure criterion*	*BCR::ABL1* KD mutation acquired	MR4 achieved by 4 years	*BCR::ABL1* genomic breakpoint coordinates			
Sample ID	Age at diagnosis	Sex	ELTS	*BCR::ABL1* transcript	Reciprocal *ABL1::BCR* transcript	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	Yes / No	*BCR*	*ABL1*	Fusion location *BCR::ABL1*	Number of reads
499	44	M	L	e13a2/e14a2		No	No	No	No	No	No	23633388	133719211	i14i1	25
504	67	M	I	e13a2	a1e14	No	No	No	No	No	No	23632120	133723113	i13i1	13
506	61	F	L	e13a2	a1e15	No	No	No	No	No	No	23632506	133591563	i13i1	7
507	36	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23633240	133590258	i14i1	6
508	42	F	H	e13a2		No	No	Yes	Yes	No	No	23631872	133591307	i13i1	32
509	26	F	L	e13a2	a1e14	No	No	No	Yes	No	No	23631880	133641682	i13i1	18
510	77	F	H	e13a2	a1e14	No	No	No	No	No	Yes	23632488	133593955	i13i1	40
511	40	F	L	e14a2		No	No	Yes	Yes	Yes	Yes				
512	31	M	I	e13e2	a1e15	No	No	No	Yes	Yes	No	23631868	133622105	i13i1	17
513	44	F	I	e13a2	a1e14	No	No	No	No	No	Yes				
515	43	F	L	e14a2	a1e15	No	No	No	No	No	No				
516	50	M	L	e14a2		No	No	No	No	No	Yes	23633331	133658839	i14i1	
517	29	M	L	e13a2	a1e14	No	No	No	No	No	Yes	23631886	133690407	i13i1	10
519	24	M	L	e14e2	a1e15	No	No	Yes	Yes	No	Yes	23633928	133603087	i14i1	5
526	30	M	L	e13a2		No	No	No	No	No	No	23631893	133699495	i13i1	41
520	52	F	L	e14a2	a1e15	No	No	No	No	No	Yes	23634455	133626239	i14i1	2

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
317					*ABL1::ARHGEF10L*	*ABL1*	9	133589843	a1e2 inv	25	Transcript inversion
						ARHGEF10L (1p36.13)	1	17907047			
					GUCD1::HMCN2	*GUCD1*	22	24950916	e1e31 inv	31	Transcript inversion
						HMCN2	9	133240100			
320											
389					*ABL1::DEPDC5*	*ABL1*	9	133589843	a1e24	51	Transcript
						DEPDC5	22	32229900			
					BCR::ABL1	*BCR*	22	23632601	e14i1 inv	26	Transcript inversion
						ABL1	9	133613842			
469					*USP20::CABIN1*	*USP20*	9	132627661	e10i25	32	Transcript
						CABIN1	22	24497370			
					USP20::CABIN1	*USP20*	9	132627661	e10i25	31	Transcript
						CABIN1	22	24497426			
325					*BCR::ABL1*	*BCR*	22	23632405	i13i1 inv	15	Genomic inversion
						ABL1	9	133635337			
505											
494	133681723	23633039	i1i14	3							
460											
348					*ABL1::LRRC8A*	*ABL1*	9	133589843	a1i2 inv	5	Transcript inversion
						LRRC8A	9	131677321			
					ABL1::intergenic	*ABL1*	9	133589843	a1-intergenic inv	4	Transcript inversion
					LRRC8A/PHYHD1	Intergenic *LRRC8A/PHYHD1* (9q34.11)	9	131680407			
					ABL1:PHYHD1	*ABL1*	9	133589843	a1i4 inv	4	Transcript inversion
						PHYHD1	9	131690134			
					ABL1::LRRC8A	*ABL1*	9	133589843	a1i2 inv	2	Transcript inversion
						LRRC8A	9	131672518			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
495											
465					*GPR107::BCR*	*GPR107*	9	132825590	i1e14	17	Genomic fusion
						BCR	22	23632544			
					GPR107::BCR	*GPR107*	9	132816353	e1e15	44	Transcript
						BCR	22	23634727			
429					*ABL1::* intergenic	*ABL1*	9	133644330	i1-intergenic	9	Genomic fusion
					IGLL5/RSPH14	Intergenic *IGLL5/RSPH14* (22q11.22)	22	23297467			
					ABL1:: Intergenic	*ABL1*	9	133589843	a1-intergenic	24	Transcript
					IGLL5/RSPH14	Intergenic *IGLL5/RSPH14* (22q11.22)	22	23322944			
439					*ARID2::BCR*	*ARID2* (12q13.11)	12	46125098	e3e15	170	Transcript
						BCR	22	23634727			
					ARID2::BCR	*ARID2* (12q13.11)	12	46153096	i3i14	4	Genomic fusion
						BCR	22	23634647			
					ABL1::ARID2	*ABL1*	9	133589843	a1e4	27	Transcript
						ARID2 (12q13.11)	12	46205200			
					BCR::BCR	*BCR*	22	23634622	i14 inv	10	Genomic inversion
						BCR	22	23634639			
474											
418					*ABL1::BCR*	*ABL1*	9	133665638	i1i13 inv	2	Genomic inversion
						BCR	22	23632299			
					BCR::BCR	*BCR*	22	23632292	i13 inv	4	Genomic inversion
						BCR	22	23632388			
					USP20::HMCN2	*HMCN2*	9	132612204	e2e17	40	Transcript
						USP20	9	133067008			
					ABL1:: intergenic	*ABL1*	9	133589843	a1-intergenic	2	Transcript
					PTGES/TOR1B	Intergenic *PTGES/TOR1B* (9q34.11)	9	132515345			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
430					*ABL1::* Intergenic Chr17q12	*ABL1*	9	133589843	a1-int inv	10	Transcript inversion
						Intergenic Chr17q12	17	36309399			
					ABL1:: Intergenic Chr17q12	*ABL1*	9	133589843	a1-int inv	8	Transcript inversion
						Intergenic Chr17q12	17	36310216			
331					*BCR::BCR*	*BCR*	22	23632205	i13 inv	36	Genomic inversion
						BCR	22	23632470			
					BCR::ABL1	*BCR*	22	23632210	i13i2 inv	8	Genomic inversion
						ABL1	9	133648336			
					ABL1:: intergenic	*ABL1*	9	133589843	a1-intergenic inv	4	Transcript inversion
					GLE1/SPTAN1	Intergenic *GLE1/SPTAN1* (9q34.11)	9	131305964			
					ABL1::SPTAN1	*ABL1*	9	133589843	a1e2	13	Transcript
						SPTAN1	9	131329016			
471											
462					*ABL1::KIF16B*	*ABL1*	9	133589843	a1e2	6	Transcript
						KIF16B (20p12.1)	20	16509086			
					ABL1:: intergenic	*ABL1*	9	133589843	a1-intergenic	5	Transcript
					KIF16B/SNRPB2	Intergenic *KIF16B/SNRPB2* (20p12.1)	20	16640932			
					ABL1:: intergenic	*ABL1*	9	133589843	a1-intergenic	2	Transcript
					KIF16B/SNRPB2	Intergenic *KIF16B/SNRPB2* (20p12.1)	20	16684475			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
461					*BCR::EXOSC2*	*BCR*	22	23632194	i13e9	74	Genomic fusion
						EXOSC2	9	133579151			
524											
350					*BCR::BCR*	*BCR*	22	23634298	i14i16 inv	12	Genomic inversion
						BCR	22	23642989			
440											
403											
501					*BCR::* Intergenic *EXOSC2/ABL1*	*BCR*	22	23632519	i13- intergenic	228	Genomic fusion
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133581710			
					Intergenic *EXOSC2/ABL1::BCR*	Intergenic *EXOSC2/ABL1* (9q34.12)	9	133581776	intergenic-i13	34	Reciprocal genomic fusion
						BCR	22	23632340			
376											
377					*ABL1::LINC02149*	*ABL1*	9	133589843	e1e3	35	Transcript
						LINC02149 (5p15.1)	5	15192559			
					ABL1:: intergenic	*ABL1*	9	133589843	e1-intergenic	25	Transcript
					ANKH/LINC02149	Intergenic *ANKH/LINC02149* (5p15.1)	5	15144796			
434					*ABL::LLGL2*	*ABL1*	9	133589843	a1e2	57	Transcript
						LLGL2 (17q25.1)	17	73539477			
					BCR::NFIX	*BCR*	22	23633094	i14i1	11	Genomic fusion
						NFIX (19p13.2)	19	13115916			
373					*BCR::* Intergenic *EXOSC2/ABL1*	*BCR*	22	23632486	i13-intergenic	56	Genomic fusion
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133582439			
336					Intergenic *DERL3/SLC2A11::BCR*	Intergenic *DERL3/SLC2A11* (22q11.23)	22	24194238	intergenic-i14	21	Genomic fusion
						BCR	22	23633925			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
490					*SWI5::SPECC1L*	*SWI5*	9	131039035	e2e14	9	Transcript
						SPECC1L	22	24807555			
					SWI5::SPECC1L	*SWI5*	9	131039035	e2e15	3	Alternate transcript
						SPECC1L	22	24808615			
398											
394											
384	133627039	23632318	i1i14	2							
437					*FUBP3::EXOSC2*	*FUBP3*	9	133495846	e9e2 inv	7	Transcript inversion
						EXOSC2	9	133570932			
					FUBP3::EXOSC2	*FUBP3*	9	133495846	e9i8 inv	9	Transcript inversion
						EXOSC2	9	133578831			
514	133708409	23632116	i1i13	2							
395											
456	133709994	23634601	i1i14	4							
369					*BCR::ABL1*	*BCR*	22	23636599	i15i1 inv	30	Genomic inversion
						ABL1	9	133707916			
					MYH9::BCR	*MYH9*	22	36705326	e15e16 inv	955	Transcript inversion
						BCR	22	23637210			
					MYH9::BCR	*MYH9*	22	36703827	i15i15 inv	7	Genomic fusion inversion
						BCR	22	23636274			
					BCR::BCR	*BCR*	22	23637343	e16e14	512	Transcript
						BCR	22	23632525			
					BCR::BCR	*BCR*	22	23646328	i16i13	8	Genomic fusion
						BCR	22	23632273			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
400					*TNRC6B::NEK6*	*TNRC6B*	22	40580783	i4i10	15	Genomic fusion
						NEK6	9	127112860			
					NEK6::TNRC6B	*NEK6*	9	127112862	i9i4	6	Genomic fusion
						TNRC6B	22	40580785			
					TNRC6B::NEK6	*TNRC6B*	22	40574145	e1e10	355	Transcript
						NEK6	9	127113115			
					TNRC6B::NEK6	*TNRC6B*	22	40552187	e4e10	130	Alternate transcript
						NEK6	9	127113115			
					ABL1:: Intergenic *URM1/CERCAM*	*ABL1*	9	133589843	a1-intergenic	21	Transcript
						Intergenic *URM1/CERCAM* (9q34.11)	9	131171211			
					ABL1::URM1	*ABL1*	9	133589843	a1e4	5	Transcript
						URM1	9	131151539			
					ABL1::CERCAM	*ABL1*	9	133589843	a1e1	3	Transcript
						CERCAM	9	131181438			
405											
503											
365											
401	133629093	23634420	i1i14	3							
355	133608009	23635249	i1i14	5	*BCR::ABL1*	*BCR*	22	23634788	e15i1 inv	43	Genomic inversion
						ABL1	9	133608207			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
450					*XPNPEP3::BCR*	*XPNPEP3*	22	41310329	e7e14	12	Transcript
						BCR	22	23632525			
					XPNPEP3::BCR	*XPNPEP3*	22	41311345	i7i13	3	Genomic fusion
						BCR	22	23632313			
356	133714435	23634135	i1i14	3							
485					*ABL1::* Intergenic	*ABL1*	9	133589843	a1-intergenic	42	Transcript
					FOXF2/FOXCUT	*FOXF2/FOXCUT* (6p25.3)	6	1562649			
					ABL1::PXDC1	*ABL1*	9	133589843	a1e2	11	Transcript
						PXDC1	6	3738383	2175734		
492	133681282	23634577	i1i14	2							
518					*USP20::BCR*	*USP20*	9	132638518	e22-3'UTR inv	42	Transcript inversion
						BCR	22	23658861			
357					*ABL1::DRICH1*	*ABL1*	9	133589843	a1i7	23	Transcript inversion
						DRICH1	22	23959666			
358					*BCR::* intergenic *EXOSC2/ABL1*	*BCR*	22	23634585	i14-intergenic	41	Genomic fusion
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133584587			
					Intergenic *EXOSC2/ABL1::BCR*	Intergenic *EXOSC2/ABL1* (9q34.12)	9	133584668	intergenic-i14	12	Reciprocal genomic fusion
						BCR	22	23634601			
					BCR:: intergenic *EXOSC2/ABL1*	*BCR*	22	23632601	e14-intergenic	24	Transcript
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133587923			
413					*BCR::EXOSC2*	*BCR*	22	23632289	i13e9	58	Genomic fusion
						EXOSC2	9	133579932			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
359					*ABL1::SPDYE2B*	*ABL1*	9	133589843	a1e2	1	Transcript
						SPDYE2B (7q22.1)	7	102292374			
					ABL1:: intergenic	*ABL1*	9	133589843	a1-intergenic	2	Transcript
					UPK3BL1::SPCYE2B	intergenic *UPK3BL1/SPCYE2B* (7q22.1)	7	102290086			
					NR_029411::BCR	*NR_029411* (7q11.23)	7	76244981	i2e15	4	Transcript
						BCR	22	23637210			
					NR_029411::BCR	*NR_029411* (7q11.23)	7	76180840	e2e15	2	Transcript
						BCR	22	23637210			
343											
482					*BCR::EXOSC2*	*BCR*	22	23631858	i13e9	8	Genomic fusion
						EXOSC2	9	133579784			
					EXOSC2::BCR	*EXOSC2*	9	133580033	e9i12	39	Reciprocal genomic fusion
						BCR	22	23631675			
522					*BCR::* intergenic *EXOSC2/ABL1*	*BCR*	22	23631828	i13intergenic	3	Genomic fusion
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133584071			
					Intergenic *EXOSC2/ABL1::BCR*	Intergenic *EXOSC2/ABL1* (9q34.12)	9	133583783	intergenici12	8	Reciprocal genomic fusion
						BCR	22	23631631			
415											
397					*BCR::* intergenic *EXOSC2/ABL1*	*BCR*	22	23631984	i13 intergenic	5	Genomic fusion
						Intergenic *EXOSC2/ABL1*	9	133584165			
					Intergenic *EXOSC2/ABL1 ::BCR*	Intergenic *EXOSC2/ABL1* (9q34.12)	9	133584167	intergenic i13	15	Reciprocal genomic fusion
						BCR	22	23631984			
					EXOSC2::BCR	*EXOSC2*	9	133579381	e9e14	4	Transcript
						BCR	22	23632525			

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	***ABL1***	***BCR***	**Fusion location *ABL1::BCR***	**Number of reads**	**Fusion**	**Gene / location**	**Chr**	**Genomic coordinates**	**Transcript or fusion type**	**Number of reads**	**Type of rearrangement**
371					*ABL1::* Intergenic chr16p	*ABL1*	9	133589843	e1intergenic 16p12.1	12	Transcript
						Intergenic chr16p12.1	16	27073527			
464					*BCR::* intergenic *EXOSC2/ABL1*	*BCR*	22	23632444	i13intergenic	20	Genomic fusion
						Intergenic *EXOSC2/ABL1* (9q34.12)	9	133582465			
318											
319											
321											
322											
323											
324	133644750	23634659	i1i14	2							
326											
327											
328	133636335	23632006	i1i14	2							
329											
330											
333											
334											
335											
337											
338	133608773	23634802	i1i14								
339											
340	133618166	23634748	i1e15								
341	133701477	23631850	i1i13	1							
342											
344	133680151	23634357	i1i14	8							
345											
346	133702202	23632192	i1i13	6							
347											
349											
351											
352											
353											
354	133685150	23632253	i1i13	12							
528											
360											
361											

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	*ABL1*	*BCR*	Fusion location *ABL1::BCR*	Number of reads	Fusion	Gene / location	Chr	Genomic coordinates	Transcript or fusion type	Number of reads	Type of rearrangement
363	133686043	23632050	i1i13	14							
364											
366											
523											
367											
368											
370											
372											
374											
378	133701637	23632419	i1i13	26							
379											
380	133709414	23632530	i1e14	25							
383											
385	133597908	23631969	i1i13	2							
386											
387											
388											
390											
391											
392											
393											
396											
399	133658564	23633882	i1i14	3							
402	133687566	23632068	i1i13								
404											
406											
407											
408	133589990	23633565	i1i14	2							
410											
411											

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	*ABL1*	*BCR*	Fusion location *ABL1::BCR*	Number of reads	Fusion	Gene / location	Chr	Genomic coordinates	Transcript or fusion type	Number of reads	Type of rearrangement
412											
414											
416											
417											
419											
420											
421											
422											
423											
425											
426											
427											
428											
432											
433											
435											
436	133697062	23633853	i1i14	2							
438	133601769	23634733	i1e15	1							
441											
442											
443	133623371	23634132	i1i14								
444											
445											
446											
447											
448											
449											
451											
452											
453	133608107	23632212	i1i13	1							
454											
455											
457											
458											
461											
463											
466											

Demogr	*ABL1::BCR* reciprocal genomic breakpoints coordinates				Philadelphia-associated rearrangements						
Sample ID	*ABL1*	*BCR*	Fusion location *ABL1::BCR*	Number of reads	Fusion	Gene / location	Chr	Genomic coordinates	Transcript or fusion type	Number of reads	Type of rearrangement
467											
468	133717921	23632152	i1i13	15							
470											
472											
473											
475											
476											
477											
478	133693344	23632192	i1i13	3							
479											
480											
481											
483	133601200	23633882	i1i14	3							
484	133646807	23632215	i1i13								
486											
487	133641243	23632114	i1i13	14							
488											
525	133692345	23632409	i1i13	2							
489											
491											
493	133627403	23632075	i1i13	2							
496	133723065	23631994	i1i13	6							
497	133634627	23632002	i1i13	5							
498											
499	133719211	23633381	i1i14	5							
504	133723115	23632121	i1i13	8							
506											
507											
508											
509	133641676	23631877	i1i13	1							
510	133593947	23632487	i1i13	4							
511											
512											
513											
515											
516											
517											
519											
526											
520											

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
317	46,XY,t(1;9;22)(p36.1;q34;q11.2) Transcript is consistent with the variant translocation. *GUCD1* is 1.3 Mb downstream of *BCR* and *HMCN2* is 0.35 Mb upstream of *ABL1* . The fusion is consistent with deletions near to *BCR* and *ABL1* and/or sequence fragmentation and inversion. *HMCN2* is not expressed in blood, however the fusion was expressed in blood.								
320		*DNMT3A*	2	25462038	ENST00000264709	Frameshift	c.2368del:p.(Arg790Glyfs*12)	23.9	
389	Consistent with an 8.6 Mb deletion downstream of *BCR* . Activation of a cryptic splice acceptor site in *ABL1* intron 1 in the inverted orientation.								T315I
469	*USP20* is 0.99 Mb upstream of *ABL1*. *CABIN1* is 0.86 Mb downstream of *BCR*. The fusions were likely generated by a deletion bridge associated with deletions adjacent to *ABL1* and *BCR* and activation of cryptic splice acceptor sites in *CABIN1* intron 25.	*DNMT3A*	2	25469598	ENST00000264709	Frameshift	c.1169dup:p.(Ser390Argfs*3)	17.2	
325									
505	t(9;22;11) No supporting fusions for the variant translocation.	*TET2*	4	106180783	ENST00000540549	Frameshift	c.3812dup:p.(Cys1271Trpfs*29)	47.0	
494		*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	29.0	
460		*IDH2*	15	90631934	ENST00000330062	Missense	c.419G>A:p.(Arg140Gln)	41.3	
348	This series of low level transcripts, that are associated with activation of cryptic splice acceptor sites, is the likely by-product of a genomic rearrangement where the breakpoints were not captured and therefore not detectable. *LRRC8A* and *PHYHD1* are 1.9 Mb upstream of *ABL1*. The rearrangements are likely associated with sequence fragmentation, inversion and imperfect sequence reassembly.								

Demogr	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
495		*ASXL1*	20	31023408	ENST00000375687	Nonsense	c.2893C>T:p.(Arg965*)	41.4	
465	*GPR107* is 0.82 Mb upstream of *ABL1.* A deletion adjacent to *ABL1* likely led to the formation of the fusion via a deletion bridge that spanned the breakpoints on chromosomes 9 and 22.								
429	The genomic break on Chr 22 is 0.34 Mb upstream of *BCR* and likely associated with sequence fragmentation and imperfect reassembly on the der 9. Activation of a cryptic splice acceptor site at the intergenic region of Chr 22.								
439	t(9;22;12) Transcripts and genomic fusion are consistent with the variant translocation. 17 bp inversion in *BCR* intron 14.								
474		*ASXL1*	20	31022245	ENST00000375687	Nonsense	c.1730C>A:p.(Ser577*)	15.6	F359I
418	96 bp inversion in *BCR* intron 13. Consistent with a 454 Kb deletion on the der 9, at 0.52 Mb upstream of *ABL1.* Activation of a cryptic splice acceptor site in the intergenic region between *PTGES* and *TOR1B.* The intergenic region is 1.1Mb upstream of *ABL1.*								

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
430	46,XY,t(9;22;17)q34;q11.2;q21)	*ASXL1*	20	31022286	ENST00000375687	Frameshift	c.1772dup:p.(Tyr591*)	7.9	Q252H
	Transcripts are consistent with the variant translocation and activation of cryptic splice acceptor sites.	*BCORL1*	X	129147354	ENST00000540052	Frameshift	c.606_607insTGGGC:p.(Ile203Trpfs*52)	4.1	Y253H
									E255K
		This patient also had a novel interchromosomal fusion at diagnosis:							T315I
		IKZF1::IGKV3-7		*IKZF1*	7:50348584				
				IGKV3-7	2:89277898				
		Plus a 34 Kb deletion involving *CLIC6* and *RUNX1* on chromosome 21 as described in reference 17							
331	265 bp inversion in *BCR* intron 13.								
	Inversion and activation of a cryptic splice acceptor site 2.3 Mb upstream of *ABL1.*								
	SPTAN1 is 2.3 Mb upstream of *ABL1* and the rearrangement was likely asociated with circularization of deleted sequence or it represents transcription from a tandem duplication.								
471		*IDH2*	15	90631934	ENST00000330062	Missense	c.419G>A:p.(Arg140Gln)	7.6	
462	The fusion suggests involvement of chromosome 20p12.1 in the translocation. A variant translocation was not reported.								
	Activation of an intergenic cryptic splice acceptor site								
	Activation of an intergenic cryptic splice acceptor site								
461									
524		*ASXL1*	20	31022233	ENST00000375687	Splice acceptor	c.1720-2A>G	91.3	
		Confirmed to be a somatic mutation that pre-existed the acquisition of *BCR::ABL1*							
350	8,690 bp inversion involving *BCR* introns 14 and 16.								Y253H

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
440		*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	6.3	
403		*BCORL1*	X	129149098	ENST00000540052	Nonsense	c.2350C>T:p.(Arg784*)	47.9	
501									
376		*CBL*	11	119148921	ENST00000264033	Missense	c.1141T>C:p.(Cys381Arg)	47.9	
		ASXL1	20	31023083	ENST00000375687	Nonsense	c.2568C>A:p.(Cys856*)	36.7	
377	t(5;9;22) Fusions are consistent with the variant translocation. Activation of an intergenic cryptic splice acceptor site								
434	46,XX,t(9;22;19)(q34:q11.2,p13.3) Fusions are consistent with a variant translocation, however, they suggest a 4 way translocation involving Chr 9, 17, 19 and 22.								
373									
336	t(1;9;22). 46,XY,t(1;9;22)(q21;q34;q11.2)[17] The orientation of the fusion suggests a deleted sequence has circularized or a tandem duplication has occurred. The intergenic sequence is 0.56 Mb upstream of *BCR* . No rearrangement associated with Chr 1 was detected.								
490	*SWI5* is 2.6 Mb upstream of *ABL1* and *SPECC1L* is 1.2 Mb downstream of *BCR*. The fusion transcript was likely generated by deletions adjacent to *ABL1* and *BCR*.								

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
398		*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	16.9	
		ASXL1	20	31022441	ENST00000375687	Frameshift	c.1934dup:p.(Gly646Trpfs*12)	5.7	
394		*RUNX1*	21	36259171	ENST00000300305	Missense	c.320G>A:p.(Arg107His)	9.8	
384		*ASXL1*	20	31021250	ENST00000375687	Nonsense	c.1249C>T:p.(Arg417*)	28	
437	Novel RNA splicing identified a 7.9 Kb intergenic deletion of *EXOSC2* exons 3 to 7. *FUBP3* is 1.1 Mb upstream of *ABL1* and *EXOSC2* is adjacent to *ABL1*. The fusions were likely associated with sequence deletion, inversion and activation of cryptic splice acceptor sites that were generated by the inversion.	*DNMT3A*	2	25467457	ENST00000264709	Missense	c.1619G>C:p.(Cys540Ser)	43.9	
514		*TP53*	17	7577538	ENSG00000141510	Missense	c.743G>A:p.(Arg248Gln)	38	
395		*DNMT3A*	2	25463289	ENST00000264709	Frameshift	c.2195_2203del:p.(Phe732_Phe734del)	37.9	
		TET2	4	106182914	ENST00000540549	Splice acceptor	c.3955-2A>C	41.2	
		Confirmed to be somatic mutations that pre-existed the acquisition of *BCR::ABL1*							
456		*ASXL1*	20	31022286	ENST00000375687	Nonsense	c.1772dup:p.(Tyr591*)	7.2	
369	*MYH9* is 13 Mb downstream of *BCR*. Copy number analysis identified a 13 Mb deletion adjacent to *BCR* that extended to *MYH9*. The orientation of the fusion indicates a deletion and inversion occurred[17]								Q252H Y253H E255K E255V

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
400	*NEK6* is 6.6 Mb upstream of *ABL1* and *TNRC6B* is 17 Mb downstream of *BCR* . Fusions are associated with a deletion upstream of *ABL1* and a deletion downstream of *BCR.* The orientation of the fusions suggests sequence fragmentation and random sequence reassembly[17].	*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	39	
	URM1 and *CERCAM* are adjacent genes approximately 2.4 Kb upstream of *ABL1* . Fusions are potentially associated with sequence fragmentation and reassembly.								
405		*RUNX1*	21	36231782	ENST00000300305	Missense	c.602G>A:p.p.(Arg201Gln)	34.6	
503		*TET2*	4	106156551	ENST00000540549	Nonsense	c.1452T>A:p.(Cys484*)	45.1	F359V
		ASXL1	20	31022263	ENST00000375687	Nonsense	c.1748G>A:p.(Trp583*)	40.5	
		The TET2 mutation was confirmed to be a somatic mutation that pre-existed the acquisition of *BCR::ABL1*							
365		*KDM6A*	X	44969431	ENST00000377967	Frameshift	c.4115_4116del:p.(Phe1372Cysfs*16)	72.4	
401		*RUNX1*	21	36252940	ENST00000300305	Nonsense	c.422C>A:p.(Ser141*)	17.4	
355	The genomic break was located within exon 15 of *BCR.* A fusion between *BCR* and *ABL1* involving an inverted sequence of *ABL1* intron 1 was identified. The breakpoints of the inversion were within a few nucleotides of the correctly oriented *BCR::ABL1* genomic breakpoints.	*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	37.3	
		IKZF1	7	50459554	ENST00000439701	Frameshift	c.720del:p.(Glu242Lysfs*15)	34.6	
450	*XPNPEP3* is 17 Mb upstream of *BCR* . The orientation of the fusion partners suggests circularization of a deleted region adjacent to *BCR,* or transcription from a tandem duplication. Potential circular DNA associated with circularization of a deleted region adjacent to *BCR* or a tandem duplication.								

Demogr	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
356		*ASXL1*	20	31021658	ENST00000375687	Nonsense	c.1657G>T:p.(Glu553*)	14.2	
485	t(1;6;9;22)	*ASXL1*	20	31022592	ENST00000375687	Nonsense	c.2077C>T:p.(Arg693*)	11.8	
	Fusions are consistent with the variant translocation, however, a rearrangment involving Chr 1 was not observed. Activation of an intergenic cryptic splice acceptor site	*ASXL1*	20	31022441	ENST00000375687	Frameshift	c.1934dup:p.(Gly646Trpfs*12)	9.5	
	PXDC1 is 2.2 Mb upstream of the other *ABL1* -Chr 6 fusion site and may indicate additional rearrangement on Chr 6.								
492		*TET2*	4	106155381	ENST00000540549	Frameshift	c.283_286del:p.(Lys95Alafs*17)	33	
518	*USP20* is 10 Mb upstream of *ABL1*. The fusion is likely associated with a deletion adjacent to *ABL1* and an inversion in the 3'UTR of *BCR* associated with activation of a cryptic splice acceptor site.								
357	*DRICH1* is 0.33 Mb downstream of *BCR*. The fusion is likely associated with a deletion, inversion and activation of an intronic cryptic splice acceptor site.								
358		*ASXL1*	20	31022289	ENST00000375687	Nonsense	c.1774C>T:p.(Gln592*)	23.6	T315I
	Unusual transcript where *BCR* exon 14 is joined to *ABL1* exon 2 in the primary *BCR-ABL1* transcript and also to exon 9 of *EXOSC2* upstream of the genomic break. *EXOSC2* is predicted to be located on the der 9. Activation of a cryptic splice acceptor site.								
413	Genomic break within *EXOSC2* e9								
359	t(7;9;22)								G250E
	Fusions are consistent with a variant translocation								
	Activation of an intergenic cryptic splice acceptor site								
	Activation of an intronic cryptic splice donor site								
	NR_029411 is located 26 Mb upstream of *SPYDE2B* , which may indicate deletion of Chr 7 sequence.								

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
343		*DNMT3A*	2	25466799	ENST00000264709	Missense	c.1904G>A:p.(Arg635Gln)	42.9	
		Confirmed to be a somatic mutation that pre-existed the acquisition of *BCR::ABL1*							
482									
522		*ASXL1*	20	31024206	ENST00000375687	Frameshift	c.3692_3693del:p.(Ser1231*)	7.3	
415		*ASXL1*	20	31023108	ENST00000375687	Nonsense	c.2593G>T:p.(Glu865*)	29	
397									E255V
	Activation of a cryptic exonic splice donor site within *EXOSC2*								
371	Karyotype not available								
464									
318									
319									
321									
322									
323									
324									
326									
327									
328									

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
329									
330									
333									
334									
335									
337									
338									
339									
340									
341									
342									
344									
345									
346									
347									
349									
351									
352									
353									
354									
528									
360									
361									E459K
363									
364									
366									
523									
367									
368									
370									
372									
374									
378									
379									
380									

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
383									
385									
386									
387									
388									
390									
391									
392									
393									
396									
399									
402									
404									
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421									
422									
423									
425									
426									
427									
428									
432									

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
433									
435									
436									
438									
441									
442									
443									
444									
445									
446									
447									
448									
449									
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470									
472									
473									
475									
476									
477									
478									
479									

Demogra	Variant Ph karyotype at diagnosis	SNVs and indels							*BCR::ABL1* KD mutation
Sample ID		Gene	Chr	Genomic coordinates (GRCh37/hg19)	Transcript ID	Effect	Variant	VAF%	
480									
481									
483									
484									
486									
487									
488									
525									
489									
491									
493									
496									
497									
498									
499									
504									
506	t(x;9;22). No supporting fusions for the variant translocation.								
507									
508	t(9;22;16). No supporting fusions for the variant translocation.								
509									
510									
511									G250E, Y253H
512									H396P
513									
515									
516									
517									
519									
526									
520									

Supplementary Table 3: Evaluation of cancer gene variants and Ph-associated rearrangements for each 4-year outcome

Variable	Cancer gene mutation			Ph-associated rearrangement		
	Not present, n=168 (%)	Present, n=33 (%)	*P* value	Not present, n=164 (%)	Present, n=37 (%)	*P* value
Failure-free survival	79%	66%	0.06	77%	70%	0.07
Acquisition of *BCR*::*ABL1* kinase domain mutations	5%	13%	0.094	3%	19%	<0.001
MMR	84%	70%	0.08	84%	69%	0.012
MR4	62%	42%	0.07	64%	31%	0.004
MR4.5	44%	35%	0.125	46%	23%	0.032
Nilotinib switch secondary to trial-defined TKI switch criteria	27%	36%	0.29	26%	40%	0.08

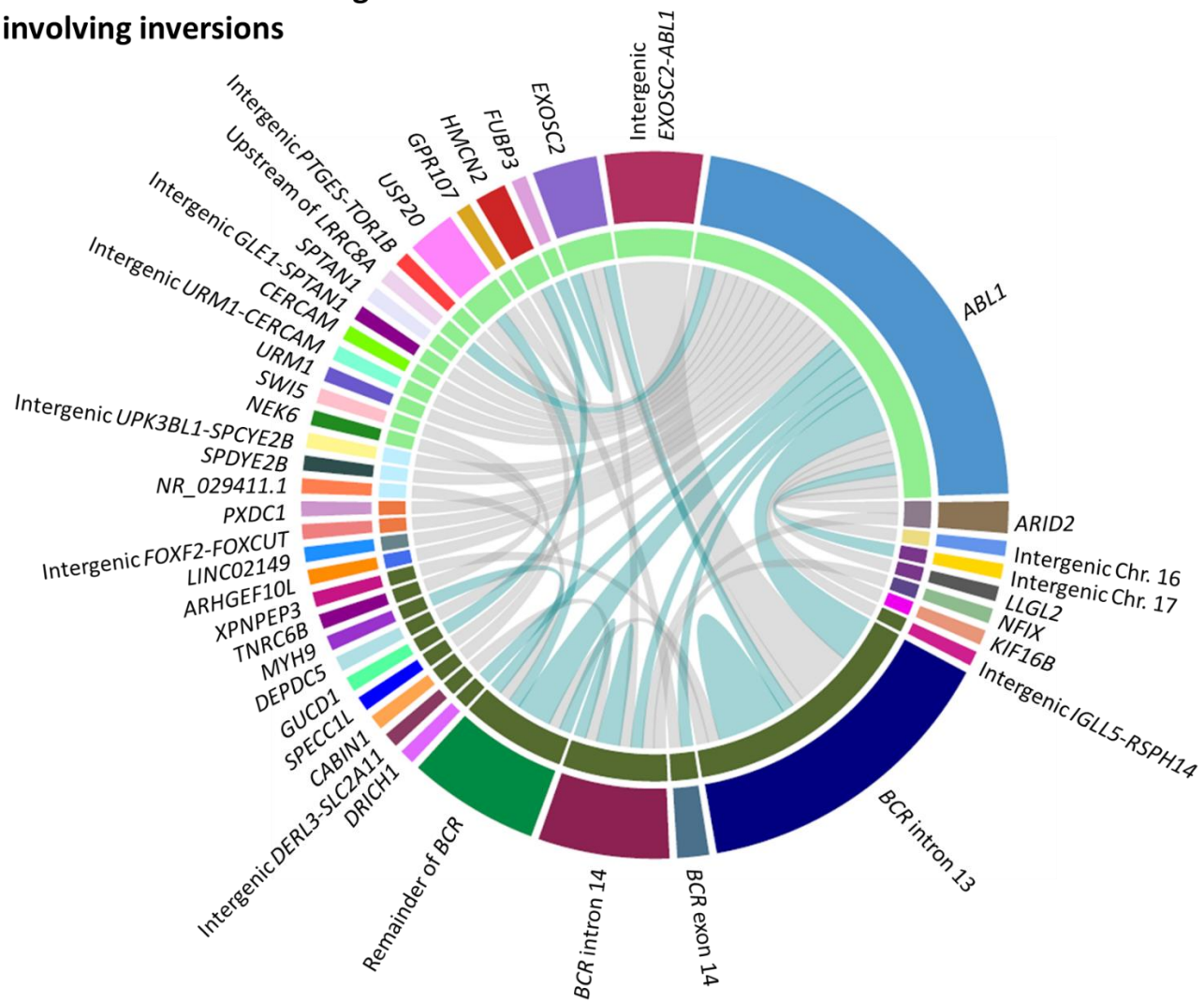
Supplementary Table 4: Breakdown of cause of nilotinib switch, other than imatinib intolerance, based on presence of AGAs

Reason for switch to nilotinib	Patients with AGAs (n=25) (%)	Patients without AGAs (n=28) (%)
BCR::ABL1 ^{IS} >10% at 3 months	10 (40)	6 (21)
BCR::ABL1 ^{IS} >1% at 6 months	6 (24)	8 (29)
BCR::ABL1 ^{IS} >0.1% at 12 months	5 (20)	10 (36)
Development of *BCR::ABL1* kinase domain mutations	3 (12)	2 (7)
Loss of response	1 (4)	2 (7)

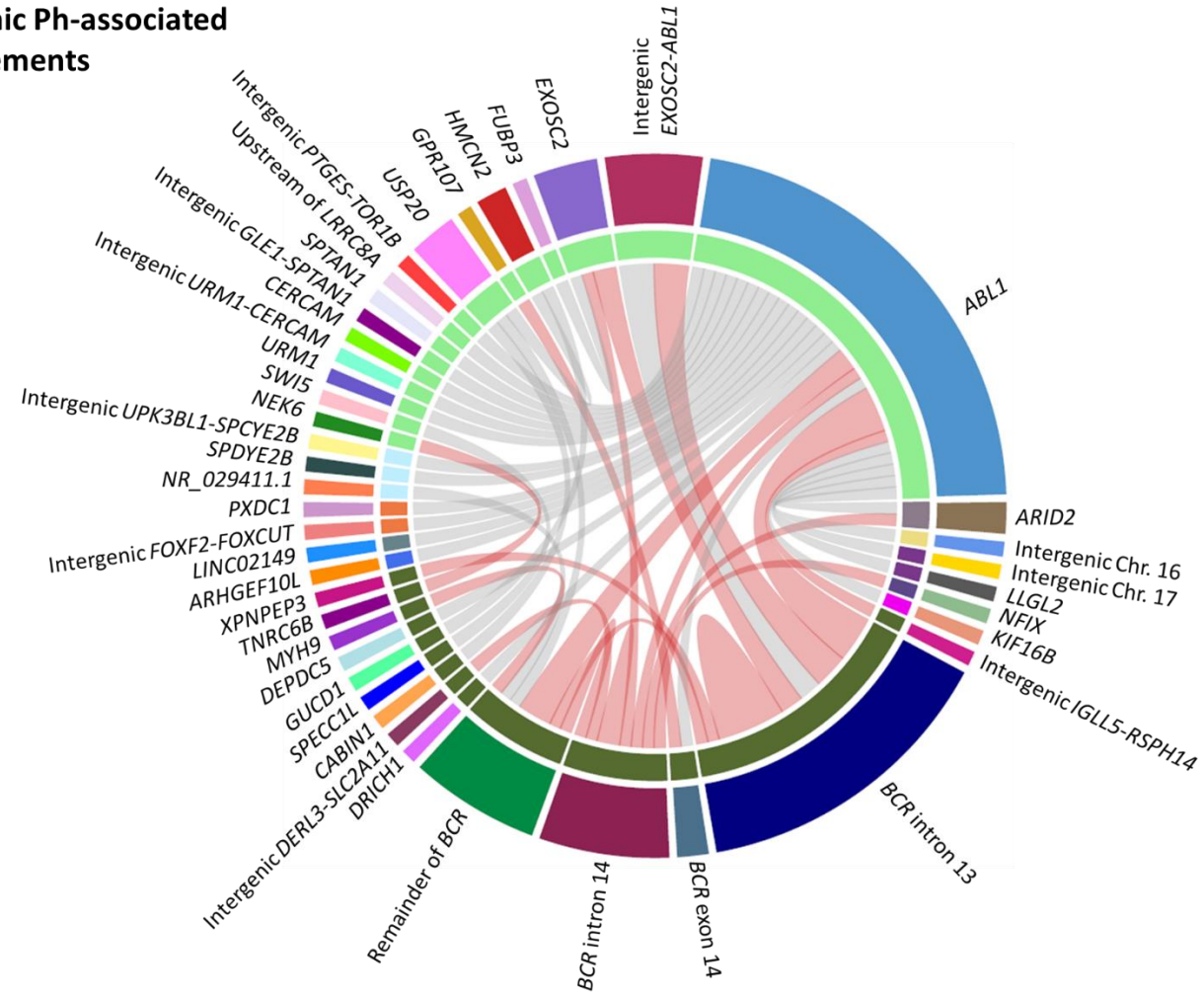
Supplementary Figures

Supplementary Figure 1: Circos plots depicting the various Ph-associated rearrangements observed in the TIDEL-II cohort
Ph-associated rearrangements illustrated in more detail, specifically indicating the number of inversion-based events, genomic rearrangements and fusion transcripts. Some patients had multiple rearrangements. BCR has been divided by location for more detailed analysis. (A) Inversion based Ph-associated rearrangements are shown by turquoise ribbons linking the relevant genes; (B) Genomic Ph-associated rearrangements, identified by intronic breakpoints, are represented by red ribbons; and (C) Fusion transcripts generated by Ph-associated rearrangements are drawn in green ribbons.

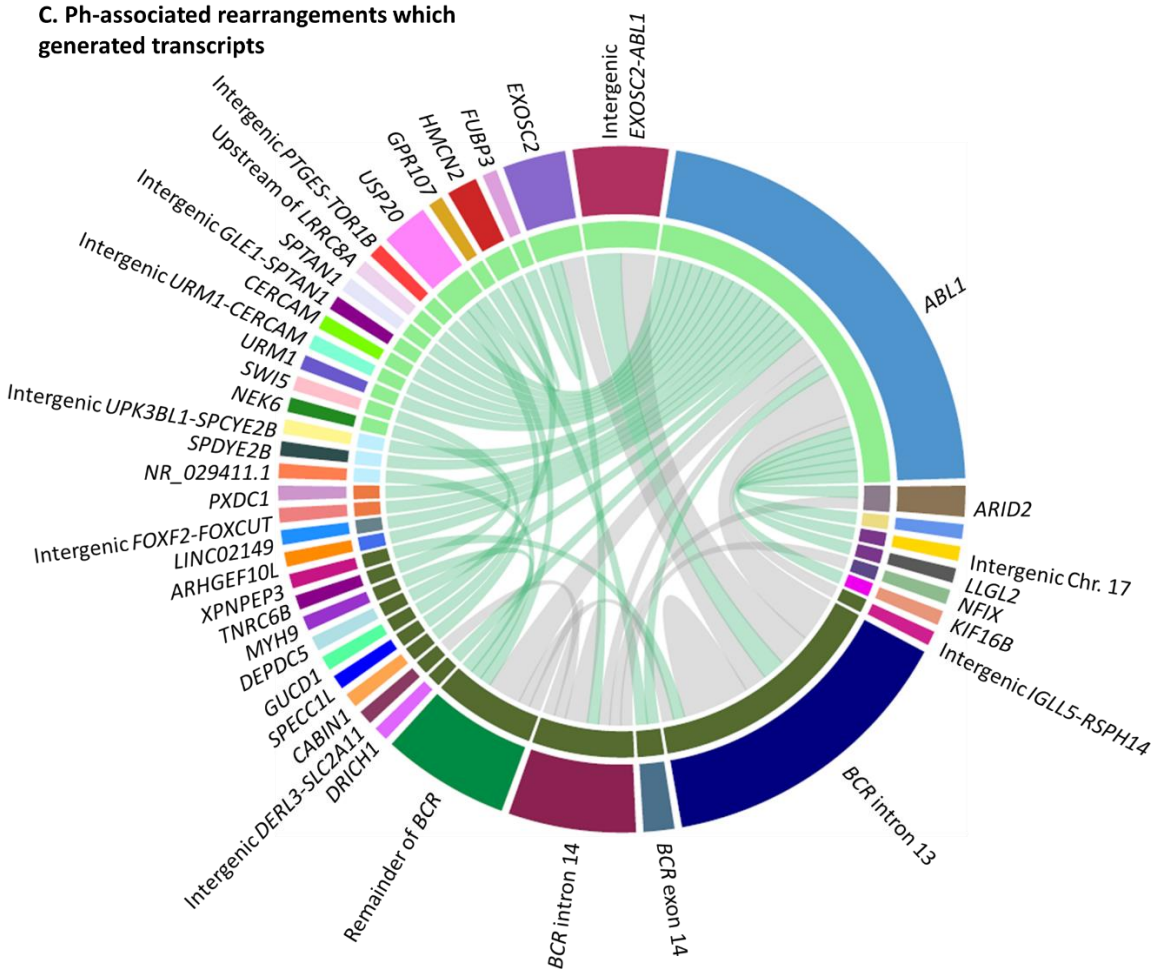
A. Ph-associated rearrangements involving inversions



B. Genomic Ph-associated rearrangements



C. Ph-associated rearrangements which generated transcripts

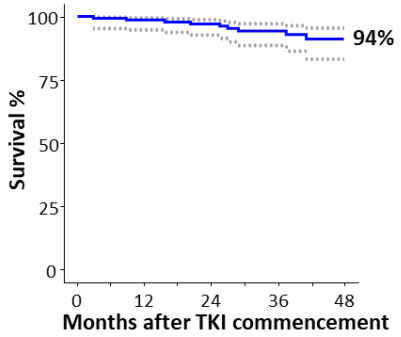


Chromosome 1 Chromosome 5 Chromosome 6 Chromosome 7 Chromosome 9 Chromosome 12
Chromosome 16 Chromosome 17 Chromosome 19 Chromosome 20 Chromosome 22

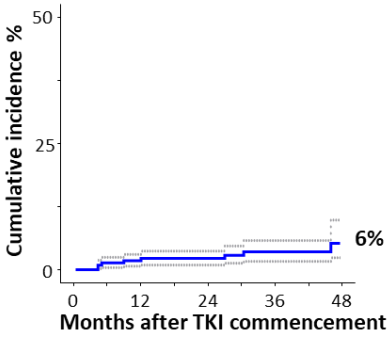
Supplementary Figure 2: Outcome and molecular responses for the study population

No patient progressed to accelerated phase. 95% confidence intervals are indicated in grey.

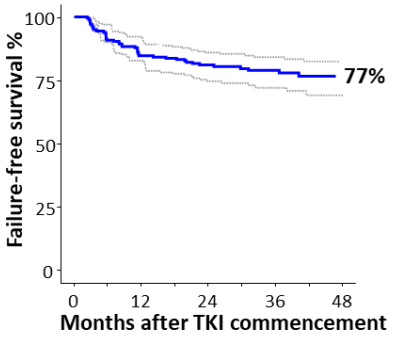
A. Overall Survival



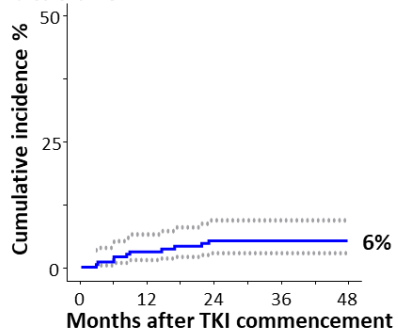
B. Progression to blast phase



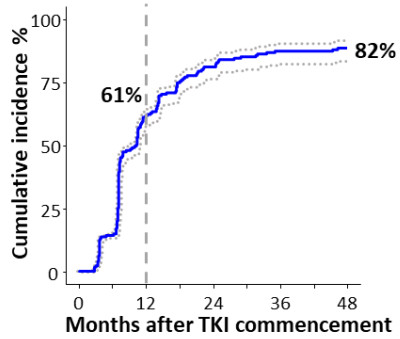
C. Failure Free Survival



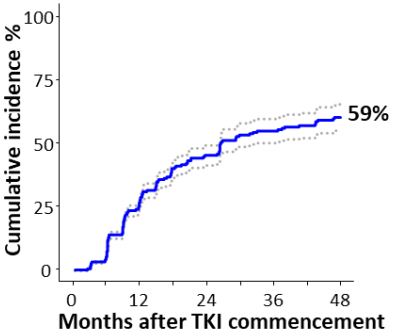
D. Acquisition of kinase domain mutations



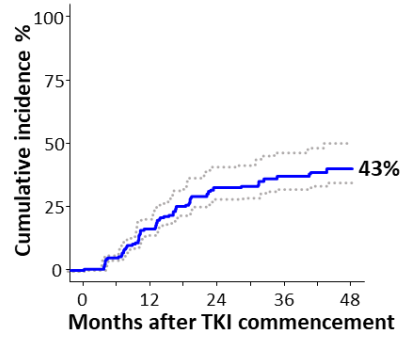
E. Major molecular response



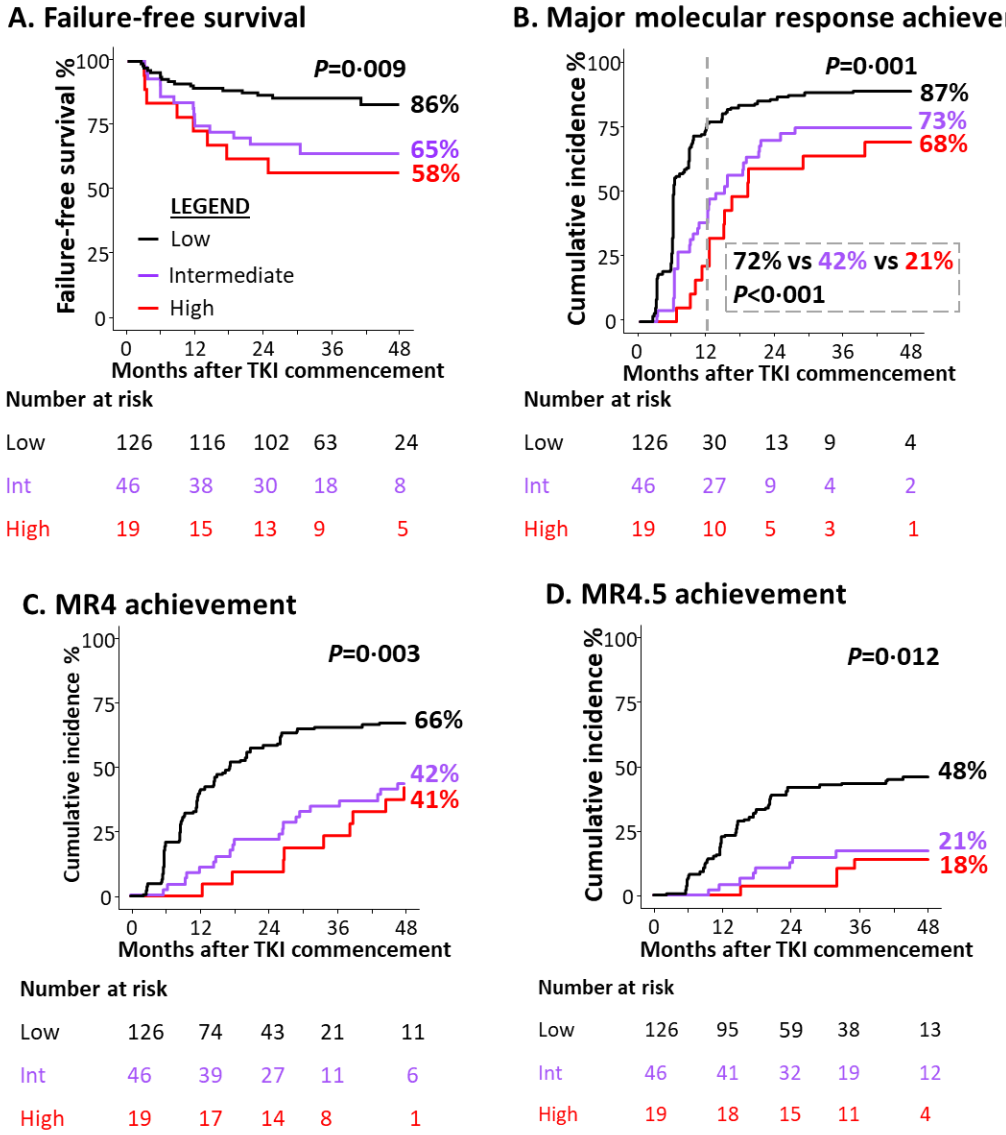
F. MR4 achievement



G. MR4·5 achievement

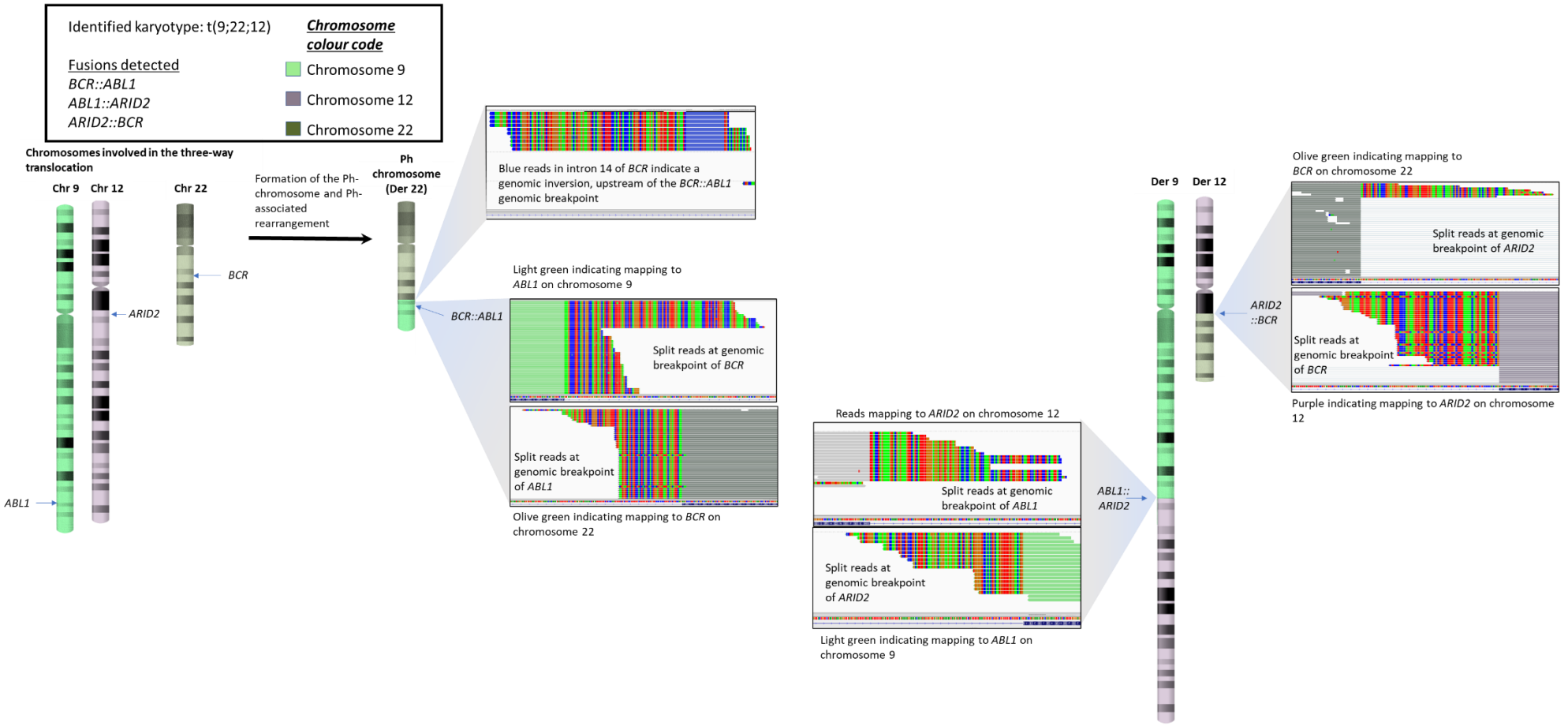


Supplementary Figure 3: Outcomes and molecular response according to the ELTS risk score

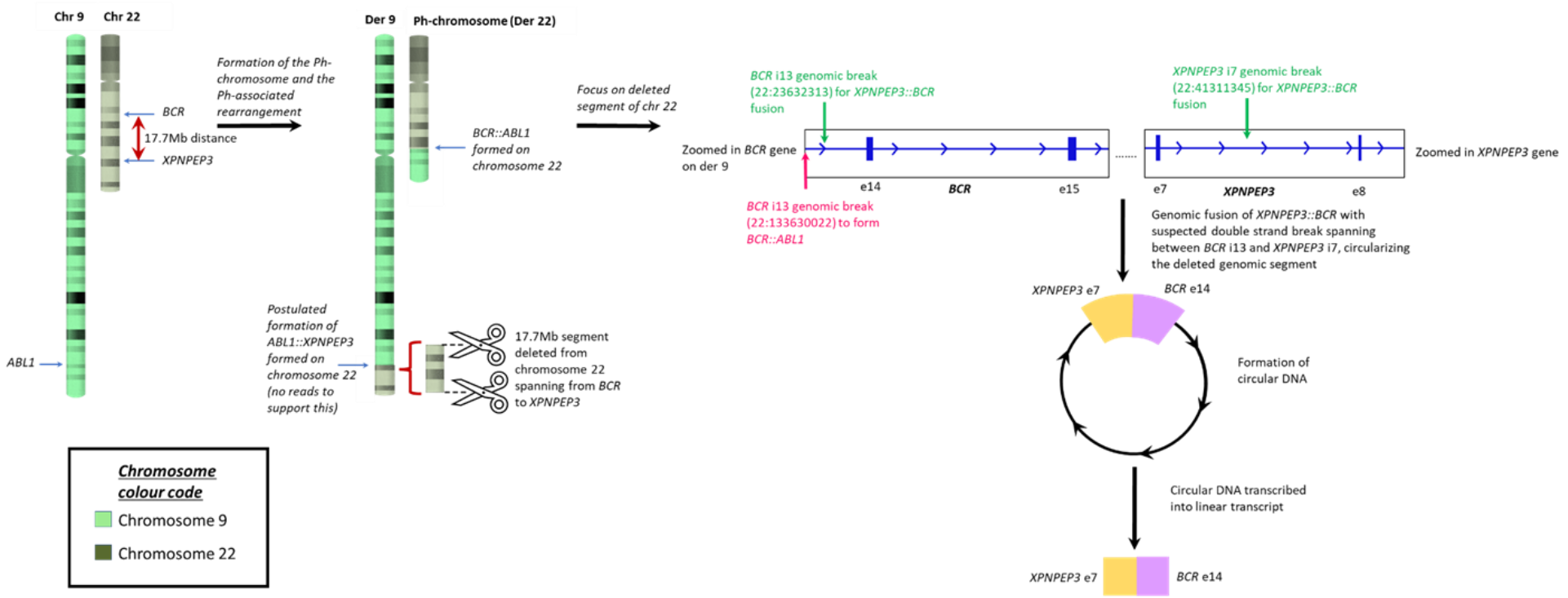


Supplementary Figure 4: Ph-associated rearrangement involving a three-way balanced translocation between chromosomes 9, 12 and 22

Patient 439 had a variant translocation involving chromosomes 9 (green), 12 (purple) and 22 (olive). The RNA-based gene panel sequencing resolved the precise genomic breakpoints and the associated gene fusions. Involvement of chromosome 12 was identified through karyotyping but RNA sequencing localized the genomic breakpoints to the ARID2 gene at nucleotide resolution. The ARID2::BCR and ABL1::ARID2 fusion transcripts were reciprocal products of the three-way chromosomal translocation. This patient also had a 17Kb genomic inversion (blue reads) within BCR intron 14, upstream of the BCR::ABL1 genomic break, which was only evident using NGS. The multicolored region of each sequence read indicates that the read spans the fusion junction and that region maps to a different location in the genome. The chromosome of origin is indicated by the solid color.


Identified karyotype: t(9;22;12)
Fusions detected
BCR::ABL1
ABL1::ARID2
ARID2::BCR
Chromosome colour code
Chromosome 9
Chromosome 12
Chromosome 22
Chromosomes involved in the three-way translocation
Chr 9
Chr 12
Chr 22
ABL1
ARID2
BCR
Formation of the Ph-chromosome and Ph-associated rearrangement
Ph chromosome (Der 22)
Blue reads in intron 14 of BCR indicate a genomic inversion, upstream of the BCR::ABL1 genomic breakpoint
BCR::ABL1
Light green indicating mapping to ABL1 on chromosome 9
Split reads at genomic breakpoint of BCR
Split reads at genomic breakpoint of ABL1
Olive green indicating mapping to BCR on chromosome 22
Reads mapping to ARID2 on chromosome 12
Split reads at genomic breakpoint of ABL1
Split reads at genomic breakpoint of ARID2
Light green indicating mapping to ABL1 on chromosome 9
ABL1::ARID2
Der 9
Der 12
ARID2::BCR
Olive green indicating mapping to BCR on chromosome 22
Split reads at genomic breakpoint of ARID2
Split reads at genomic breakpoint of BCR
Purple indicating mapping to ARID2 on chromosome 12

Supplementary Figure 5: Ph-associated rearrangement associated with formation of possible circularized DNA in patient 450
The orientation of the genes associated with the XPNPEP3::BCR e7e14 gene fusion transcript in this case suggests that a 17.7 Mb deleted sequence adjacent to the BCR::ABL1 genomic breakpoint that extended to the XPNPEP3 gene (downstream on chromosome 22), had possibly circularized. The XPNPEP3::BCR genomic breakpoints were resolved at nucleotide resolution and a corresponding XPNPEP3::BCR fusion transcript was detected that was likely generated by transcription of linear RNA from the circularized DNA. A corresponding ABL1::XPNPEP3 genomic fusion may have occurred on the derivative 9 chromosome but would not be detectable if the breakpoints occurred in regions not targeted by probes. i13 indicates BCR intron 13 and i7 indicates XPNPEP3 intron 7. e=exons.


Chr 9
Chr 22
BCR
17.7Mb distance
XPNPEP3
ABL1
Formation of the Ph-chromosome and the Ph-associated rearrangement
Der 9
Ph-chromosome (Der 22)
BCR::ABL1 formed on chromosome 22
Postulated formation of ABL1::XPNPEP3 formed on chromosome 22 (no reads to support this)
17.7Mb segment deleted from chromosome 22 spanning from BCR to XPNPEP3
Chromosome colour code
Chromosome 9
Chromosome 22
Focus on deleted segment of chr 22
BCR i13 genomic break (22:23632313) for XPNPEP3::BCR fusion
Zoomed in BCR gene on der 9
e14
BCR
e15
BCR i13 genomic break (22:133630022) to form BCR::ABL1
XPNPEP3 i7 genomic break (22:41311345) for XPNPEP3::BCR fusion
e7
XPNPEP3
e8
Zoomed in XPNPEP3 gene
Genomic fusion of XPNPEP3::BCR with suspected double strand break spanning between BCR i13 and XPNPEP3 i7, circularizing the deleted genomic segment
XPNPEP3 e7
BCR e14
Formation of circular DNA
Circular DNA transcribed into linear transcript
XPNPEP3 e7
BCR e14

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7 Conclusion

7.1 Summary

Our understanding of the mechanisms underpinning resistance in chronic myeloid leukaemia are limited. *ABL1* kinase domain mutations have been the best appreciated resistance pathway, but kinase domain mutations are only identified in approximately 50% of patients deemed resistant.[1] Furthermore, predictors of the various outcomes in CML such as achievement of optimal molecular targets and even likelihood of successful treatment-free remission (TFR) have been lacking.

In this thesis, I have presented studies that represent an integrated investigation of novel biomarkers for the prediction of outcomes in CML, providing a personalized approach to risk prognostication. Moreover, the methodologies required for the investigation of resistance pathways associated with adverse outcomes in CML were also presented in this body of work. This research provides a platform for modifying recommendations from international guidelines into the investigation and management of CML in addition to planning exploratory research to further the existing knowledge base. This concluding chapter illustrates the key scientific findings identified in the thesis while exploring the clinical impact of the data presented.

7.2 Key findings and significance

7.2.1 The personalized prediction of a successful TFR

1. The data published here is the first evidence that examined the strong relationship between the 3-month *BCR::ABL1* halving time and the possibility of TFR. Currently, the halving time is the strongest discriminator of TFR outcomes.

2. Patients with a more rapid *BCR::ABL1* decline, manifested by a shorter halving time, were more likely to enter a successful TFR. The halving time quartiles defined in this study were derived from a predominantly imatinib-treated patient cohort.

3. Similarly, patients with a shorter halving time had an increased likelihood of achieving TFR eligibility more rapidly compared to patients with longer halving times.

4. In addition to the *BCR::ABL1* halving time, the *BCR::ABL1* transcript type and the duration of tyrosine kinase inhibitor (TKI exposure) were also associated with TFR outcome.

5. A prediction algorithm was developed and presented to assist clinicians in calculating the probability of TFR based on these three variables.

7.2.2 Reducing the frequency of BCR::ABL1 monitoring in TFR

1. The current *BCR::ABL1* testing strategy within the first 12 months of a TFR attempt is conservative but will safely capture all patients with molecular relapse, defined as a *BCR::ABL1* value exceeding 0.1%^{IS}.

2. The monitoring strategies presented in chapter 3 illustrated that the frequency of *BCR::ABL1* testing can be safely reduced within the first 12 months of a TFR attempt. The delay in TKI recommencement was dependent on the strategy utilized with strategy 2, involving 7.5 tests/patient, having no delay to molecular relapse detection or TKI recommencement. However, strategy 3, averaging 5.8 tests/patient, was associated with a one-month delay in TKI recommencement in a patient who failed TFR.

3. Reducing the frequency of *BCR::ABL1* testing following a TFR attempt is possible, enabling the wider adoption of TFR especially in resource-poor settings where TFR may not have been previously possible due to the testing requirements.

4. An update to the European LeukemiaNet recommendations for the clinical management of CML is currently underway and includes a discussion regarding the adoption of less frequent *BCR::ABL1* testing following a TFR attempt.

7.2.3 The highly sensitive detection of kinase domain mutations, including novel myristate site mutations, is possible with mass spectrometry

1. The SABER-based mass spectrometry technique can detect low-level kinase domain mutations to a level of 0.5%, including novel mutations generated by use of the new STAMP inhibitor, asciminib.

2. Low-level mutation detection has relevance for optimal TKI selection as expansion of the resistant low-level clone occurs if that specific TKI has known resistance against the clone.

3. The existing mass spectrometry assay for detection of low-level *BCR::ABL1* mutations was modified to enable detection of asciminib-specific mutations.

4. The SABER-based mass spectrometry method for *BCR::ABL1* kinase domain mutation detection is highly sensitive and reproducible. It is at least 5 times more sensitive for *BCR::ABL1* kinase domain mutation detection in comparison to current next-generation sequencing techniques.

5. The modified technique is currently utilised for detection of low-level *BCR::ABL1* kinase domain mutations in two large multinational clinical trials investigating the efficacy of asciminib in resistant and intolerant CP-CML patients and in CP-CML patients who have failed to reach a major molecular remission following frontline imatinib therapy.

7.2.4 Development of a hybridisation capture technique for the detection of various mutation subtypes relevant for CML

1. A number of different variant subtypes have been reported to contribute to resistance in CP-CML, including single nucleotide variants, small insertions and deletions, focal gene deletions, fusions (genomic and transcripts) and other structural variants in addition to aberrant splicing.

2. Almost all blast phase (BP) patients investigated with NGS have had at least one identifiable variant of potential or known clinical relevance in blood cancer-related genes.

3. DNA is the genetic material preferred for NGS given the stability of the medium and the ease of utilization, whereas RNA has a higher propensity for degradation and extraction is more laborious. Regardless, RNA has the added benefit of being able to identify fusion transcripts and aberrant splicing.

4. A hybridisation capture technique to detect various types of mutations that have proved clinically relevant for patients with CML, including single nucleotide variants, small insertions and deletions, focal gene deletions and gene fusions was developed and validated.

5. The performance criteria of the assay was established with both DNA and total RNA as the source material, demonstrating high accuracy and reproducibility for variant detection using either material.

6. The utility of total RNA enabled the detection of intronic variants from pre-spliced RNA in addition to genomic breakpoints. This also enabled the detection of variants subject to nonsense mediated decay, which may not have been otherwise identified by RNA sequencing using poly-A selection.

7. The expression of variants in RNA can be influenced by allele specific expression or nonsense mediated decay, resulting in discordance of VAF between RNA and DNA. This was frequently observed with kinase domain mutations.

8. Additional genetic variants were also identified using total RNA in the form of fusion transcripts and aberrant splicing, which were not detectable using DNA alone.

9. The impact of splice region variants on splicing was only evaluable using RNA, whereas DNA would rely upon in silico prediction models and additional testing to identify the true functional impact of splice region variants.

10. Both DNA and RNA were able to capture novel rearrangements associated with the formation of the Philadelphia chromosome.

11. Total RNA-based sequencing increased the yield of variant detection for patients with CML compared to DNA.

7.2.5 The impact of NGS at the diagnosis of chronic phase CML

1. The RNA-based hybridisation capture technique demonstrated that additional genetic abnormalities (AGAs) were identified at diagnosis in approximately 30% of chronic phase CML patients. These events encompass variants in cancer genes as well as complex rearrangements involving the formation of the Philadelphia chromosome, termed Ph-associated rearrangements

2. This study was the first report of genomic profiling of a consecutive cohort of imatinib-treated patients that were not selected for sequencing specifically based on their known outcome.

3. Despite a highly proactive treatment intervention strategy, patients harbouring AGAs experienced a higher rate of failure and an increased propensity for kinase domain mutation acquisition.

4. Ph-associated rearrangements had a very strong association with kinase domain mutation acquisition.

5. AGAs negatively impacted the achievement of major molecular response, MR4 and MR4.5 despite initiation of 600mg daily of imatinib, a highly proactive strategy of imatinib dose escalation or nilotinib switch in the event of failure to achieve pre-determined molecular milestones. The effect of AGAs will have likely been more pronounced if patients had been treated with more conventional dosing of imatinib,

i.e 400mg daily of imatinib, without the proactive dose escalation or TKI switch strategies.

6. TKI switch to nilotinib due to failure to achieve ELN-defined milestones did not completely rescue responses if patients harboured AGAs.

7. The presence of AGAs, together with the ELTS score, predicted for a high rate of failure with inferior molecular outcomes despite a highly proactive strategy in the event of failure to meet strict molecular targets.

8. *ASXL1* was the gene most frequently mutated in this cohort, similar to other published reports, and was observed in 9% of the study cohort. Failure-free survival was inferior for patients with an *ASXL1* mutation compared with other AGAs and was also associated with a higher rate of kinase domain mutation development.

9. While *ASXL1* variants are associated with inferior outcomes, they may require acquisition of additional variants for transformation to BP as evidenced by patient 430 where the *ASXL1* variant was lost with expansion of a single clone harbouring three variants (*BCORL1* frameshift, *RUNX1* deletion and *IKZF1* fusion) at the time of BP.

10. This work confirms the potential of NGS in CML, especially at diagnosis, for prognostication and response assessment. Appropriate treatment for modifying the negative impact associated with the additional genetic abnormalities will need to be determined.

7.3 Future directions

The research presented in this thesis illustrates the importance of precision medicine in the prediction of outcomes in CML. Based on existing literature and results from the thesis, some of the key areas of future focus are presented.

7.3.1 Maximizing the potential of the BCR::ABL1 halving time

While the potency of second-generation TKIs is known to be higher than imatinib with more rapid achievement of molecular milestones,[2,3] the biological rationale for justifying upfront therapy with a second-generation TKI with regards to TFR achievement is lacking. Our unpublished data demonstrates that the median halving time of imatinib-treated patients is

longer than those treated with second-generation TKIs. This data may suggest that patients treated with upfront second-generation TKIs will achieve TFR eligibility more rapidly than if treated with imatinib. While further work is required to confirm these findings, frontline use of a second-generation TKI specifically for earlier achievement of TFR eligibility and improved TFR success will need further exploration.

7.3.2 Developing a diagnostic grade NGS assay

The validation of an RNA-based next-generation sequencing hybridization capture assay was demonstrated in chapter 5. This method has proved capable of detecting the relevant mutation subtypes associated with resistance in CML including *BCR::ABL1* kinase domain mutations, Ph-associated rearrangements and cancer gene variants. While this assay is being deployed for various research projects, it requires modification prior to being implemented in regular clinical testing as a diagnostic tool and for resistance monitoring. The current method is only validated for mutant detection with a variant allele frequency of ≥5%.[4]

A factor for the early prediction of resistance is the capacity to detect an emerging mutant clone while *BCR::ABL1* transcript levels remain low. However, the *BCR::ABL1* level where mutations in other cancer genes can be reliably detected using next-generation sequencing is unknown. I received the 2022 John Goldman Research Award of €80,000 to develop a diagnostic grade method based on the RNA-based capture technique with enhanced sensitivity and workflow. This will involve limiting the number of genes that the panel investigates from >100 to 30-40 that are the most relevant in CML. The modified panel will also have the potential to detect emerging mutant clones, including gene fusions, deletions and those altering RNA splicing that may be mediating TKI resistance, enabling longitudinal serial monitoring of low-level variants, similar to minimal residual disease monitoring that exists in other malignancies, such as acute myeloid leukaemia.[5,6] This will facilitate the inclusion of NGS into current international clinical guidelines for CML at key timepoints, such as diagnosis, frank resistance and at 'warning' *BCR::ABL1* responses.

7.3.3 Targeting AGAs with more potent TKIs

The impact of AGAs, together with the ELTS score, in a highly proactive imatinib-treated patient cohort was demonstrated in chapter 6. The next question is whether frontline use of more potent second-generation TKIs can modify the adverse risk associated with these negative risk factors. The second-generation TKIs (dasatinib and nilotinib) are already known to provide deeper and more rapid molecular responses compared to imatinib[2,3] but whether this response is ameliorated by the presence of AGAs is unknown and investigation is currently underway. Whether the novel STAMP inhibitor, asciminib, will have a different response profile to the additional genetic abnormalities will also need exploration. Assuming the more potent TKIs can modify the adverse risk associated with additional genetic abnormalities, this may enable personalised TKI selection based on clinical risk scores and the individual genomic profile.

7.4 Concluding remarks

In summary, the results presented in this thesis contribute to the understanding of the complexity of risk prediction involved in CML and has identified potential predictive biomarkers. This provides a foundation for personalized medicine in CML for improved outcomes and for the achievement of TFR. It also delivers a platform for future research and studies in order to guide international management guidelines.

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Appendix A

Early management of CML

Shanmuganathan N, Hughes TP: Early Management of CML. Current hematologic malignancy reports 2019, 14(6):480-491.

Shortened title: Early CML management

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Abstract

Purpose of review: The marked improvement in clinical outcomes for patients with chronic myeloid leukemia (CML) can be solely attributed to the introduction of targeted therapies against the fusion oncoprotein, *BCR-ABL1*. However patient responses, although generally positive, remain heterogenous. Careful drug selection, ensuring the optimal TKI is chosen for each patient, involves a complex decision process which incorporates consideration of numerous factors.

Recent findings: For some patients, with disease characteristics that indicate adverse intrinsic disease biology, more potent *BCR-ABL1* inhibition is often appropriate, whereas other patients with major co-morbidities will benefit from a less aggressive approach to avoid life-shortening toxicities. For the vast majority of patients, the long-term goal of therapy will be the achievement of a deep molecular response and subsequent treatment-free remission and this consideration will play a large part in the drug selection process.

Summary: We explore early management of CML, from the first presentation through to frontline therapy selection.

Introduction

Since the introduction of imatinib, the first targeted *BCR-ABL1* inhibitor, newer, more potent tyrosine kinase inhibitors (TKIs) have been vital additions to the therapeutic landscape of chronic phase chronic myeloid leukemia (CP-CML) management. TKIs bind to the fusion chimeric protein, *BCR-ABL1*, via the ATP-binding site, inhibiting kinase activity and thus leukemogenesis, through reduction in proliferation, regulation of differentiation, and restoration of the impaired apoptotic pathway.[1] However despite the excellent treatment options available to newly diagnosed CML patients, optimal TKI selection is not straightforward despite the well-established benefits and risks associated with each individual drug. Upfront treatment selection, assuming availability of imatinib, nilotinib, dasatinib and bosutinib, should ideally entail consideration of multiple clinical and laboratory factors. While the primary purpose of CML therapy is to prevent disease progression, we recommend early determination of the long term goals of therapy, specifically what priority should be given to achieving deep molecular response (DMR) as a platform for the eventual achievement of treatment-free remission, as it will influence TKI selection. The dramatically improved survival[2] now observed in CP-CML signifies that newly diagnosed patients may be required to endure decades of TKI therapy, mandating optimal upfront drug selection to maximize molecular response and safety outcomes.

First presentation

The diagnosis of CML is frequently incidental, with a large proportion of patients identified with asymptomatic leucocytosis on routine investigation. The recommended baseline investigations are listed in Table 1.[3] Peripheral blood demonstrates an abundance of mature granulocytes, especially in CP, with a myelocyte peak.[4] Eosinophilia and basophilia are common while dysplastic features are infreqeunt.[4] Thrombocytosis may be the sole

hematological abnormality in rare cases and CML should be considered as a differential diagnosis to patients with unexplained persistent thrombocytosis, especially in the setting of *JAK2*, *MPL* and *CALR* negativity. Active questioning and assessment for features of leukostasis, especially when white cell counts exceed 100 x 10^9/L, should be conducted. Evidence of hyperviscosity affecting pulmonary (dyspnoea), neurological (headache, tinnitus and visual changes) and vascular (including priapism and cardiac ischemia) systems necessitate urgent cytoreduction via hydroxyurea and/or leukopheresis.[3, 5] Physical examination should focus on evaluation for splenomegaly and documentation of actual length it is palpable below the costal margin, important for prognostic scoring models, exclusion of extramedullary leukemia and sites of leukostasis involvement including fundoscopic evaluation for retinal haemorrhage. While the diagnosis of CML can be confirmed via peripheral blood cytogenetic analysis or *BCR-ABL1* quantitative PCR, we recommend performing a diagnostic bone marrow biopsy to confirm the disease stage as per either the ELN or WHO criteria.[6, 7] Differentiating between CP, accounting for 90-95% of new diagnoses,[4] and the more aggressive accelerated phase (AP) and blast crisis (BC) CML is imperative to ensure appropriate management is instituted. The significance of marrow fibrosis, although previously considered to be a predictor of therapy failure, is unclear.[8, 9] Potential transplant candidates diagnosed with more aggressive disease (AP/BC CML) need to be tissue typed with early consideration of an allograft,[10] although more recent data suggests that not all AP patients necessarily have the same adverse outcomes.[11] Patients with cytogenetic abnormalities as the sole criterion for AP classification have a more favourable prognosis with TKI therapy than patients categorised as AP due to other features.[11]

Fertility and family planning

Fertility and family planning discussions need to occur in female patients with child-bearing potential prior to the commencement of any TKI. Despite the limited data in this setting, TKI exposure during pregnancy is associated with increased rates of miscarriage and fetal malformations[12-14] We recommend interruption of TKI therapy prior to conception and alternative methods for disease control utilized throughout the pregnancy in female CML patients.[15] The impact of TKIs on male fertility is less clear with conflicting reports on the effect on spermatogenesis and male sex hormones, although the frequency of fetal malformations and stillbirths appears to be no higher than their healthy contemporaries.[14, 16] The general consensus is that TKI therapy does not need to be discontinued in male patients prior to conception.[10] Referral to fertility specialists may be warranted prior to TKI commencement for patients wishing to explore potential options for fertility preservation.

Current first-line therapeutic landscape

Prior to exploring the various factors important in deciding the optimal TKI for newly diagnosed CML patients, a review of the available first-line options is essential with important molecular and survival measures documented in Table 2.

Imatinib

Imatinib, the first-generation TKI, revolutionized CML management with its introduction. With almost two decades of therapeutic availability, imatinib is the TKI that clinicians have had the longest familiarity with – through the first-in-human clinical trials to the current local drug access programs specific to each country. Long-term safety and toxicity data from the earliest clinical trials are readily available with published molecular and response measurements. Moreover, with the addition of generic imatinib to the pharmaceutical market in recent years, the declining costs of treatment to the patient and governmental bodies have also improved accessibility.

While imatinib maintains the safest toxicity profile of all TKIs, it is also the least tolerated, with toxicities often impacting on quality of life (QOL) measures.[17]. Gastrointestinal events, especially diarrhea, were reported in almost 30% of patients in the International Randomized study of Interferon and STI571 (IRIS) study, although only 2% were reported to be grade 3-4 severity.[18] Fatigue and muscle cramps have been consistently reported as the main culprits negatively impacting QOL.[17-19] Concerns regarding long-term cardiotoxicity have proved to be largely unfounded with the incidence of grade 3-4 cardiac events in the 10-year follow-up of IRIS being 7.1%.[20] Reductions in bone mineral density have also been reported in a small population of patients, indicating the importance of close monitoring of bone health.[21] Long-term imatinib therapy is also associated with a reduction in glomerular filtration rate and slight increase in the frequency of chronic renal failure, indicating the importance of regular assessment of renal function.[22, 23] Apprehension regarding second malignancy risk with imatinib treatment has not materialized as although 11.3% of the imatinib cohort in IRIS[20] developed a second cancer, this may be attributed to the slightly increased malignancy risk associated with CML as opposed to a specific TKI.[24, 25]

The pivotal IRIS trial demonstrated the dramatic survival benefit associated with targeted *BCR-ABL1* inhibition by imatinib, later corroborated by the German CML-IV study.[20, 26] Disease progression was also less likely with imatinib when compared to the control arm of interferon alpha and cytarabine, predominantly occurring early in therapy.[18] The 10-year rates of major molecular response (MMR, equating to a *BCR-ABL1* ≤ 0.1% IS) and MR4.5 (equating to a *BCR-ABL1* ≤ 0.0032% IS) were 93.1% and 63.2% respectively.[20] However, kinase domain mutations develop in almost 20% of patients, a higher frequency compared to treatment with second-generation TKIs.[26-28] Additionally almost 30% of imatinib-treated patients will require a TKI switch due to intolerance or resistance, highlighting the need for early optimal treatment selection.[20, 26]

The optimal dose of imatinib has been a contentious issue. The German CML-IV study had 5 treatment arms, 2 of which were 400mg and 800mg of imatinib.[26] While the 10-year OS and molecular measurements were largely similar between the 2 groups, the 800mg cohort achieved MMR and MR4 (equating to a *BCR-ABL1* ≤ 0.01% IS) more rapidly.[26] Higher rates of MMR at 12 and 24 months of TKI therapy were demonstrated by the Australian group in patients maintaining 600mg of imatinib compared to those tolerating <600mg daily.[29] In contrast, data from the Tyrosine kinase inhibitor OPtimization and Selectivity (TOPS) trial did not reveal a significant difference in MMR achievement between a daily dose of 400mg compared to 800mg of imatinib. Furthermore, higher doses of imatinib have a clear association with an increased incidence of adverse events,[19, 30] especially those impacting

quality of life such as gastrointestinal toxicities.[17] Due to the difficulties in maintaining higher doses of imatinib secondary to toxicities together with the lack of proven survival benefit,[26] 400mg daily of imatinib has become the accepted standard dose.[10]

Nilotinib

Nilotinib, a second-generation TKI, has significantly higher affinity for binding to the kinase domain of *BCR-ABL1* compared to imatinib, leading to increased potency.[31] Although nilotinib, like other second- and third-generation TKIs, can rescue some patients with imatinib-resistant disease, this falls out of the purview of this review and will be explored elsewhere.[32-35]

The Evaluating Nilotinib Efficacy and Safety in clinical Trials–Newly Diagnosed (ENESTnd) study, the head-to-head comparison of imatinib 400mg daily to nilotinib in newly diagnosed CP-CML patients, recently published a 5-year update.[28] Early molecular response (*BCR-ABL1* ≤10% IS at 3 months), a predictor of molecular milestone achievement, was significantly higher with either nilotinib dose.[28] By 5 years, MMR achievement was 77% with 300mg twice daily of nilotinib compared with 60% of imatinib-treated patients.[28] Similarly, 54% of nilotinib-treated patients achieved MR4.5 compared to only 31% of imatinib patients.[28] Additionally, not only was molecular milestone achievement higher with nilotinib, they were reached more rapidly with early divergence of the cumulative incidence curves.[28] Transformation risk and *BCR-ABL1* kinase domain mutation development is also significantly lower with nilotinib.[28] Despite the dramatic molecular benefit observed with nilotinib, the study failed to prove a survival benefit compared to imatinib, perhaps due to the added toxicity burden of nilotinib.[28]

Despite nilotinib being relatively well tolerated, it is associated with serious toxicities which patients need to be aware of prior to frontline selection. A concerning signal for cardiovascular toxicity, including ischemic heart disease, cerebrovascular events and peripheral arterial disease, emerged on the nilotinib studies. The risk was relative to dose, as 400mg twice daily of nilotinib (13.4%) had almost double the incidence of events compared with 300mg twice daily (7.5%) by 5 years.[28] Only 2.1% of imatinib treated patients developed similar toxicity.[28] High Framingham scores at study entry, primarily due to pre-existing risk factors (such as smoking, hypercholesterolemia and hypertension) had a high association with cardiovascular events whereas patients deemed low risk had a similar rate of events compared to low risk patients receiving imatinib.[28] Other grade 3-4 toxicities observed with both nilotinib doses include hypertension (8.3-10.4%), hypercholesterolemia (26.7-27.6%) and grade 3-4 elevated glucose (6.9-7.2%), requiring regular monitoring to minimize additional cardiovascular events.[28] QT prolongation and pancreatitis has also been observed with nilotinib. There is no discernible difference in the incidence of second malignancies between nilotinib and imatinib.[28]

Together with the increased toxicity burden associated with the higher dose and in conjunction with the minimal differences in molecular responses observed in the randomised study, 300mg twice daily of nilotinib is the recommended dose in first-line therapy.[10] The dosing schedule is twice daily with recommended administration in a fasting state[10] which can lead to compliance difficulties, emphasizing the importance of adequate patient education prior to drug commencement. The schedule of drug administration may also

influence TKI selection for patients in certain settings, such as shift workers who may struggle complying with the strict twice daily regime of nilotinib therapy.

Dasatinib

Dasatinib, providing dual tyrosine kinase inhibition against both *SRC* and *ABL*, has similar responses and outcomes to nilotinib with clear superiority in efficacy compared to imatinib. The landmark trial proving efficacy in upfront treatment was the DASatinib vs. Imatinib Study In Treatment-Naïve CML patients (DASISION), comparing 100mg dasatinib to 400mg imatinib. By the end of the 5-year follow-up, dasatinib demonstrated higher rates of achievement of molecular targets including MMR (76% vs. 64%) and MR4.5 (42% vs. 33%) compared to imatinib.[27] Fewer patients progressed to AP/BC with dasatinib (4.6%) compared to imatinib (7.3%).[27] However like ENESTnd with nilotinib, there was no associated survival benefit observed with dasatinib.[27]

Hematological toxicity is frequently observed with dasatinib, occasionally necessitating treatment interruption. There is a minor risk of bleeding secondary to platelet dysfunction which patients need to be aware of.[36] Cardiovascular events in DASISION were only slightly increased compared with imatinib therapy by 5 years, although more recent data suggests the risk may be similar to nilotinib.[27, 37, 38] Pulmonary toxicities are the hallmark of dasatinib therapy, manifesting as pleural effusions or the more serious complication of pulmonary hypertension. Up to 28% of patients developed a pleural effusion by 5 years of dasatinib exposure, the incidence linearly increasing with each year of therapy.[27] The risk appears to be relative to age with 60% of patients ≥65 developing an event.[27] Additionally, elevated dasatinib levels (≥ 3nmol/L) are associated with an increased risk of effusion development.[39] In comparison, pulmonary hypertension is infrequent, occurring in less than 5% of patients. If detected however, dasatinib should be ceased.[27] The pulmonary hypertension generally resolves with drug discontinuation, but one-third of patients showed evidence of persistence.[40, 41] Although the standard dose of dasatinib is 100mg daily, lower doses of dasatinib have been associated with good clinical response. Clinical trials investigating dasatinib dose adjustment according to therapeutic drug monitoring are underway to evaluate the balance between molecular response and drug toxicity.[39, 42]

Bosutinib

Restricted availability of bosutinib has limited familiarization compared to the other first-line TKIs but like dasatinib, it is a potent dual SRC and ABL kinase inhibitor.[43] The recently published BFORE study, comparing 400mg daily of bosutinib to 400mg daily of imatinib, revealed similar efficacy to the other second-generation agents.[43] Although long-term experience with bosutinib is still accruing, progression to AP/BC are less frequent compared to imatinib.[43, 44] Molecular milestone achievement by 12 months is comparable to the other second-generation TKIs.[43] The toxicities frequently observed with both nilotinib and dasatinib, including cardiovascular and pulmonary events, are also less prominent with bosutinib therapy.[43] Gastrointestinal toxicities, specifically diarrhea, are more characteristic with grade ≥3 diarrhea developing in almost 8% of patients treated with 400mg daily of bosutinib in BFORE, frequently resulting in drug interruption.[43]

What is old is now new – the reintroduction of interferon to CML therapy

Attempts to further improve the molecular responses observed with TKIs has led to a resurgence of interest in interferon, the historical gold-standard treatment of CML. Combining pegylated interferon with TKI therapy is the focus of a number of ongoing clinical trials. Superior rates of MMR achievement was observed with the addition of pegylated interferon Alfa-2a to imatinib.[45] Preliminary data from combining nilotinib with pegylated interferon is indicative of comparable, if not superior, molecular responses to nilotinib monotherapy.[46-48] Similar early results are reported from dasatinib studies.[49] While the toxicity profile of combination therapy appears to be relatively manageable, the data requires maturation and longer-term follow up is needed before pegylated interferon can be incorporated into standard practice.[45-49]

First-line therapy selection

Goals of therapy

As the OS of patients diagnosed with CML approaches the general population, CML patients may anticipate decades of TKI exposure for disease control with concurrent accumulation of TKI associated toxicities. However, select patients are also able to achieve treatment-free remission (TFR) after achievement of a durable DMR. On average, 50-60% of eligible patients attempting TKI discontinuation successfully achieve TFR.[50-59] Although second-generation TKIs have not been proven to increase TFR success to date, earlier achievement of DMR with these agents allows for earlier TFR attempts, potentially greatly shortening the duration of TKI exposure and thus limiting toxicities. Consequently, strategies to maximize early achievement of DMR, definition dependent on institution (MR4 or MR4.5), in addition to maximizing stability of depth of response should be at the forefront of consideration. For females of child-bearing age, timely achievement of stable DMR will allow for TKI discontinuation either as a TFR attempt or temporary interruption, enabling conception and pregnancy and minimizing the need for CML-specific management.[15]

Patient comorbidities

While the effectiveness of TKIs in treatment of CML is indisputable, appropriate treatment selection is warranted to achieve the right balance between toxicity and molecular response. TKIs are generally well tolerated but as described earlier, each drug has a relatively unique toxicity profile requiring consideration of suitability for each patient prior to selection. While patient comorbidities do not affect achievement of molecular responses or risk of progression, higher scores on the Charleston comorbidity index correlated with inferior OS, even with exclusion of the age-dependent factors.[60]

The risk of vascular events is a major concern and should be a key factor when considering optimal therapy for each patient. Epidemiological data has demonstrated that the incidence of major arterial events (per 100/patient-years) were lowest with imatinib (0.1), incidence increasing with the more potent agents - bosutinib (0.4), dasatinib (1.1) and nilotinib (2.8).[61]

The risk of peripheral arterial occlusions remains highest for nilotinib compared with the other first-line agents.[61] Similarly, nilotinib had an increased rate of ischaemic heart disease with at 1.4/100 patient-years with fewer events observed with the other TKIs (dasatinib 0.6, bosutinib 0.3, imatinib 0.1).[61] However other studies suggest that the cardiovascular risk associated with nilotinib and dasatinib are actually similar.[37, 38] Cerebrovascular events were only observed in 0.5% of patients treated with first-line agents.[61] Moreover, upfront use of second-generation TKIs is associated with less cardiovascular risk compared with utility second-line or beyond, perhaps due to shorter treatment duration or due to inherent bias regarding therapy selection.[62]

Cardiovascular risk should be evaluated at diagnosis with initiation of appropriate therapies to minimize CV risk.[63] Risk models such as the Framingham and the systemic coronary risk evaluation (SCORE) have been utilised to predict patient risk of developing cardiovascular toxicity; higher scores correlating with higher risk of cardiovascular events. [28, 64] Hypertension should be controlled according to local guidelines, appropriate diabetes therapy should be applied while hyperlipidemia should be treated with statin therapy where indicated. Statins least likely to interfere with TKI metabolism by CYP3A4 (i.e. rosuvastatin and pravastatin) are preferred.[65] Evidence for primary prophylaxis with aspirin is inconclusive[62] and may actually be detrimental in patients treated with dasatinib due to the slight increase in intrinsic bleeding risk.[36, 65] A harmonious balance between treatment goals, disease risk and cardiovascular risk should be sought. For example, a second-generation TKI would be preferred in patients with low cardiovascular but high CML risk. If TFR is a goal of therapy, then a second-generation TKI may be preferred for early achievement of DMR. If cardiovascular risk is deemed to be high, then avoiding nilotinib and possibly dasatinib would be prudent. Guideline-driven monitoring of cardiovascular risk is recommended for all patients[63] while appropriate lifestyle changes (such as smoking cessation and establishment of a healthy weight) is strongly advised.

Pleural effusions are a major complication associated with dasatinib, advanced age being the only consistent risk factor associated with development.[66] Preliminary data suggests that elevated dasatinib levels are linked with an increased risk of effusion development with a clinical trial in accrual, investigating dasatinib dose adjustment secondary to therapeutic drug monitoring and incidence of pleural effusions.[39, 42] In the interim, careful patient selection prior to dasatinib commencement is advised. Given the risk of pulmonary hypertension with dasatinib, patients with pre-existing pulmonary hypertension should be considered for an alternate TKI.[67] Due to concomitant platelet dysfunction, dasatinib should be prescribed with caution in patients on anticoagulants due to the risk of hemorrhagic complications.[68]

Hepatitis B reactivation has been reported secondary to TKI therapy leading to the recommendation for serological screening prior to TKI commencement.[69]

Disease risk scores

Risk stratification in CML incorporates simple disease parameters to calculate the risk of CML-related death, OS or cytogenetic response utilizing models including the Sokal, Hasford, EUTOS or ELTS.[70-73] Although the Sokal and the Hasford scores were developed in the pre-TKI era, they remain predictive of molecular response.[20, 28, 43, 74] High Sokal scores is associated

with poorer 10-year OS rates (68.6%) compared to intermediate (80.3%) or low scores (89.9%).[20] High Sokal risk patients also have inferior achievement of MMR and stable MR4.5.[75, 76] Treatment with second-generation TKIs improved molecular responses regardless of risk score compared with imatinib but failed to completely nullify the adverse effect of a high risk Sokal or Hasford score.[27, 28, 43] These results are indicative that more potent *BCR-ABL1* inhibition via second-generation TKIs, while not able to eliminate the adverse prognostic implications of high Sokal or Hasford-risk, still provide significant benefit compared to imatinib therapy. Arguably their greatest benefit is seen in the high-risk categories.

The EUTOS score, developed and validated in an imatinib-treated cohort, is felt to be a simpler and more refined prognostic model, having removed factors in the prior risk calculators that fail to predict imatinib response.[72] Like the Sokal and Hasford scores, high-risk calculation as per the EUTOS score predicted for inferior MR4 achievement compared to a low-score despite first line nilotinib therapy.[77] This led to the development of the ELTS score, using the same variables as the Sokal score with different weighting, to improve long-term survival prediction, specifically for CML-related death.[73] Recently, a comparative analysis has shown that the Sokal score overestimates the number of high-risk patients compared to the ELTS leading to inaccuracy in long-term survival prediction.[78, 79] However, only the Sokal and Hasford scores are incorporated into the current NCCN guidelines although the EUTOS score is included in the 2013 ELN recommendations.[6, 10] Regardless of which score is utilized, all of these scoring systems will provide risk discrimination and improved TKI selection can then follow although the ELTS score is probably more accurate in the TKI era.

Cytogenetic abnormalities

Additional chromosomal abnormalities (ACAs) in the Ph+ clone at diagnosis is present in about 5% of patients with increasing incidence in the more aggressive phases of disease.[80-82] Identification of major route ACAs (defined as a second Ph+, trisomy 8, isochromosome 17q or trisomy 19) heralds an inferior survival rate while foreshadowing progression to AP/BC.[80] Recognition of major route ACAs from the German CML IV and Italian studies indicated slower MMR achievement with inferior OS and PFS, despite upfront imatinib therapy.[80, 81] However more recent data suggests that ACAs, irrespective of major route abnormalities, do not predict for inferior molecular or survival outcomes, although these findings may be confounded by treatment with a second-generation TKI.[83] ACAs remain an indicator of poor prognosis in AP/BC CML.[82, 83] To date, there is no clarity as to whether a second-generation TKI is truly beneficial in this scenario although it remains our standard practice.

Transcript type

The most common transcripts identified in patients are the e13a2 (b2a2) or e14a2 (b3a2); any other transcript, termed “atypical”, are infrequently observed (less than 2% of cases).[84] Early studies demonstrated that the e14a2 transcript was associated with better cytogenetic responses.[85] This data was corroborated by other studies while also demonstrating more rapid achievement of MMR and DMR with either the e14a2 or both transcripts compared with e13a2 alone in additional to improved OS and PFS.[84, 86] In contrast, German data demonstrated that while inferior cumulative molecular responses are associated with e13a2

transcripts, there was no significant impact on OS.[87] While the impact of transcript type on survival is uncertain, more recent data has emerged suggesting that patients with the e13a2 transcript who achieve a deep molecular response and attempt to achieve TFR are less likely to be successful TFR.[88, 89] At this stage, the impact of the transcript type on response and outcome is not sufficiently clarified to be included in frontline treatment consideration.

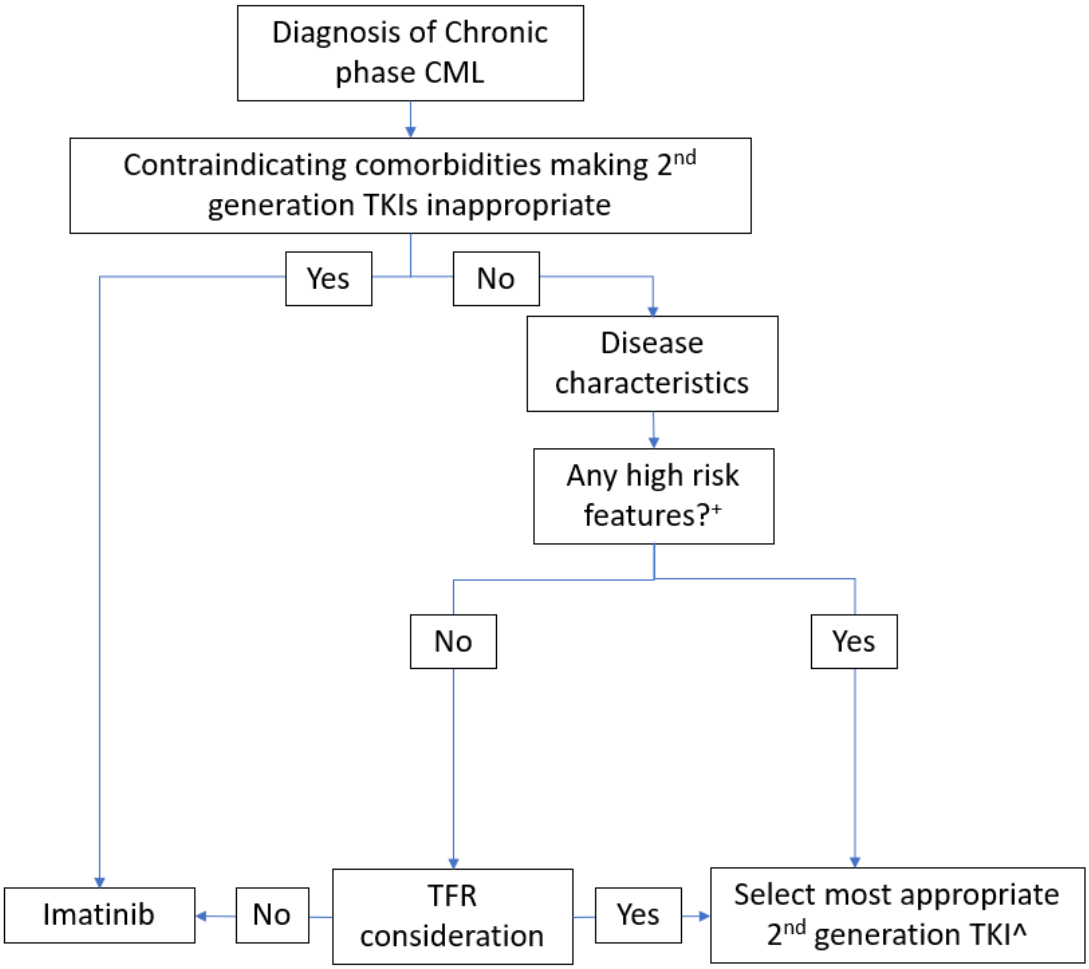
The first conversation

Initiating a dialogue with patients is mandatory to ensure that they are aware of all the available options, the potential advantages of second generation TKIs in terms of response, but also understand the common toxicities associated with each drug to inform drug selection (Figure 1). Issues that need to be addressed in younger patients include fertility and family planning. In all patients, but particularly in older patients, comorbidities must be identified and considered, because they will often lead to the physician and patient favouring one TKI above others. The importance of drug adherence as well as compliance with monitoring requirements must be clearly outlined. The goals of therapy should be introduced including the longer-term goals of deep molecular response and TFR. The potential to eventually stop therapy rather than commit to lifelong therapy is very attractive for many patients and will likely influence frontline therapy selection.

TKI Adherence

Good TKI adherence is vital to achieve molecular milestones[90] and as such, clinicians should ensure that patients are appropriately educated regarding the importance of drug adherence. Suboptimal responses are often associated with poor compliance with the prescribed TKI[90, 91] Addressing patient concerns regarding dosing schedules, drug toxicity and concomitant medications may assist with improving compliance.[92] Strategies to improve inadvertent non-compliance should be encouraged including electronic reminders, mobile phone applications or dose administration aids.[93] Tackling deliberate drug non-adherence requires directed discussion for potential factors influencing non-compliance while reinforcing the hazards of poor disease control.[93]

Figure 1: Decision tree for TKI selection



+ High score on risk model (Sokal, Hasford, EUTOS, ELTS), Additional cytogenetic abnormality

^ Avoid nilotinib in patients with high cardiac risk scores by Framingham or SCORE models

Conclusion

The goals of therapy have shifted from a sole focus on prevention of disease progression to toxicity minimization and achieving a deep molecular response as a platform for future attempts at drug discontinuation. We have presented an overview of the various first-line TKI options with disease characteristics and considerations that we anticipate will inform drug selection in the hematology clinic. Early discussion of goals of therapy will enable TKI selection to encompass long-term plans while the focus for some patients will remain on disease control and toxicity prevention. Individualizing CML therapy by tailoring TKI selection to each patient's comorbidities, disease biology and expectations will ideally maximize outcomes, walking the fine line between optimal molecular response and minimal toxicity.

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Conflict of Interest Statement

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Tables

Table 1: Baseline evaluation recommended in a newly diagnosed CML patient[6, 10, 94]

History
- Comorbidity assessment
Physical examination
- Spleen size – measured, not estimated
- Evaluation for extramedullary leukemia
- Evaluation for leukostasis (including fundoscopy)
- Vascular assessments including blood pressure and peripheral pulses
Peripheral blood investigations
- Full blood count
- Biochemistry including urate, renal and liver function
- Fasting lipid studies
- Fasting glucose +/- HbA1c
- Lipase/amylase
- Thyroid function tests
- Vitamin D
- Parathyroid hormone
- Hepatitis B serology
- *BCR-ABL1* qPCR
Bone marrow biopsy
- Blast count evaluation
- Cytogenetic analysis: standard karyotyping or fluorescence in situ hybridization (FISH)
Other investigations
- Chest xray
- ECG
- Tissue typing (if an allogeneic transplant is anticipated or patient is diagnosed in accelerated or blast crisis)
Optional
- Ankle brachial index

Table 2: Molecular responses based upon first line TKI studies

	IRIS[20]		CML IV[26]		ENESTnd[28]			DASISION[27]		BFORE[43]	
	Cytarabine/IFN	IM 400mg	IM 400mg	IM 800mg	NIL 300mg twice daily	NIL 400mg twice daily	IM 400mg	DAS 100mg	IM 400mg	BOS 400mg	IM 400mg
MMR											
- 1 year		27.7%	36.7%	55.6%	55%	51%	27%	46%	28%	47.2%	36.9%
- 2 year					71%	67%	44%	64%	46%		
- 3 year			80.6%	83.2%	73%	70%	53%	67%	55%		
- 4 year		42.5%			76%	73%	56%	73%	60%		
- 5 year		50.3%	86.3%	86.8%	77%	77%	60%	76%	64%		
- 10 year		34.4%	92.2%	89.1%							
MR4.5											
- 1 year		0.7%	4.8%	9.2%	11%	7%	1%	5%	3%	8.1%	3.3%
- 2 year					25%	19%	9%	19%	8%		
- 3 year			34.6%	43.1%	32%	28%	15%	24%	13%		
- 4 year		9.0%			40%	37%	23%	34%	23%		
- 5 year		23.0%	49.4%	58.4%	54%	52%	31%	42%	33%		
- 10 year		23.3%	67.2%	70.6%							
EFS											
- 1 year										96.3%	93.6%
- 5 year					95%	96.9%	92.6%				
- 10 year	56.6%	79.6%									
PFS											
- 5 year					96.5%	98.3%	94.7%	85%	86%		
- 10 year			83%	77%							
OS											

- 5 year - 10 year	78.8%	83.3%	80%	79%	93.7%	96.2%	91.7%	91%	90%		
Progression to AP/BC - 1 year - 5 year - 10 year		6.9%			3.7%%	2.2%	7.9%	4.6%	7.3%	1.6%	2.5%

IFN: Interferon; IM: Imatinib; NIL: Nilotinib; DAS: Dasatinib; BOS: Bosutinib; MMR: Major molecular response; EFS: Event-free survival; PFS: Progression-free survival; OS: Overall survival; AP: Accelerated phase; BC: Blast crisis

Appendix B

Molecular Monitoring in CML: How Deep? How Often? How Should It Influence Therapy?

Shanmuganathan N, Hughes TP. Molecular monitoring in CML: How deep? How often? How should it influence therapy? *Blood*. 2018;132(20):2125-2133.

Shortened title: Molecular monitoring in CML

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Abstract

With the advent of tyrosine kinase inhibitors (TKIs), the goals of therapy in chronic myeloid leukemia (CML) are steadily shifting. Long-term disease control on TKI therapy has been the goal and expectation for most patients. More recently, treatment-free remission (TFR) has entered mainstream practice and is increasingly being adopted as the main goal of therapy. This therapeutic shift not only influences TKI selection, but has necessitated the refinement and dissemination of highly sensitive and accurate molecular monitoring techniques. Measurement of BCR-ABL1 mRNA expression through reverse-transcription quantitative polymerase chain reaction (RT-qPCR), reported according to the International Scale (IS), has become the primary tool for response assessment in CML. Achieving specific time-dependent molecular milestones, as defined by global therapeutic guidelines, has been established as critical in maximizing achievement of optimal outcomes while identifying patients at risk of therapy failure. Depth and duration of a deep molecular response (DMR) has become the new therapeutic target in patients considered for TFR. Consequently, molecular monitoring in CML has become even more critical to ongoing response assessment, identifying patients with TKI resistance and poor drug adherence, as well as enabling TFR to be attempted safely and effectively.

Learning objectives

- Molecular monitoring is assuming increasing importance in ensuring patients meet treatment milestones, maximizing survival and deep molecular responses.
- Sensitive *BCR-ABL1* RT-qPCR testing, calibrated to the International Scale, is the gold standard of molecular testing.
- Achieving and maintaining deep molecular response is crucial to treatment-free remission success.
- Strategies to attain treatment-free remission should direct therapeutic decisions in motivated patients.

What constitutes molecular monitoring?

After achievement of a complete cytogenetic response (CCyR), equating to a *BCR-ABL1* RT-qPCR of ~1% (IS), patient monitoring via conventional bone marrow metaphase analysis has minimal value unless CCyR is lost. Qualitative PCR has limited value except at diagnosis where it should be used to identify patients with atypical BCR-ABL1 transcripts. Quantitative PCR assays of peripheral blood, measuring the level of *BCR-ABL1* mRNA relative to an internal control gene, are the gold standard for monitoring in CML with the degree of leukemic cell burden translating to critically important prognostic information. Real time RT-qPCR is the primary tool for monitoring CML response and has the advantage of reliability, sensitivity, ease of replicability and generates relatively rapid results. Other quantitative strategies previously utilised include nested PCR which is more sensitive than RT-qPCR but more labour intensive, requiring days to generate a result.[1] Multiplex PCR is useful at diagnosis to identify the *BCR-ABL* breakpoint, enabling atypical transcript detection.[2] More novel techniques such as digital and DNA-based PCR can improve testing sensitivity but are not widely available and are currently research tools only.[3, 4] Establishment of the concordance of *BCR-ABL1* RT-qPCR measurements in blood with bone marrow cytogenetics assessment[5]

and BCR-ABL1 values[6] has reduced the requirement for invasive testing while development of the IS allowed for harmonization of testing between individual institutions.[7]

On the IS, patient results are expressed relative to the standardized baseline of 100%, for example, 0.1% BCR-ABL1 (IS) is a 3-log reduction equating to a major molecular response (MMR).[7] IS reporting by individual laboratories of BCR-ABL1 RT-qPCR is dependent on a predetermined conversion factor, generally derived from sample exchange with reference laboratories. While IS development removed many variables leading to inter-laboratory discrepancy, other factors exist. Utility of different control genes (i.e., *ABL* vs. *GUSB*) may influence the consistency of the conversion factor at high BCR-ABL transcript levels.[7] The sample quality is crucial in ensuring accuracy and specimens should be processed rapidly to minimize RNA degradation.[8] Appropriate quality assurance should be undertaken to maximize accuracy and precision, while testing should be performed in replicate to minimize error.[8]

Case 1
A 52-year-old man presented with marked leucocytosis with associated neutrophilia, eosinophilia, basophilia with a myelocyte peak. Bone marrow biopsy confirmed CP-CML with karyotype analysis demonstrating the Philadelphia chromosome. Molecular studies of peripheral blood revealed a BCR-ABL1 RT-qPCR result of 90% (IS). Sokal score was high and he was commenced on nilotinib therapy at 300 mg twice daily. Complete hematological response was achieved within 6 weeks of therapy. BCR-ABL1 values at 1 and 3 months following nilotinib commencement were 80% and 25% respectively. Is this response adequate? If not, should this patient be considered for a switch in TKI therapy?

Early responses (0-18 months)

Response to TKI therapy is an important prognostic measurement, irrespective of which TKI is commenced in the first-line setting. The importance of these time dependent milestones (Table 1) has been highlighted by several clinical trials and have been incorporated in global guidelines such as those published by the European LeukemiaNet (ELN)[9], National Comprehensive Cancer Network (NCCN)[10] and European Society of Medical Oncology (ESMO).[11] Response criteria have been divided into 3 categories with "optimal" responses associated with the best long-term outcomes. Patients falling into the "failure" category should, with rare exception, switch therapy to minimize risk of disease progression and death. However, in some cases, a switch may not be appropriate for instance if poor adherence or drug interruption has contributed to the poor response, although differentiating non-compliance from TKI resistance may not be straightforward. Mutation analysis should be performed in "failure" patients as *BCR-ABL1* kinase domain (KD) mutations are the primary known cause of TKI resistance.[12] However, controversy remains about patients falling into the "warning" category, previously termed "suboptimal", particular those falling into the warning category at early time points.

Table 1: Critical milestones in first line CML therapy as defined by ELN[9], NCCN[10] and ESMO[11]

Month		**Optimal response**	**Warning**	**Failure**
3 months	ELN	BCR-ABL1 ≤10% +/- Ph+ ≤35%	BCR-ABL1 >10% +/- Ph+ 36-95%	No CHR or Ph+ >95%
	NCCN	BCR-ABL1 ≤10%	BCR-ABL1 >10%	NA
	ESMO	BCR-ABL1 <10% +/- Ph+ ≤35%	BCR-ABL1 >10% +/- Ph+ 36-95%	No CHR or Ph+ >95%
6 months	ELN	BCR-ABL1 <1% +/- Ph+ 0	BCR-ABL1 1-10% +/- Ph+ 1-35%	BCR-ABL1 >10% +/- Ph+ >35%
	NCCN	BCR-ABL1 ≤10%	NA	BCR-ABL1 >10%
	ESMO	BCR-ABL1 <1% +/- Ph+ 0%	BCR-ABL1 1-10% +/- Ph+ 1-35%	BCR-ABL1 >10% or Ph+ >35%
12 months	ELN	BCR-ABL1 ≤0.1%	BCR-ABL1 >0.1-1%	BCR-ABL1 >1% +/- Ph+ >0
	NCCN	BCR-ABL1 <1%	BCR-ABL1 1-10%	BCR-ABL1 >10%
	ESMO	BCR-ABL1 <0.1%	BCR-ABL1 0.1-1%	BCR-ABL1 >1% +/- Ph+ ≥1
18 months	ESMO	BCR-ABL1 <0.01%	BCR-ABL1 0.1-1%	NA
At any time >12 months	ELN	BCR-ABL1 ≤0.1%	CCA/Ph- (-7 or 7q-)	Loss of CHR Loss of CCyR Confirmed loss of MMR* Mutations CCA/Ph+
	NCCN	BCR-ABL1 <0.1%	BCR-ABL1 0.1-1%	BCR-ABL1 >1%
Clinical considerations[9, 10]		Monitor response and side effects. No change to patient therapy	Evaluate for patient compliance and drug interactions.	Evaluate for patient compliance and drug interactions.

		Consider mutation analysis	Consider mutation analysis. Recommend TKI switch if no compliance issues or drug interactions identified

Ph+: Philadelphia positive cells, CHR: Complete hematological response, NA: Not applicable, CCA/Ph-: Clonal chromosome abnormalities in Ph- cells, CCyR: Complete cytogenetic response, MMR: Major molecular response (BCR-ABL1 ≤0.1%), CCA/Ph+: Clonal chromosome abnormalities in Ph+ cells

All *BCR-ABL1* values expressed using International Scale (IS)

* In 2 consecutive tests, one of which BCR-ABL1 transcripts ≥1%

Early molecular response (EMR)

Differentiation of patients with poor outcomes from the majority with optimal responses has been a major area of research. The prognostic implication of early predictors such as EMR, traditionally defined as *BCR-ABL1* ≤10% (IS) 3 months following TKI commencement, has been demonstrated by numerous trials. This molecular threshold, developed in parallel by Hanfstein *et al.*[13] and Marin *et al.*,[14] was established as being predictive of superior overall survival (OS), progression-free survival (PFS), CCyR and molecular responses in patients treated with frontline imatinib. As a predictor for future eligibility for TFR, patients who fail to achieve EMR have the poorest achievement of stable MR4.5 (*BCR-ABL1* ≤0.0032% (IS)) following 8 years of imatinib therapy in an Australian series of 423 patients (2/84 patients; 2%), highlighting the link between EMR and TFR eligibility.[15]

The value of EMR has also been established in patients treated with second-generation TKIs with data from the 5-year update from ENESTnd revealing that EMR achievement was associated with superior rates of MR4.5 at 5 years compared to those with EMR failure.[16] Survival measurements (OS, PFS) were also superior in patients successfully achieving EMR.[16] Analysis of the dasatinib cohort of the SPIRIT 2 study demonstrated that patients failing to achieve EMR had significantly poorer achievement of CCyR, MMR and MR4.5 by 24 months.[14] In the 5-year update of the DASISION study, patients achieving EMR had a significantly higher likelihood of reaching molecular targets, including MR4.5, with lower transformation rates and superior survival outcomes.[17]

However, while achieving EMR clearly is an important predictor of outcome, insufficient evidence exists to mandate a switch of therapy based on this single result as some patients who fail to achieve EMR still reach optimal outcomes.[18] The NCCN recommends interpreting the 3-month BCR-ABL1 value with caution in patients with values only slightly >10%, especially if there has been a rapid decline from baseline.[10] Combining the 3 and 6-month disease reassessments may further assist in differentiating patients[19, 20] but may also delay an effective switch to more potent therapy in some cases.

Another differentiator for patients failing to achieve EMR is the kinetics of *BCR-ABL1* decline. Branford *et al*. described a halving time of <76 days at 3 months in patients not achieving EMR was associated with superior outcomes compared to those with slower rates of decline (OS 95% vs. 58%, PFS 92% vs. 63%, FFS 59% vs. 6%, MMR 54% vs. 5% respectively).[18] A time-specific differentiator was not identified in the CML IV study but OS and PFS were superior in patients with a minimum of half-log reduction in BCR-ABL1 values between baseline and 3 months.[21] While EMR remains an important early landmark for clinicians, there is a paucity of consensus agreement regarding TKI switch in the event of EMR failure.

Case 2
A 34-year-old lady, diagnosed with CML 6 years ago, is considering pregnancy. Her Sokal score at diagnosis was low and she was treated with first-line imatinib 400 mg daily with reasonable tolerance. She achieved and maintained MMR after 18 months of imatinib, but has not yet been able to achieve sustained deeper responses, despite excellent medication compliance. She would like to discuss TFR and her likelihood of achieving this. What is your advice?

Timing of major molecular response (MMR) achievement

While the ELN and ESMO have classified MMR achievement at 12 months as optimal, the NCCN diverges from this recommendation by accepting a molecular response <1% at this time point. Conflicting data contributes to the lack of clarity regarding the timing of MMR achievement. Long term follow-up from the IRIS study determined that imatinib-treated patients with MMR achievement at 12 months had superior 10-year OS (91.1% vs. 85.3%) with fewer CML-related deaths (97.8% vs. 89.4%) compared to those failing this target.[22] Similarly, the 10-year follow-up of the CML IV study demonstrated MMR achievement at 12 months had statistically significant positive differences in OS, PFS, MR4 (BCR-ABL1 ≤0.01% (IS)) and MR4.5 attainment.[23]

While the 3-year follow-up of the DASISION study demonstrated that patients with MMR or CCyR achievement at 12 months had significantly better PFS compared with those who did not, this was not statistically significant.[24] Furthermore, achievement of CCyR at 12 months was predictive of OS irrespective of MMR status.[24] Similar findings were established by Marin *et al.* in a cohort of 224 CP CML patients treated at a single institution where CCyR achievement at 12 months conferred a benefit in 5-year OS and PFS whereas MMR failed to demonstrate any statistically significant difference.[25] Evaluation by Jabbour *et al.* of 167 patients treated with second-generation TKIs in CP CML revealed that event free survival (EFS) was similar in patients achieving CCyR by 12 months, regardless of molecular response.[26] Further analysis of the CML IV study cohort validated the landmark of 12-15 months for achievement of a BCR-ABL1 value of 1% while therapy failure should be considered if patients have failed to achieve MMR by a cut-off of 2.5 years, perhaps providing a deadline for when to consider TKI switch for patients persistently in the warning zone.[27]

While it is unclear whether MMR achievement by 12 months affects survival, early achievement of MMR does predict subsequent achievement of MR4.5, important for

treatment discontinuation consideration. Utilizing data from the CML IV study, Hehlmann *et al.* demonstrated that MMR achievement at 3, 6, 12 and 18 months had a 5-year cumulative incidence of MR4.5 of 83.3%, 69.2%, 56.7% and 51.1% respectively (Table 2).[28] Comparatively, a BCR-ABL1 value of 0.1 – 1% at 12 and 18 months had a 5-year cumulative incidence of MR4.5 of only 14.5% and 6% respectively.[28] Similar results were observed by the Australian group, emphasizing the relationship between the rate of MR4.5 and early MMR achievement (Table 2).[15] Early MMR achievement was also conducive to maintaining stable MR4.5 (i.e., sustained MR4.5 for 2 years), important for TFR success.[15]

Table 2: Cumulative incidence of MR4.5 depending on time point specific molecular responses

	Month	**Molecular response**			
		< 0.1%	**0.1-1%**	**1-10%**	**>10%**
German CML IV results **5-year cumulative incidence of MR4.5[28]**	3	83.3%	52.9%	30.8%	22.1%
	6	69.2%	28.8%	21.9%	20.1%
	12	56.7%	14.5%	15.4%	21.6%
	18	51.1%	6%	7.7%	22.9%
Australian Data **8-year cumulative incidence of stable MR4.5[15]**	3	78.2%	52.7%	29%	8.6%

Frequency of molecular monitoring

While *BCR-ABL1* RT-qPCR is not necessarily mandated at baseline as subsequent quantification is aligned with the IS, the rate of *BCR-ABL1* decline is useful for prognostication.[18, 21] Consequently, we recommend *BCR-ABL1* quantification at baseline for all patients. Following TKI initiation, 3-monthly *BCR-ABL1* RT-qPCR testing is generally recommended until stable MMR is achieved; followed by 3-6 monthly testing thereafter.[9, 11] More frequent molecular monitoring (3-4 tests per year) has been associated with increased TKI compliance, suggesting 3-monthly *BCR-ABL1* testing might encourage improved medication adherence.[29] Our recommendation is to maintain a 3-monthly testing schedule where possible especially in select populations: (1) Patients with compliance concerns despite the achievement of MMR, (2) Patients being considered for future TFR attempts to ensure sustained DMR and (3) patients switching to a new TKI. We recommend monthly monitoring in specific scenarios: (1) Female patients who have ceased TKI for pregnancy, (2) Patients entering a TFR attempt for the first 6 months following TKI discontinuation and (3) Patients attempting TFR who are beyond 6 months and have lost MR4.5 but maintain MMR.

Transcript type

The transcript generated from the *BCR-ABL* fusion gene is dependent on the site of breakage in *BCR*. Numerous studies have attempted to differentiate the effect of the common transcripts (b2a2 or e13a2; b3a2 or e14a2) on long-term outcomes. Although

patients with b3a2 transcripts (alone or in combination with b2a2) achieve ELN-defined milestones more rapidly and have superior EFS,[30] patients with b2a2 still have excellent outcomes. Whilst the majority of patients have common transcripts, approximately 5% have atypical transcripts which has implications for monitoring and long-term therapeutic decisions.[2] The majority of laboratories cannot quantify atypical transcripts and so response assessments based on the pre-specified milestones are difficult to apply. Cytogenetic assessments are useful until CCyR is achieved; at subsequent time points qualitative PCR may be utilized. By comparing the intensity of the PCR amplicon on gel electrophoresis to the sample's low-level controls, an approximate value may be provided. TFR should generally be avoided in this setting due to an inability to accurately monitor transcript levels.

Mutation analysis

KD mutations are the best described mechanism of TKI resistance and mutation screening should be performed in patients failing TKI therapy. Detection of a KD mutation should direct subsequent TKI selection based on known resistance patterns to available TKIs.[31, 32] Failure to achieve milestones or loss of response should trigger investigation for mutations. Direct (Sanger) sequencing is the currently accepted technique for investigation of KD mutations but has relatively low sensitivity and can only detect mutations present in samples with ≥10-20% of Philadelphia positive cells, assuming the BCR-ABL1 value is ≥1%.[33] This level of detection is generally adequate in characterizing the relevant mutation due to the rapid expansion of the resistant clone leading to a rise in BCR-ABL1 transcripts. Mutation analysis should be performed prior to stopping the TKI that has failed to maximize detection success. This is because, once the TKI is stopped, mutations can rapidly become difficult to detect. More sensitive techniques such as mass spectrometry, digital droplet PCR and next generation sequencing can improve the detection limit to ~0.2%, but these techniques are not widely available.[33] Presence of multiple low-level mutations in patients with proven TKI failure has been predictive of poorer outcomes in the setting of inappropriate TKI selection.[34, 35] However, the significance of low-level mutations at diagnosis or in the absence of suspected resistance is unknown and testing is not indicated outside of a clinical trial. KD domain mutations are only identified in about half of the patients with imatinib failure and patients do not always respond to the appropriately selected agent; indicating the presence of other resistance pathways in many cases.

Deep molecular response (DMR)

The duration and depth of molecular response has become synonymous with TFR eligibility. The term "complete molecular response", implying undetectable BCR-ABL1 transcripts, has largely been abandoned due to significant variability in assay and sample sensitivity. "Deep molecular response" is the preferred expression and the various definitions used to represent DMR describe the depth of response relative to the standardized baseline (e.g., MR4 is ≥4-log reduction or ≤0.01% BCR-ABL1).[4]

Selection of more potent kinase inhibitors upfront can accelerate achievement of DMR and increase the percentage of patients who achieve it. The 5-year follow-up of ENESTnd demonstrated that 31% of the imatinib-treated group achieved MR4.5 while 54% and

52% of the 2 nilotinib dosing arms reached MR4.5 by this time point.[16] Similar results were observed at the 5-year update of DASISION where dasatinib treatment was associated with higher rates of MR4.5 compared to imatinib (42% vs. 33%).[17] High dose imatinib (i.e., 800 mg daily as opposed to 400 mg daily) demonstrates comparable rates of MR4.5 to second-generation TKIs although the higher dose is associated with more adverse events and is rarely used today.[36] Regardless, TKI selection for DMR achievement should be tempered with toxicity consideration as each drug has a relatively unique adverse effect profile. Aiming for MMR or CCyR as opposed to DMR in patients who don't regard TFR as a high priority (i.e., the elderly, patients with significant comorbidities) is appropriate and therapy selection can be tailored to reflect this.

Switching to second-line therapy due to failure to achieve DMR with frontline imatinib has been proven to be a successful strategy. The 48-month analysis of ENESTcmr demonstrated that for patients treated with long-term imatinib with detectable molecular disease despite reaching CCyR, switching to nilotinib significantly increased the likelihood of MR4.5 achievement.[37] For imatinib-treated patients where the best response has been CCyR but not MMR, switching to nilotinib can also induce MR4.5 whereas patients randomized to ongoing imatinib had little prospect of achieving MR4.5.[37] While robust data are not yet available for second-line dasatinib, switching to dasatinib following imatinib intolerance or failure allowed for MR4 achievement and eventual successful TFR attempts.[38]

Case 3
A 50-year old man, treated for CML with upfront imatinib at 400 mg daily for the past 14 years, stopped imatinib as part of a supervised TFR attempt. At diagnosis, his Sokal risk score was intermediate. He achieved EMR by 3 months and MMR by 12 months. He struggled with severe fatigue and gastrointestinal toxicity secondary to imatinib which was a significant motivator for the TFR attempt. He eventually achieved MR4.5 after 12 years. After maintaining MR4.5 for 24 months he ceased imatinib in a TFR attempt. Within 3 months he had lost MMR, requiring TKI restart. By the time he restarted imatinib, he had lost CCyR. CCyR was rapidly regained following imatinib recommencement but he had not yet achieved MMR after 6 months of treatment. Is this an appropriate response? What should your approach be?

Treatment free remission (TFR)

Several trials have demonstrated the safety and viability of TFR in selected patients and TFR is now incorporated into global guidelines (Table 3).[9, 10] TFR success has a number of health and economic implications, including eliminating the morbidity associated with TKI toxicity and a reduction in therapy costs for either the patient, the drug funder, or both. The potential savings is illustrated by the drug costs saved in the Euro-Ski study, which were estimated at €22 million.[39] Successful TFR has been achieved in ~40-60% of patients enrolled in the various stopping studies and most patients rapidly regain molecular control if TKI recommencement is required. Achieving and maintaining DMR is currently the fundamental requirement for TFR eligibility. The French STIM1, the first of the 2 pivotal stopping studies, ceased imatinib in 100 patients in sustained MR5 for ≥2 years with the trigger to TKI recommencement being ≥1-log increase in BCR-ABL1

transcripts or loss of MMR. At 6 months, the rate of TFR was 43% but this fell to 38% by 60 months.[40] Similar results were observed in the Australian TWISTER study, where the entry criteria was ≥2 years of undetectable BCR-ABL transcripts with comparable retreatment criteria to STIM1; the 24 month successful TFR rate was 47%.[41]

Table 3: Summary of selected larger clinical trials of treatment-free remission relative to depth and duration of deep molecular response

Clinical study	Patient number	TKI at time of cessation	Depth of DMR	Duration of DMR	TKI restart criteria	TFR success (follow-up)
STIM1[40]	100	Imatinib	UMRD	≥2 years	Loss of MMR or ≥1-log increase in BCR-ABL1	38% (60 months)
TWISTER[41]	40	Imatinib	UMRD	≥2 years	Loss of MMR or rising BCR-ABL1 on 2 consecutive tests	47.1% (24 months)
KID[42]	90	Imatinib	UMRD	≥2 years	Loss of UMRD on 2 consecutive tests of MMR loss	58.5% (24 months)
STOP 2G-TKI[43]	60	Dasatinib or nilotinib	UMRD	≥2 years	Loss of MMR	53.57% (48 months)
A-STIM[44]	80	Imatinib	UMRD (occasional weakly positive samples also eligible)	≥2 years	Loss of MMR	61% (36 months)
ENESTFreedom[23]	190	First line Nilotinib	MR4.5	≥1 year	Loss of MMR	51.6% (48 weeks)
ENESTop[45]	126	Second line nilotinib after imatinib	MR4.5	≥1 year	Loss of MMR or confirmed loss of MR4	53% (96 weeks)

ISAV[3]	112	Imatinib	MR4-MR4.5	≥18 months	Loss of MMR	48.1% (21.6 months)
DADI[38]	63	Second line dasatinib	MR4 or 0.0069% (IS)	≥1 year	Loss of DMR	44% (36 months)
D-STOP[46]	54	Dasatinib	MR4	≥1 year	BCR-ABL1 >0.0069% on 2 consecutive results	57% (24 months)
EURO-SKI[39]	755	Imatinib>nilotinib/dasatinib	MR4	≥1 year	Loss of MMR	50% (24 months)
Interim results						
STIM2 (interim)[47]	124	Imatinib	UMRD	≥2 years	Loss of MMR or ≥1-log increase in BCR-ABL1	59% (12 months)
DASFREE (interim)[48]	84	Dasatinib (first or second-line)	MR4.5	≥1 year	Loss of MMR	49% (12 months)
DESTINY[49]	117	Imatinib> dasatinib/nilotinib	MR4	≥1 year	Loss of MMR	77% (24 months)

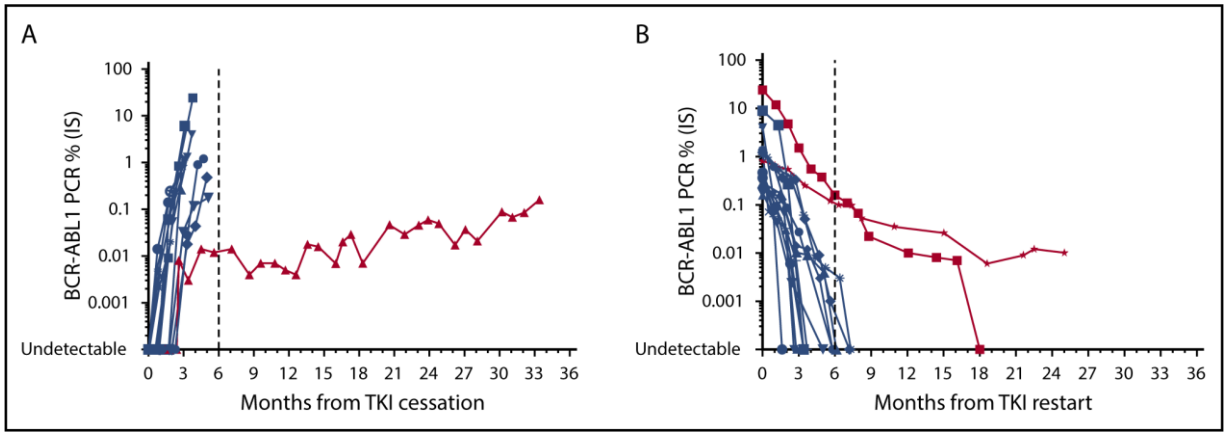
UMRD: Undetectable molecular residual disease

Subsequent stopping studies have had less stringent enrolment and restarting criteria. A-STIM, accepting loss of MMR as the trigger to TKI recommencement, demonstrated a TFR rate of 61% at 36 months; establishing MMR as a safe threshold for TKI recommencement.[44] Euro-Ski, the largest cessation study to date, allowed patients with MR4 for ≥1 year to cease TKI. At 24 months, 50% of patients attempting TFR remained in MMR off TKI.[39] Discontinuation of second-generation TKIs provide similar rates of success.[23, 38, 43] While the optimal depth and duration of DMR prior to TKI cessation is unclear, there is evidence to suggest ≥24 months of sustained DMR is associated with superior TFR success.[50]

Molecular monitoring in TFR

Monitoring in the setting of DMR establishment and TFR should employ high quality standardized RT-qPCR[51] (detection limit of at minimum MR4.5 if possible). The limit of detection of the method utilised needs to be clarified because "PCR negativity" in a laboratory with a poor detection limit may lead to inappropriate TFR attempts. Cessation studies have predominantly incorporated monthly *BCR-ABL1* testing for the first 6-12 months followed by 3-monthly testing thereafter. The timing of molecular relapse following TKI discontinuation is mainly within the first 6 months (Figure 1); reinforcing the requirement for the intensity of monitoring during this timeframe. Beyond 6 months, less frequent monitoring (i.e., 2-3 monthly) could follow as late molecular relapses are infrequent. However, monthly monitoring should ensue in the event of rising BCR-ABL1 transcripts. Loss of MR4.5 three months after TKI cessation has been shown to predict TFR failure[43] although there is a small subset of patients who lose DMR but maintain MMR with fluctuating BCR-ABL1 transcript levels (Figure 1). While late relapses are infrequent, there are reports of patients losing MMR and requiring TKI recommencement >36 months post-drug cessation.[43] This demonstrates the importance of continued molecular monitoring every 3-6 months over the long term despite apparent TFR success.

Figure 1: Molecular profile of 15 patients at our institution attempting and failing TFR. (A) Molecular relapse following TKI cessation; (B) Molecular response following TKI recommencement. Blue: Typical molecular responses showing rapid rise or decline; Red: Atypical molecular response showing slow rise or decline



TKI recommencement in TFR failure

In the event of molecular relapse and MMR loss, we recommend 1-2 monthly *BCR-ABL1* testing until MMR has been re-established. In nearly all cases, patients recommencing TKI following TFR failure remain sensitive to TKI therapy with rare reports of KD mutation development.[23, 45] There has been a single report of progression to lymphoid blast crisis following TFR failure, 9 months after imatinib recommencement despite regaining MMR.[44] However the majority of patients rapidly regain MMR and DMR. Median time to both targets was 2 and 3 months respectively in the interim analysis of STOP 2G-TKI.[43] Comparable data has been generated from other stopping studies[23, 39, 40, 42, 44, 52] demonstrating that while most patients rapidly regain MMR and DMR there remains a small subset of patients who fail to do so (Figure 1). Failure to regain MMR within 6-12 months of treatment initiation should prompt KD mutation analysis and patients could appropriately be considered for a switch in TKI therapy.

Predictors of TFR success

Duration of TKI exposure prior to TFR has been linked to TFR success. Multivariate analysis of the STIM study revealed that imatinib treatment of >54 months was associated with lower risk of detectable BCR-ABL1 transcripts following TKI cessation[40] with similar results observed in other clinical trials.[39, 42, 45] History of imatinib resistance in patients who have attempted TFR while on second-generation TKIs has also been associated with an increased rate of molecular relapse.[38, 43] Prognostic models, such as the Sokal risk score, developed in the pre-TKI era, remain useful as an indicator of the underlying biological characteristics at diagnosis and have been utilized to predict TFR success; low Sokal risk being associated with reduced likelihood of molecular relapse compared with those deemed high risk[40, 41] but this has not been observed in all studies.[39, 46]

Longer duration of DMR has been correlated with TFR success in both the Euro-Ski (>3.1 years[39]) and KID studies (>36 months[42]) although this has not been replicated in other cessation trials.[23, 46] Furthermore in Euro-Ski, each additional year of DMR was associated with a 3% increase in the probability of remaining in MMR at 6 months in Euro-Ski.[39] Stability of DMR prior to TKI cessation may be an important variable in TFR success but limited published data exist to date.[44, 53] Although no individual variable has been consistently predictive of TFR success, they all need to be considered prior to TFR attempts and patients need to be counselled about their risk of molecular relapse prior to ceasing TKI.

Pitfalls associated with TFR

Careful patient selection is vital in maximizing TFR success but also minimizing risk of adverse outcomes. Inappropriate cessation in patients with atypical transcripts can result in failure to detect molecular relapse. Inadvertent monitoring of the incorrect transcript can delay detection of relapse until hematological relapse.[45] TFR attempts require reliable laboratory facilities able to provide rapid *BCR-ABL1* results to ensure clinicians can promptly intervene in cases of molecular relapse. Patient compliance remains vital as adherence to the proposed monitoring schedule will minimize delayed

interventions. Systems should exist to identify patients failing to comply with the monitoring regime and to ensure timely clinician review of each *BCR-ABL1* result.[51]

Pregnancy

Patients and their families need to be counselled against conception while on TKI therapy and fertility preservation should be discussed with those of child-bearing age prior to TKI commencement. Due to risk of birth deformities and miscarriage, TKIs should be ceased before conception and not recommenced until after delivery. The timing of pregnancy should ideally be when the patient is eligible for a TFR attempt (i.e., reached and maintained DMR for at least 12 months) but delaying TKI cessation to achieve this may not always be possible. The risk of disease progression and the importance of molecular monitoring during pregnancy needs to be extensively discussed. We recommend monthly monitoring in pregnant patients and once the BCR-ABL1 value approaches 1%, alfa interferon can be utilized until delivery has occurred. TKI can be promptly recommenced after delivery as long as breast feeding is avoided.

Case discussion

Case 1

It was decided to persist with nilotinib rather than switch therapy. This was based on the significant fall in BCR-ABL level over the first 3 months, and the fact that he was already on a potent TKI. Given identical molecular results in the context of imatinib therapy, we would have elected to switch to a second-generation TKI.

This patient continued on nilotinib 300 mg twice daily and at 6 months, his BCR-ABL1 value was 14% (IS). Having failed nilotinib therapy, the options were to switch to another second-generation TKI, to switch to ponatinib, or to proceed with an allogeneic transplant. Based on the low rate of response to a second-generation TKI after failure of another second-generation TKI[54], ponatinib was the preferred choice. However he was not eligible for ponatinib under the local funding scheme so he was switched to dasatinib. He had minimal response to dasatinib, and within 3 months his BCR-ABL1 increased to 53% (IS). Mutation analysis revealed that the pan-TKI resistant T315I mutation had developed and the patient was switched to ponatinib with an allograft planned. Ponatinib failed to achieve a better response and 3 months later, the patient proceeded to a sibling-matched allogeneic stem cell transplant following which his BCR-ABL1 transcripts became undetectable. This patient is a rare example of primary resistance to all TKI therapy in CML and regular monitoring was key in identifying failure of milestone achievement, mutation development and vital in early recognition that allogeneic transplantation was the only option for this patient.

Case 2

This patient, although having achieved MMR by 18 months, would like to achieve TFR before contemplating pregnancy. Data presented above demonstrates that this patient is unlikely to achieve DMR on imatinib within a reasonable time.[15, 28] Since safe drug cessation is paramount and her time-frame is relatively short, either nilotinib or

dasatinib would be a better option for her, with current evidence demonstrating TFR success with second-line second-generation TKI.[23, 43, 45] This patient was switched to nilotinib and rapidly achieved MR4.5. She was able to attempt TFR within 2.5 years and has remained off therapy for over 12 months.

Case 3

While the majority of patients regain MMR and DMR rapidly, there remains a small subset who have delayed response to TKI recommencement, which this case illustrates. The approach here could be to either persist with the current TKI, after ensuring no KD mutation has developed, or to switch to a different TKI, especially if a second TFR attempt is being considered. Given his initial very slow decline in BCR-ABL after restarting TKI, it was decided to switch to a more potent agent. This patient had no evidence of KD mutations and was switched to dasatinib 100 mg daily. He achieved MMR and MR4.5 within 3 months and has remained in MR4.5 for >2 years.

Conclusion

Because of the abundance of therapies available for patients with CML and the excellent outcomes achieved in most cases, there is a danger that CML may be regarded as an easy disease to treat, even by the non-expert. This attitude can leave the high-risk and complex patient vulnerable to inadequate attention to toxicity issues, delayed therapeutic responses resulting in non-optimal management and inferior outcomes. To ensure outcomes are optimised, regular high-quality molecular monitoring is absolutely vital in maximizing the number of patients who meet milestones that minimize the risk of progression and maximize the opportunity for TFR. Early detection of rising BCR-ABL1 values or failure to achieve pre-determined milestones should trigger a thorough evaluation regarding compliance. KD mutation analysis may be required and if patients fail therapy, a switch to a more potent TKI is required. The current awareness of TFR as a safe and feasible option has resulted in a shift in therapeutic goals for many patients; introducing the potential for an “operational cure” for CML. Achievement of sustained DMR correlates with TFR success and maximizing DMR through appropriate therapy selection has become an increasingly important therapeutic target.

Conflict of interest disclosure

N.S. has received honoraria from Novartis and Bristol-Myers Squibb, travel and accommodation expenses from Novartis and Janssen. T.P.H. holds a consultancy role and has received research funding and honoraria from Novartis, Bristol-Myers Squibb and Ariad

Authorship

This paper was written and reviewed by both authors.

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Appendix C

Multiplex technologies for the assessment of minimal residual disease and low-level mutation detection in leukaemia: Mass spectrometry vs. next generation sequencing

Shanmuganathan N, Branford S. Multiplex technologies for the assessment of minimal residual disease and low-level mutation detection in leukaemia: mass spectrometry versus next-generation sequencing. *British Journal of Haematology*. 2022;196(1):19-30.

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Summary

With the focus of leukaemia management shifting to the implications of low-level disease burden, increasing attention is being paid on the development of highly sensitive methodologies required for detection. There are various techniques capable of identification of minimal residual disease (MRD) either evidencing as relevant mutation detection (e.g. *NPM1* mutation) or trace levels of leukaemic clonal populations. The vast majority of these methods only permit detection of a single clone or mutation. Mass spectrometry and next generation sequencing (NGS), however, enable the interrogation of multiple genes simultaneously, facilitating a more complete genomic profile. In this review, we explore the methodologies of both techniques in conjunction with the important advantages and limitations associated with each assay. We also highlight the evidence and the various instances where either technique has been used and propose future strategies for MRD detection.

Keywords:

Mass spectrometry, next generation sequencing, minimal residual disease, sensitive mutation detection, clonal population

1. Introduction

The rapid evolution of technologies over the last two decades has enabled dramatic improvements in diagnostics, risk prognostication and monitoring of patients with haematological malignancies. The development of highly sensitive testing methods in conjunction with the identification of key unique characteristics of various haematological malignancies, especially leukaemias, has permitted targeted monitoring but also correlation to disease burden, response and in some instances, early relapse. Consequently, patient therapy can now be individually tailored based on precision-based technologies.

Conventional disease monitoring has generally focused on relatively insensitive morphological assessment of disease on peripheral blood or bone marrow, assessing 200-500 cells to a leukaemic detection limit of ~1-5% (i.e. sensitivity of ~1:20 cells).[1, 2] Sanger sequencing, the current gold standard for mutation detection, has a limit of detection of ~10-20% mutant, making this method insensitive for the detection of low-level mutations. Moreover, increasing evidence is accumulating regarding the importance of detection of residual leukaemic disease beyond the 5% threshold identifiable on morphological assessment. Defining minimal residual disease, or preferentially termed measurable residual disease (MRD), refers to the detection of leukaemic disease at a level of a single cell in 10,000-1,000,000 white blood cells, which is beyond the sensitivity of cytomorphology. Accumulating evidence regarding MRD detection has enabled risk adaptive approaches in leukaemias[3, 4] with the earliest proof being demonstrated in chronic myeloid leukaemia (CML).[5] More recently, MRD detection has enabled therapy modification in acute lymphoblastic leukaemia (ALL)[6] and acute myeloid leukaemia (AML) while also influencing survival prediction and relapse outcomes.[7] Furthermore, sustained MRD negativity also permits the selection of patients to attempt a trial of therapy discontinuation termed 'treatment-free remission' in CML. Re-emergence of *BCR-ABL1*-positive leukaemic cells beyond a pre-defined threshold triggers therapy recommencement.[8, 9]

1.1. Current methods of MRD detection

There are multiple techniques available for MRD assessments such as polymerase chain reaction (PCR) based methods utilised for quantitation of either fusion gene transcripts or commonly mutated genes (*NPM1* frameshift mutations in AML) or detection of individual patient specific molecular markers (immunoglobulin/T-cell receptor gene rearrangements in ALL).

Commonly occurring fusion transcripts in leukaemia include *BCR-ABL1*, which is observed in CML and 25-30% of B-ALL, the disease defining *PML-RARα* in acute promyelocytic leukaemia and *ETV6-RUNX1* which occurs in 25-30% of childhood ALL. In addition to being diagnostic, detection of these fusions can enable targeted therapies (e.g. *BCR-ABL1* tyrosine kinase inhibitors) but also inform regarding prognosis and should be sought for at diagnosis. The genomic breakpoints of these events are varied and can encompass large intronic regions, which is difficult to span with DNA PCR. Therefore, RNA fusion transcript detection of specific exon junctions using reverse transcription PCR (RT-PCR) is the preferred and simpler fusion transcript detection technique. Highly sensitive primer sets can be optimized for all patients

with the same fusion to a level of 10^{-4}-10^{-6}.[10] Quantification of amplified products can be achieved using real-time PCR and comparison to a standard curve derived from a dilution series of a positive control.[11-14]

Some PCR techniques are highly individualised necessitating development of allele specific oligonucleotide (ASO) primers to identify specific mutations, which although highly sensitive, are also labour intensive and therefore costly[15] Normal lymphocyte development is dependent on somatic rearrangement of the immunoglobulin and T-cell receptor genes resulting in individualised sequences exclusive to each lymphocyte. In lymphoid leukaemia, the neoplastic process results from expansion of a dominant clonal population with a unique rearranged immunoglobulin/T cell receptor sequence. Detection of the immunoglobulin/T cell receptor rearrangement of the malignant clone exploits the unique re-assembly process, enabling development of a highly sensitive monitoring method. Clonal PCR fragments[16] are obtained from PCR amplified diagnostic genomic DNA prior to Sanger sequencing to derive individualised ASO primers. These primers enable target enrichment through the specific design of the ASO primers, frequently due to single base mismatches at the terminal 3' residue of the PCR primers, which cannot be corrected by DNA polymerase. Thus, amplification will only occur in genomic DNA exactly complementary to the ASO primer, excluding all other sequences. MRD monitoring is generally performed by real-time quantitative PCR with the amplification conditions and sensitivity of the assay established on serial dilutions of diagnostic material, which enables a detection limit of 1 leukaemic cell in 100,000 normal lymphoid cells.[15] This method is able to generate at least one sensitive molecular probe suitable for MRD analysis in >90% of ALL cases.[6, 17] Sensitivity is hinged upon mutant enrichment by the specific design of the ASO primers.

More recently, the development of digital droplet PCR (ddPCR), a modern reiteration of the conventional PCR based assay, has introduced a more sensitive method of monitoring relevant mutations compared with Sanger sequencing. The methodology introduces sample partitioning into droplets prior to quantification of targeted mutations.[18, 19] The added sensitivity of ddPCR compared with conventional quantitative PCR techniques is due to individual assessment of the target sequence amplification in each partitioned droplet, which eliminates the amplified differences in reaction efficiency in quantitative PCR and improves the false positive rate of MRD detection.[18, 20, 21] While studies have demonstrated the equivalence of ddPCR measurements with quantitative PCR for MRD detection in lymphoid malignancies,[22, 23] no established guidelines yet exist regarding the analysis and interpretation of ddPCR derived MRD values and is currently being standardized across a number of clinical trials.[24] Moreover, ddPCR is time consuming and costly as each mutation requires individual design and validation of customised primers. Consequently, this assay is ideal for MRD monitoring for patients with recurrently observed point mutations,[25] such as *IDH1* and *IDH2* in AML, but will be technically demanding if individualized assay design is required for less common variants.

Multi-parametric flow cytometry (MFC) is another tool utilized to assess MRD in leukaemia,[2, 26] measuring antigenic epitopes expressed on either cell membranes or in the nucleus or cytoplasm. Antigenic expression evolves through cell development with unique profiles associated with different cell lineages and stage of maturation. MFC relies on antibody-

fluorochrome conjugates binding to the complementary antigen on individual cells prior to laser interrogation of a single cell stream. Unique colours are emitted by the conjugated fluorochromes as they pass through the laser, signaling that the corresponding antigen is present. The minimum MRD quantification threshold is generally defined as 0.1%, equating to the detection of 1 leukaemic cell in 10,000 cells.[7] This technique can generate results within hours, which is a substantial benefit compared to many molecular techniques but is conditional on rapid analysis following sample collection to avoid cellular degradation.

While these techniques are frequently used for MRD detection and monitoring in leukaemia, it is difficult to multiplex these reactions. Mass spectrometry and next generation sequencing (NGS) are two techniques that are able to be multiplexed, enabling high-throughput testing of multiple variants and patient samples simultaneously in addition to sensitive analysis for pre-defined targets. In this review we discuss and contrast these methods of mutant detection.

2. Mass spectrometry

Developing a method that had higher level of sensitivity as standard techniques but enabled multiplexed mutation detection was a vital diagnostic tool, especially for detection of low-frequency mutations. Mass spectrometry allows the detection of multiple clinically relevant variants in a single workflow. The principle of mass spectrometry is based on single base extension of a primer at a variant site and measurement of mass-to-charge ratio. This allows interrogation of multiple variants within a single reaction. This method is ideal for mutations involving single nucleotide variants (SNVs) but less applicable for small insertions and deletions (indels) or structural variants (SVs) including fusions.

2.1. Methodology

The MassARRAY® System (Agena Bioscience) is a mass spectrometer for variant detection. Isolated genomic material (either DNA or cDNA) will undergo PCR amplification spanning the region of the targeted mutations using forward and reverse primers. Prior to primer extension, dephosphorylation of excess deoxynucleotides left over from the prior reaction is mandatory to prevent interference with the subsequent reaction.[27] Individual unique extension primers for each targeted variant are added to the amplified genomic template in addition to customized dideoxynucleotide terminators that only permit single base extension of probes, thus terminating the reaction.[27] The generated products are then transferred to a SpectroCHIP® array prior to interrogation by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyser for data acquisition.[27] Laser irradiation of the SpectroCHIP® ionizes the analytes prior to acceleration of the positively charged molecules through a detector.[28] Molecular separation is detected, which is directly proportional to the mass of the individual molecules. This is converted onto a spectrum and intensity of the reaction is plotted against the analyte mass prior to analysis with custom software. Optimization will be required to ensure adequate concentration and separation of the extension primers and resultant products to minimise error. The limit of detection with this particular method is 5-10%.[27]

Challenges associated with sensitive mutation detection using mass spectrometry include the masking of low-level mutations in the presence of abundant wild-type alleles. Consequently, excluding the wild-type allele is crucial to improving sensitivity. Grouping the mutations into reaction mixtures based on the terminator nucleotide required for single base extension permits the exclusion of the wild-type terminator. Exclusion of the wild-type means that only the mutated base is detected and dramatically improves the limit of detection to 0.05% mutant.[29] Extension primers can be designed to extend in either the forward or the reverse direction with adequate spread of extension product masses to minimize interference on the spectrum. This method, termed SABER or single allele base extension reaction,[29] prevents extension products being generated from unmutated sequences. Exact quantification of the mutant allele will not be possible due to lack of a wild-type comparison. Therefore, inclusion of standards with defined mutant concentrations is necessary for estimation of relative mutant percentage.[29]

Modification to the SABER chemistry enabled development of another sensitive mass spectrometry method, the UltraSEEK®,[30] which has a reported limit of detection of 0.1-5%.[30-32] The major difference to the SABER technique is replacement of the standard terminator with a biotin-labelled terminating nucleotide as well as incorporation of streptavidin-coated magnetic beads for product capture.[30] This is followed by solid phase capture with additional washing steps to eliminate contaminants.[31] The UltraSEEK® assay is also not quantitative since the wild-type allele is purposefully excluded to improve sensitivity and in the absence of mutant dilution standards, mutant estimation will also not be possible.

2.2. Limitations

Performance of the mass spectrometry assay requires substantial sample preparation and processing prior to data acquisition. Appropriate sample controls will need to be generated and run for each test in addition to dilution standards if mutation estimation is to be executed. Mutations to be targeted by the mass spectrometry assay require *a priori* knowledge of frequently occurring mutations of interest within the samples in question in order to design mutant specific extension primers. For example, monitoring of the hot spot point mutations *IDH1* R132H or *IDH2* R140Q/R172K in patients with acute myeloid leukaemia or detection of *FLT3* tyrosine kinase domain (TKD) mutations D835/I836,[33, 34] which confer resistance to *FLT3* inhibitor therapy, could be performed by mass spectrometry. This tool cannot be used for novel mutation discovery and as mentioned earlier, only point mutations are ideal targets for mass spectrometry detection.

2.3. MRD detection by mass spectrometry in leukaemia

2.3.1. Resistance monitoring with mass spectrometry

The best example of mass spectrometry use for MRD analysis is in the setting of CML. *BCR-ABL1* kinase domain (KD) mutations are the best understood mechanism of resistance in CML and each mutation confers variable resistance to *BCR-ABL1* directed tyrosine kinase inhibitors (TKIs).[35] Inappropriate TKI selection in patients with KD mutations conferring resistance to the particular TKI will result in rapid clonal expansion of the KD mutation.[29] Imatinib was the first of the TKIs for patients with CML and more than 70 different mutations within the *BCR-ABL1*

KD confer imatinib resistance.[35] Nilotinib and dasatinib are second-generation inhibitors and are less likely to induce KD mutations.[35] Most of the imatinib-resistant mutants are sensitive to these inhibitors but there is still a limited set of KD mutations that confer resistance.[35] One mutation, T315I, is considered a 'gate-keeper' mutation and confers resistance to imatinib, nilotinib and dasatinib.[36]

We conducted a retrospective study of imatinib-resistant patients (n=220) who were subsequently treated with either nilotinib or dasatinib therapy. Sanger sequencing and mass spectrometry analysis was performed at the time of TKI switch.[29] *BCR-ABL1 KD* mutations (n=169) were detected in 128 of the 220 patients using Sanger sequencing. Thirty-one of these patients had 2 to 4 KD mutations. In comparison, mass spectrometry, which targeted 31 of the most frequently occurring KD mutations, detected 281 mutations in 131 of 220 patients. Multiple KD mutations (up to 10 mutations) were identified in 60 of these 131 patients. Of the 281 KD mutations identified by mass spectrometry, 132 mutations in 64 patients were not detected by Sanger sequencing, secondary to the fact that the mutant abundance was below the limit of detection of Sanger sequencing. Of the 64 patients with these low-level mutations (defined as below the ~10% mutant threshold detectable by Sanger sequencing), 53 also had mutations identifiable by Sanger sequencing and 11 only had low-level KD mutations. Importantly, we identified 25 low-level KD mutations in 22 patients that were known to confer resistance to the subsequent TKI that these patients were treated with. The pan-TKI resistant T315I mutation was identified only by sensitive mass spectrometry at switchover to the subsequent TKI in 12 patients. This resistant mutation subsequently expanded and became detectable by Sanger sequencing in 11 of these 12 patients when they switched to either nilotinib or dasatinib and the patients failed therapy. This was an important finding because nilotinib and dasatinib should not be used for patients with the T315I mutation due to their known lack of efficacy and demonstrated the power of more sensitive mutation detection for appropriate drug selection. Of the remaining 13 low-level mutations, 10 mutations eventually expanded to be detectable by Sanger sequencing and these were mutations known to confer resistance to nilotinib or dasatinib. Only 2/22 (9%) patients with low-level mutations eventually achieved a complete cytogenetic response and the remaining patients either experienced disease progression or had minimal/no cytogenetic response.[29] The incidence of KD mutations detectable at the time of switching to another TKI that would influence subsequent treatment choice was 23% using Sanger sequencing compared with 32% (P=.03) using mass spectrometry. This indicates that the improved sensitivity of mass spectrometry allows more informed treatment decisions for improved outcomes.

We also found that mass spectrometry could identify more patients with multiple *BCR-ABL1* KD mutations (≥2) than Sanger sequencing. These multiple mutations predicted for lower rates of complete cytogenetic response and major molecular response.[37] A major molecular response is represented by a *BCR-ABL1* transcript value of ≤0.1% on an international reporting scale and indicates a safe-haven where patients are essentially protected from disease progression. In a further study, mass spectrometry performed prior to commencement of the third-generation TKI ponatinib in 363 TKI-resistant patients, identified low-level mutations in 15% of patients. Importantly, the gate-keeper mutation T315I is sensitive to inhibition by ponatinib. Unlike our prior observation for nilotinib and dasatinib treated patients, in general there was no impact on response to ponatinib for patients with multiple mutations. However, patients with multiple KD mutations in combination with T315I had a poorer outcome

compared to those with T315I alone. Use of mass spectrometry in patients with CML resistance can be used to refine the therapeutic approach.

2.3.2. Potential targets for MRD monitoring using mass spectrometry

A possible application of mass spectrometry is in the setting of AML. Pre-leukaemic founder mutations such as *DNMT3A*, *TET2* and *ASXL1* are not suitable targets for MRD analysis as these variants can persist at high levels at remission due to clonal haematopoiesis and do not reliably reflect disease remission.[7] *NPM1* mutations are identified in up 35% of patients with AML[38] and due to distinct biological characteristics and clinical behaviour, *NPM1* mutated AML has been categorized into a distinct entity by the WHO.[39, 40] The majority of these mutations are restricted to exon 12 (98%) and frequently consist of a 4bp insertion between nucleotides 960 and 961.[41] The mutational burden of *NPM1* mutation correlates with leukaemic disease and disappearance or rising levels of the mutation being a powerful prognostic factor, especially in conjunction with other associated mutations.[42] At present, MRD analysis of *NPM1* mutations is reliant on competitive allele-specific TaqMan (CAST) PCR[42, 43] once the exact mutation is identified by Sanger sequencing, with a minimal sensitivity of 10^{-5}-10^{-6}.[43] Over 70% of *NPM1* mutations are described to be type A mutations, involving insertion of TCTG at position 960 of exon 12, followed by type D (~9%, insertion of CCTG) and type B (~5%, insertion of CATG) mutations. Consequently almost 90% of *NPM1* frameshift mutations could be targeted by mass spectrometry if primers were designed to identify the first nucleotide insert.

IDH1 (R132) and *IDH2* (R140/R172) mutations are currently not recommended to be used as sole MRD markers[7] despite being identified in 15-20% of AML.[44, 45] These mutations are considered to be driver events but reliable sensitive methods to monitor these mutations have proven challenging given their potential involvement in clonal haematopoiesis. *IDH1/2* mutations can also occasionally develop in the setting of clonal haematopoiesis,[46] which adds to the complexity of using *IDH1/2* as MRD markers.[47] While there is paucity of data regarding the use of mass spectrometry for hot spot *IDH1/2* mutations in leukaemia, these variants have been successfully detected by mass spectrometry in glioblastoma samples.[48] Developing a sensitive mass spectrometry method to detect *IDH1/2* MRD has substantial potential in leukaemia as other methods have demonstrated the significance of monitoring these variants to a low detection level. A French study investigating 103 *IDH1/2* mutated AML patients demonstrated the feasibility of monitoring the mutation with digital droplet PCR. Achievement of MRD negativity (defined as VAF<0.2%) following treatment in conjunction with a normal karyotype and a concurrent *NPM1* mutation strongly correlated with disease free survival.[47] Another study investigating combination therapy with ivosidenib (*IDH1* inhibitor) and azacitidine in *IDH1*-mutated AML also demonstrated high correlation with *IDH1* mutation clearance by digital droplet PCR and MRD negativity by MFC.[49] Another French study confirmed that *IDH1/2* MRD mutation status correlated with relapse risk in 31 *NPM1*-mutated AML patients.[50] *IDH1/2* mutations are currently detectable on the MassARRAY® system although this will not achieve the sensitivity required for MRD detection in the absence of either the SABER or the UltraSEEK® techniques.

Almost 30% of newly diagnosed AML patients will have mutations in *FLT3*. The majority occur as internal tandem duplications (ITD) with the remainder manifesting as point mutations in

the TKD region.[34] *FLT3* mutation in AML is an inferior prognostic marker, and while patients may initially respond to chemotherapy, these patients have a high rate of leukaemic relapse which is often driven by the underlying mutated *FLT3*. However, these mutations are not current recommended MRD targets[7] in the absence of another MRD marker due to difficulty in devising sensitive testing methods that can identify rising mutation levels prior to overt relapse. *FLT3*-ITD mutations manifest as replicated sequences either in the juxtamembrane or the TKD domain of the *FLT3* receptor and the location and length vary, generally requiring detection via PCR or fragment-based analysis. However, the TKD point mutations, D835/I836,[33, 34] would be suitable for sensitive detection using mass spectrometry. Other mutations that can be targeted by mass spectrometry are the recently described *BCL2* mutations G101V/D103Y which occur in up to 50% of venetoclax-resistant CLL. *BTK* mutations conferring resistance to ibrutinib therapy,[51] such as the C481S point mutation, can also be targeted by mass spectrometry. Development of a mass spectrometry assay that can target mutations relevant to leukaemia and resistance incorporates all the mutations of interest may be a future method for sensitive multiplexed mutation detection, which may be more cost effective than alternative options.

Mass spectrometry can also be utilised for sensitive longitudinal monitoring of relevant mutations that are below the detection limit of Sanger sequencing. Mutations such as those associated with clonal haematopoiesis[46, 52] generally have a variant allele frequency (VAF%) <10% which can be difficult to monitor with simple PCR and sequencing based methods. Serial monitoring may be warranted for specific mutations as rising VAF% to >10% in clonal haematopoiesis-associated mutations is associated with increased risk of leukaemic transformation.[46, 53] Expansion of specific mutations, such as *TET2*, also correlates with cardiovascular risk[52, 54] and monitoring permits prophylactic risk factor modification and appropriate counselling.

3. Next generation sequencing

The explosion of technological advancement has enabled increasing availability of next generation sequencing (NGS) in diagnostics and prognostication, but also permits risk adaptive treatment modifications such as decisions regarding allogeneic stem cell transplantation and targeted therapies. Multiple platforms utilizing different sequencing technologies are available, all dependent on bioinformatics pipelines to process big data and map the individual reads to the reference human genome. The big advantage of NGS is the potential to examine the whole genome but this can be prohibitive due to cost and limited sensitivity. Capturing specific genes/regions of interest permits target enrichment, allowing for improved sensitivity. NGS also permits mutation discovery and multiple different mutation subtypes can be detected, including SNVs and indels but also structural variants such as deletions, tandem duplications and gene fusions. The mutations of interest will influence the suitability of either DNA or RNA as the source material as certain variants will be easier to identify with one or the other. For example, as RNA encodes for exons, it is the medium of choice for detection of splicing variants and their functional consequences, whereas large gains or deletions of genetic material are preferentially detected by copy number variation analysis using DNA.

3.1 Methodology

The initial step of NGS involves extracting, isolating and purification of the nucleic acid of choice prior to library preparation fragmentation and library preparation where sample specific indexes and adaptors are added. Adaptors are platform specific sequences used for fragment recognition. There are various NGS techniques for massive parallel sequencing and detailed methodology review is beyond the scope of this paper and has been reviewed elsewhere.[55] Following loading of the libraries onto a sequencing platform, amplification of the genomic material occurs through complementary extension of fluorescent-tagged nucleotides onto the genomic template. The reads will then require bioinformatics processing to convert the data into a workable format to facilitate analysis. Whole exome sequencing generally has a reported limit of detection of 10% mutant while target enrichment with capture sequencing can detect down to 5% mutant.

Enhanced sensitivity NGS techniques are available to improve the detection of low-level mutations.[56] A major problem with NGS for MRD is the higher false positive rate compared with Sanger sequencing. Paired end sequencing facilitates improved accuracy of the reads to enable detection of more complex variants with sequential reading of both the forward and reverse strands.[57] Inclusion of customized adapters marked with unique molecular identification (UMIs), which permit single molecule tracking and removal of duplicate reads can also improve sensitivity.[58]

3.2 Limitations

One of the barriers to more widespread application of NGS to regular diagnostic work has been the high financial burden associated with the technology. However, increasing availability has substantially reduced the cost of performing NGS, although it is still more expensive compared to standard PCR based methods. NGS also requires sophisticated bioinformatics systems and computational infrastructure for big data processing and large data storage capabilities, which adds to the costs associated with NGS. The turnaround time has also historically been a limitation to widespread application, but results can now be obtained with a short turnaround for specific platforms. While NGS can be utilised as a mutation discovery tool, the clinical significance of many identified variants is unknown and may not contribute to current diagnostics or prognostication. NGS has also not routinely been utilised for MRD detection due to lack of sensitivity with the limit of detection generally reported to be 5-10% depending on the platform. However, customized methodologies have enabled dramatic improvements in sensitivity, permitting detection of clinically relevant low-level mutations and clones which could be utilized for MRD detection.

3.2 MRD detection by NGS in leukaemia

3.2.1 MRD monitoring using NGS

While use of NGS for MRD detection is yet to be widely adopted, there are multiple methodologies that have the potential to become standard of care techniques for MRD assessments. For example, clonoSEQ has demonstrated sensitive tracking of malignant clones in ALL[59] and CLL.[60] Cancer clonotype sequences are characterized on a baseline diagnostic

sample with abundant malignant material though locus-specific multiplex PCR with primer mix targeting the V,D and J regions of the *IgH*, *IgK*, *IgL*, *BCL1/IgH* and *BCL2/IgH* genes.[61] Reaction specific barcoding is performed by a second PCR to aid sample identification which also amplifies the targeted genomic regions.[61] Sequencing libraries are then constructed by pooling the barcoded amplicons prior to quantitative PCR confirming the adequacy of DNA amplification. Sequencing is performed on an Illumina NextSeq 500 system and data is processed utilizing customised bioinformatics pipelines. Sequenced reads for each sample are categorized to rearranged B cell receptors and clustered into clonal receptor sequences which are assessed for pathogenicity and suitability as a MRD marker.[61] Factors contributing to suitability as a MRD marker include percentage of overall fraction of all B cell receptor sequences and nucleated cells, degree of separation from background repertoire, adequate representation by gDNA templates and uniqueness.[61] Suitable disease-associated clonotypes are then tracked and measured in subsequent MRD samples, minimizing for the impact of somatic hypermutation which can interfere with accurate MRD monitoring.[62] Approximately 500ng of DNA is required for the initial clonotype selection but MRD assessments rely on substantially larger quantities of DNA being sequenced, targeting up to 20-40 micrograms of DNA for detection of a single malignant clone in 1,000,000 cells.[61, 62]

A comparative assessment of the power of clonoSEQ versus MFC was performed in 25 adult patients diagnosed with B-ALL.[63] Approximately 91% of pre-treatment samples had at least one immunoglobulin clonal sequence identified, which could be tracked by clonoSEQ analysis.[63] In subsequent follow-up samples analysed by both clonoSEQ and MFC, concordant MRD status assessment was achieved in 82% of samples.[63] However, clonoSEQ detected MRD in an additional 17% deemed MRD-negative by MFC, whereas only 2% of samples had detectable MRD on MFC after being classified as MRD-negative using clonoSEQ.[61] Patients classified as MRD-negative by both clonoSEQ and MFC had reported rates of overall and relapse-free survival >80%. However, patients with MRD-detectable disease only by clonoSEQ had inferior survival outcomes.[14] A similar study compared clonoSEQ to MFC in 619 paired pre-treatment and post-induction bone marrow samples in paediatric B-ALL.[64] Utilizing a MRD threshold of 0.01%, both clonoSEQ and MFC predicted similar 5-year event-free and overall survival in patients that were either MRD-positive or negative by both methodologies.[64] However, clonoSEQ identified an additional 39% of patients as being MRD-positive compared to MFC and these patients had inferior outcomes compared with the remainder of patients classified as MRD-negative using MFC.[64] ClonoSEQ has also been successful in MRD detection for patients with CLL, where MRD negativity was found for patients with relapsed CLL post allogeneic stem cell transplantation who were salvaged with ibrutinib.[65] These studies clearly demonstrate the utility of clonoSEQ in the armamentarium of MRD technology.

An ultradeep sequencing method for quantitative *NPM1* mutation assessment has been developed for MRD monitoring in AML which can be integrated into existing amplicon-based sequencing panels. Single amplicon amplification of exon 11 of *NPM1* is performed with an assay sensitivity of 10^{-4}-10^{-5}.[66] Comparison of the standard *NPM1* MRD PCR method to NGS detection in 40 *NPM1*-mutated AML patients showed 98% (n=39/40) concordance in detection of the *NPM1* mutation.[66] The single discordant result was secondary to RNA degradation, adversely affecting the sensitivity of the PCR-based method. Another 48 samples comparing MFC to *NPM1* detection by NGS demonstrated only 48% concordance between the two methods.[66] However, MFC failed to detect an abnormal blast population in 46% of

samples which had identifiable *NPM1* mutations by NGS, reflecting the enhanced sensitivity of the NGS-based method.[66] Similarly, another amplicon-based NGS method has been developed for sensitive detection of *CEBPA* with high concordance with conventional capillary sequencing.[67] Data regarding NGS monitoring of other MRD targets important in AML such as *RUNX1-RUNXT1* or *CBFB-MYH11* fusions is currently limited and for now, will be dependent on the current PCR-based assays for MRD monitoring.[68]

An ultrasensitive NGS panel adapted from single molecule molecular inversion probe (smMIP) technology was developed to interrogate common genetic lesions observed in AML.[69] This strategy applies multiplexed targeted sequencing of key genomic regions of 32 genes implicated in AML, covering almost 50kb, with a reported detection sensitivity of 1 mutant cell in 1,500.[69] The standard sensitivity of NGS is limited by the sequencing error rates associated with the multiple rounds of PCR amplification required to prepare the NGS libraries.[69] Incorporation of UMIs eliminate random sequencing errors and function as an error correction strategy.[70] The smMIP capture panel integrates UMI barcoding, potentially increasing the detection limit to 1 in 60,000 mutant alleles.[69] When applied to patient samples, the panel was able to detect MRD at earlier timepoints compared to standard of care methods.[69]

Evolving techniques can also monitor specific mutations relevant in AML such as *FLT3-ITD* mutations where MRD detection may assist in decisions regarding allogeneic stem cell transplantation and whether *FLT3* inhibitors should be used as maintenance therapy. As discussed previously, *FLT3-ITD* mutations have been difficult to measure using sensitive techniques despite being commonly identified in AML. Furthermore, *FLT3* may not be an ideal MRD monitoring target as relapsed disease can be *FLT3*-negative, especially if *FLT3*-targeting inhibitors have been used. However, sensitive PCR based NGS methods have been developed which have the potential to detect 1 in 10,000 *FLT-ITD* positive cells.[71, 72] Amplified segments of the *FLT3* gene, specifically exons 14 and 15, are barcoded and bound to sequencing adaptors, which minimizes amplification bias.[71] Libraries are prepared with purified PCR products prior to sequencing on an Illumina MiSeq platform. Data is then processed with proprietary software.[71] MRD detection (defined as VAF% $>10^{-4}$) in patients considered to be in complete remission who had been treated with gilteritinib, a novel *FLT3* inhibitor, predicted for inferior overall survival.[71] Another study demonstrated the potential of deep-sequencing monitoring of *RUNX1* mutation levels in AML patients. NGS was able to discriminate between 'good' and 'poor responders' based on MRD levels.[73] 'Good responders', defined as having a mutation load of <3.61%, had an event free survival of 21 months compared with 5.7 months in 'poor responders' (mutation load of ≥3.61%).[73] Similarly, 'good responders' also had a superior overall survival (56.9 months) compared with 'poor responders' (32.0 months).[73]

3.2.2. Resistance monitoring using NGS

With the common emergence of targeted drug resistance in multiple blood cancers due to acquisition of secondary mutations at drug binding regions, sensitive multi-gene mutation detection is desirable for clinical laboratories. However, low-level detection methods should not restrict sequencing only to regions of known mutation sites. Drug resistance drives the development of new inhibitors that can bind different target regions.[74] An approach for sensitive sequencing across broad regions of the drug target is wise.

BCR-ABL1 KD mutation detection for TKI resistance assessment in CML has also been investigated with sensitive NGS methods.[75-77] Sensitive NGS has the potential to supersede Sanger sequencing due to the clinical relevance of the early detection of low-level *BCR-ABL1* mutations for timely treatment intervention. However, it is not necessary to test all patients at regular intervals. *BCR-ABL1* mutation-driven resistance occurs in <10% of first-line imatinib treated patients[78] and patients are selected for *BCR-ABL1* mutation analysis based on specific criteria, such as failure to reach time-dependent *BCR-ABL1* transcript levels or a rise in *BCR-ABL1*.[79] Kizilors et al. interrogated a retrospective unselected cohort of 99 consecutive newly diagnosed CML patients and 22 patients identified at the time of TKI resistance using a customized NGS method.[76] The amplicon-based NGS protocol underwent the International Organization for Standardization validation steps in order to implement the method into the diagnostic laboratory.[76] The established limit of detection and quantification was 1% and 3%, respectively.[76] Mutations were detected as early as 3 months of first-line TKI therapy,[76] although this has been reported previously using Sanger sequencing.[78] The NGS method was able to detect a higher proportion of KD mutations compared to Sanger sequencing, although the low-level mutations were not always those that are known to confer TKI resistance. Presence of any mutation (including low-level) was associated with inferior progression-free and event-free survival.[76] The authors suggest that *BCR-ABL1* KD mutation screening should occur at 6 month intervals until a major molecular response is achieved.[76] However, careful interpretation of the TKI-resistance conferring capacity of low-level *BCR-ABL1* KD mutations identified using NGS is critical before treatment intervention proceeds.

An Italian multi-centre study prospectively investigated 236 CML patients using a common amplicon-based NGS protocol to screen for *BCR-ABL1* KD mutations: the NEXT-in-CML study.[75] Patients enrolled in the study met a criterion for treatment failure or warning according to the 2013 European LeukemiaNet recommendations.[79] Importantly, an optimised pipeline for data analysis was established and the accuracy and inter-laboratory reproducibility across 4 centres was assessed. A lower limit of detection of 3% was defined to exclude false positive variant detection. Almost twice as many patients were positive for any *BCR-ABL1* KD mutation using the NGS method compared with Sanger sequencing. Overall, a third of patients had NGS-detectable low-level mutations.[75] Prospective monitoring also confirmed the clonal expansion of KD mutations if patients were treated with the TKI conferring reduced activity against the mutation.[75] A critical aspect of this study was that the resistance profile of individual low-level mutations was carefully assessed against a large internal database of known resistant *BCR-ABL1* mutations. Furthermore, variants with an unknown resistance profile were confirmed by repeat analysis in an independent laboratory. Reproducibility does not however rule out the possibility that some low-level mutants are passenger variants and the resistance profile of all mutants should be carefully evaluated before treatment intervention. Ideally, repeat analysis on subsequent occasions is warranted and in the absence of other evidence of TKI resistance, such as rising *BCR-ABL1* transcripts, low-levels mutants with an unknown resistance profile should not trigger therapy change. The NEXT-in-CML study confirmed the clinical relevance of low-level mutations that was previously demonstrated using mass spectrometry.[29, 37, 80, 81] The study also demonstrated the feasibility of sensitive and standardised NGS assessment for *BCR-ABL1* KD mutations across multiple laboratories. The benefit of *BCR-ABL1* KD mutation screening using NGS awaits further clinical evaluation before incorporation into international recommendations.

Alternative customized methods for ultra-sensitive mutation detection have been developed based on error correction strategies using single-molecule consensus sequencing.[56] These methods use molecular barcode labelling of individual molecules before NGS, followed by bioinformatic deconvolution after sequencing to distinguish likely sequencing errors from true low-level variants. True low-level variants will be present in all sequencing reads derived from the same molecule with the same barcode tag. Any variation observed in individual reads derived from the same molecule represent errors introduced during the sequencing process. These methods substantially reduce the NGS error rate and enhance the sensitivity of mutant detection. The approaches include single-strand consensus sequencing and duplex consensus sequencing. Duplex sequencing involves barcode labelling of each strand of individual DNA duplexes, which allows distinction of reads derived from each strand of DNA and further enhances sensitivity of variant detection.[77] True low-level variants are seen at the same position of both DNA strands. However, duplex sequencing is generally the most-expensive error-correction approach because a higher sequencing depth is required.

Duplex sequencing has been used for ultra-sensitive sequencing of the *BCR-ABL1* KD.[82] Interestingly, this study demonstrated that isolation of the leukaemia specific *BCR-ABL1* allele using RT-PCR prior to sequencing introduced a substantial level of background errors. These were eliminated by directly sequencing the *ABL1* KD using DNA and without RT-PCR. Duplex sequencing was also used for sensitive detection of *ABL1* KD mutations in a cohort of TKI-refractory *BCR-ABL1* positive patients treated with the third-generation TKI ponatinib.[82] In 23% of patients who gained high-level KD mutations at the end of treatment, duplex sequencing to a sensitivity level of ~0.01%[82] demonstrated that the mutations pre-existed prior to commencing ponatinib.

Another study investigating 63 newly diagnosed Ph+ ALL patients demonstrated that although duplex sequencing could identify 78% of patients harbouring KD mutations pre-treatment, these mutations did not clonally expand nor correlate to relapse potential.[83] Furthermore, duplex sequencing at diagnosis failed to identify the TKI-resistant KD mutation that eventually emerged in patients experiencing disease relapse despite a validated sensitivity of 0.005% mutant.[83] These mutations identified at relapse were however detected by duplex sequencing up to 5 months prior to florid disease relapse.[83] These studies demonstrate a role for duplex sequencing for sensitive detection of *ABL1* KD mutations although the sequencing costs may be prohibitive. Furthermore, removal of the RT-PCR step that specifically isolates the *BCR-ABL1* allele could mean that the method is only applicable for patients with high leukaemic burden, otherwise sensitivity of mutant detection could be reduced due to the abundance of the non-mutated *ABL1* allele.

4. Comparison of mass spectrometry and next generation sequencing

With the increased relevance of MRD to the monitoring of leukaemia, customized sensitive methodologies are required to not only detect significant mutations but also identify low-level leukaemia that has the potential to alter prognosis and management. Mass spectrometry can reliably detect pre-specified SNVs to a mutation burden of 0.05% whereas NGS has the potential for mutation discovery in addition to monitoring relevant clones in

lymphoid leukaemia. The same level of sensitive mutation detection using NGS would be costly. Another advantage of mass spectrometry is turnaround time, but a dedicated instrument is required. Some NGS techniques require substantial data processing and infrastructure, but many laboratories now have various NGS instruments available, facilitating the development of the sensitive techniques required for MRD detection. The evolving nature of NGS and the widespread utility of the technology for multiple disease processes, including leukaemia, may make mass spectrometry redundant in all but a few select scenarios.

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Author contribution

NS wrote and reviewed the manuscript and SB reviewed and proof read the manuscript.

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Tables

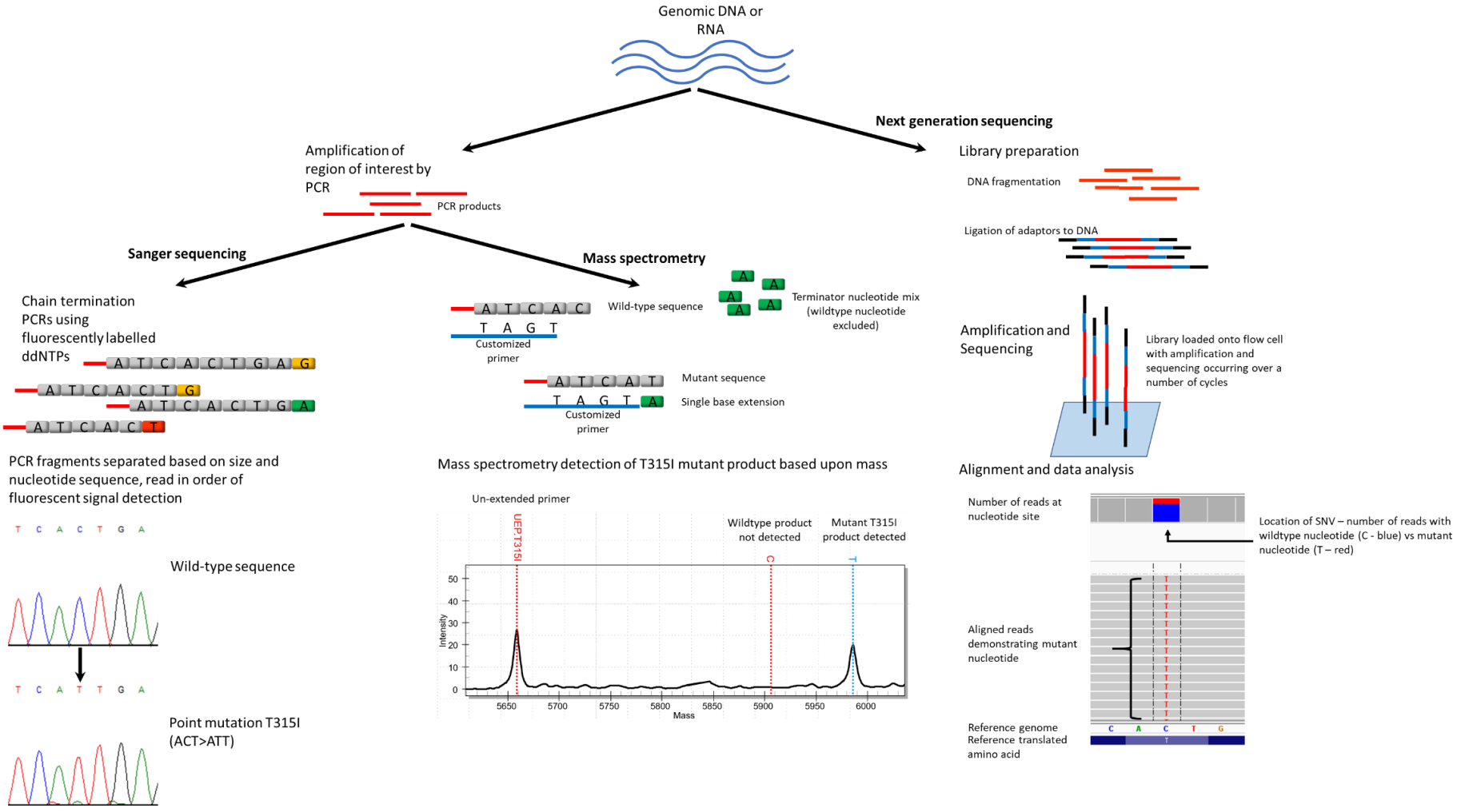
Table 1: Comparison between mass spectrometry and next generation sequencing

	Mass spectrometry	**Next generation sequencing**
Limit of detection	0.05-0.1%	Highly platform dependent, 0.1% or below
Targetable mutations	SNVs	Multiple types
Mutation discovery	No	Yes
Sensitive mutant quantification	Mutant estimation if appropriate dilution standards are used	Yes
Cost	Inexpensive relative to NGS	Highly variable depending on the platform. *BCR-ABL1* KD mutation detection has been reported to be cost equivalent to Sanger sequencing[75]
Labour	Substantial sample preparation required prior to data acquisition	Substantial sample preparation required prior to data acquisition
Data analysis	Rapid	Customized bioinformatics pipelines required to assist with data analysis
Turn-around time	1 week	Up to 4 weeks but reported turnaround time for certain panels [75] is days
Key infrastructure	MALDI-TOF analyser	Sequencing platform, computer capacity and storage required for big data
Quantity of nucleic acid required	2μg RNA/DNA	500ng-1μg of DNA/RNA required to establish clonality but up to 20-40μg of genetic material required for MRD analysis

Figures

Figure 1: Differences between Sanger sequencing, mass spectrometry and next generation sequencing for detection of the ABL1 kinase domain mutation, T315I, which is a common mutation identified in CML.

Sanger sequencing depends on the detection of fluorescently tagged nucleotides whereas mass spectrometry relies on the mass of the generated PCR products. Simplified illustration of next generation sequencing also demonstrated.


Genomic DNA or RNA
Next generation sequencing
Amplification of region of interest by PCR
PCR products
Sanger sequencing
Mass spectrometry
Chain termination PCRs using fluorescently labelled ddNTPs
PCR fragments separated based on size and nucleotide sequence, read in order of fluorescent signal detection
Wild-type sequence
Point mutation T315I (ACT>ATT)
Wild-type sequence
Customized primer
Terminator nucleotide mix (wildtype nucleotide excluded)
Mutant sequence
Single base extension
Customized primer
Mass spectrometry detection of T315I mutant product based upon mass
Un-extended primer
UEP.T315I
Wildtype product not detected
Mutant T315I product detected
Intensity
Mass
Library preparation
DNA fragmentation
Ligation of adaptors to DNA
Amplification and Sequencing
Library loaded onto flow cell with amplification and sequencing occurring over a number of cycles
Alignment and data analysis
Number of reads at nucleotide site
Location of SNV – number of reads with wildtype nucleotide (C - blue) vs mutant nucleotide (T – red)
Aligned reads demonstrating mutant nucleotide
Reference genome
Reference translated amino acid

Appendix D

What's NEXT for CML – NGS mutation screening

Shanmuganathan N, Hughes TP. What's NEXT for CML-NGS mutation screening. *Blood*. 2020;135(8):515-516.

Authors: Dr Naranie Shanmuganathan and Professor Timothy P. Hughes
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In this issue of Blood, Soverini *et al*. illustrate the potential utility of next generation sequencing (NGS) based BCR-ABL1 kinase domain (KD) mutation analysis of CML patients experiencing either failure or warning responses.[1]

Patients with resistance or disease progression do not always have an overt resistance mechanism identified. KD mutations, the best understood mechanism of resistance in CML, are only identified in ~50% of imatinib-failure patients,[2] although this may be an underestimate, due to the limited sensitivity of Sanger sequencing. The resistance profile of specific KD mutations can be utilized to select subsequent TKI therapy as expansion of the resistant clone can be expected if the inappropriate TKI is continued or selected for next-line therapy. Development of a more sensitive mass spectrometry assay for KD mutations lowered the detection limit to 0.2% for the majority of mutations[3] and was highly clinically predictive, but this assay is not widely available.

The Next-in-CML study,[1] led by Soverini *et al*., is a prospective assessment of NGS compared to Sanger sequencing, the current gold standard method, in identifying KD mutations in CML patients with molecular responses to TKI therapy classified as either warning or failure.[3] The first phase of the study involved viability assessments of the NGS-based mutation analysis across collaborating laboratories, confirming the accuracy and reproducibility of the assay. While Sanger sequencing was able to identify KD mutations in 25% of patients in the clinical phase of the study, the NGS method detected low-level KD mutations in a further 22%. NGS also identified additional low-level mutations in 48% of the patients already found to have KD mutations on Sanger sequencing. Moreover, patients classified as 'failure' were more likely to harbor multiple mutations and had a higher risk of failing subsequent therapy, supporting previous observations.[4] Longitudinal assessments of the kinetics of low-level mutations demonstrated persistence and expansion of the mutation known to be resistant to the selected TKI, as predicted.

This comprehensive prospective study provides an alternative method for sensitive KD mutation testing, one that is perhaps easier to adopt given the rate and number of institutions establishing NGS testing for other purposes. Earlier detection of low-level KD mutations will inform clinicians of the need to alter the current therapy prior to disease progression to blast crisis CML, which is a real possibility as shown in this study. The dismal outcomes associated with blast crisis CML[5] may be circumvented by an earlier switch with optimal TKI selection, reducing transformation rates and improving survival.

However, several limitations that are associated with NGS make routine application of this technology for the proposed purpose challenging. Although Soverini *et al*. were able to provide results within an average of 11 days (range 7-24 days), many laboratories would struggle to achieve this rapid turn-around time,[6] thwarting the feasibility of obtaining an actionable result prior to overt treatment failure or disease progression. Moreover, the costs associated with NGS can be prohibitive in a diagnostic setting, requiring batch testing to reduce the expense associated with each sample, adding to the delay in obtaining results. In comparison, Sanger sequencing is fast and cheap although with a mutation detection limit of 10-20%,[7, 8] negating its applicability in low-level mutation testing. Prior to adoption of this assay into mainstream diagnostics, these issues will need to be addressed by each institution intending to implement an NGS-based assay for KD mutation testing

A question to address for the future will be the actual consensus definition of a low-level mutation. This NGS-based assay classified low-level KD mutations as being undetectable by Sanger sequencing with a variant allele frequency of 3-20%. However, as technology improves, it is likely that the sensitivity of NGS will increase, with the potential to detect mutations with an allele frequency considerably less than 3%. What should be the actionable threshold to recommend a TKI switch? Furthermore, should we be screening patients deemed high risk for failure at earlier timepoints, even before a warning response is recognized? This could conceivably include screening selected patients at diagnosis, to detect low-level actionable KD mutations that would optimize TKI selection and prevent the development of TKI resistance. While these issues remain considerations for the future, the findings from this manuscript make a strong argument to support the use of NGS in patients not achieving optimal molecular responses, facilitating rational therapy selection and ideally preventing overt resistance and progression.

Conflict of interest disclosures:

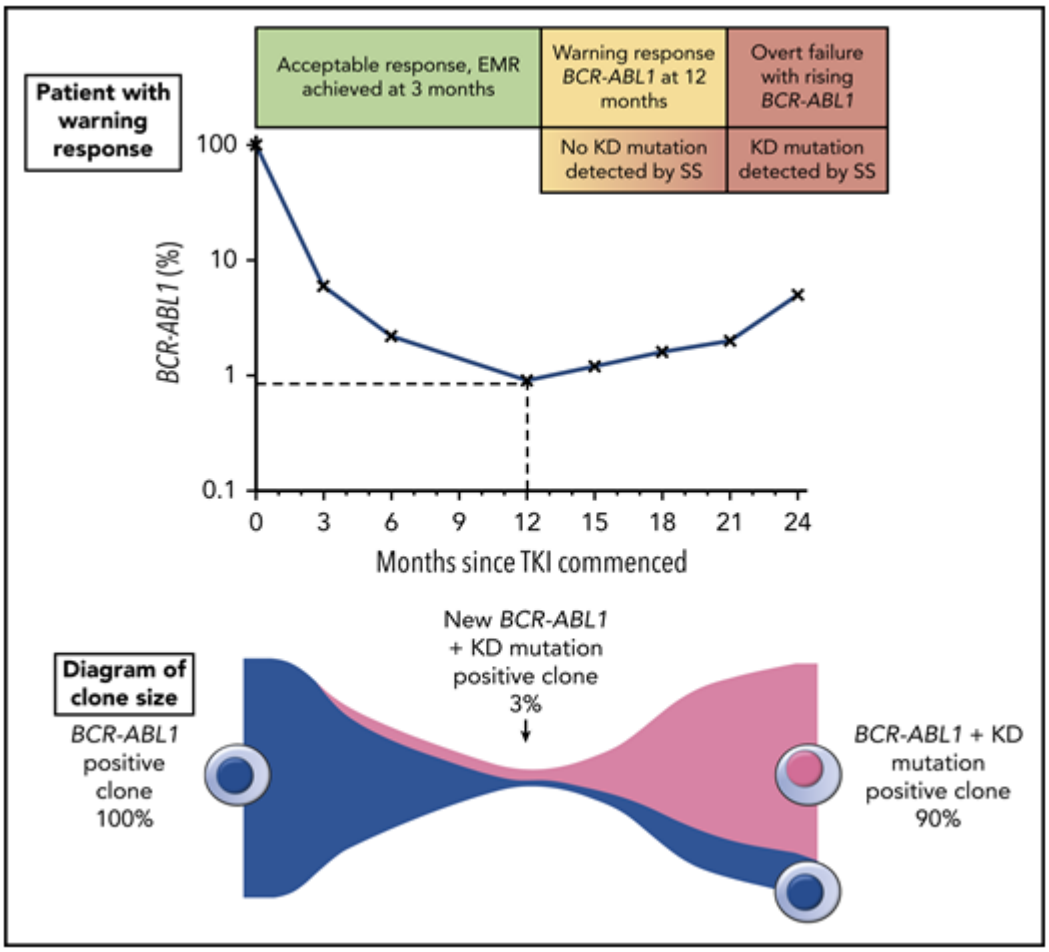
N.S. received honoraria from Novartis and travel and accommodation expenses from Novartis, Gilead, Amgen, and Janssen. T.P.H. holds a consultancy role in, and has received research funding and honoraria from, Novartis and Bristol-Myers Squibb.

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Figure 1

The figure illustrates a hypothetical patient diagnosed with chronic phase CML and commenced on TKI. They had a warning response to TKI, based on the 2013 ELN criteria, due to failure to achieve a major molecular response[9] (*BCR-ABL1* ≤0.1%) by 12 months. While the patient's response plateaued for a number of months, there was a subsequent progressive *BCR-ABL1* rise consistent with overt treatment failure. The second panel illustrates the clone sizes at various stages of therapy. At the time of warning response, there is emergence of a KD mutation with an allele frequency of ~3% but as this is not detectable by Sanger sequencing, the same therapy is continued until there is significant expansion of the resistant clone until it is eventually detectable by Sanger sequencing. Earlier intervention would likely prevent overt treatment failure through initiation of an appropriate therapy switch at the time of detection of the new low-level KD mutation. KD, kinase domain; SS, Sanger sequencing


Patient with warning response
Acceptable response, EMR achieved at 3 months
Warning response BCR-ABL1 at 12 months
No KD mutation detected by SS
Overt failure with rising BCR-ABL1
KD mutation detected by SS
BCR-ABL1 (%)
100
10
1
0.1
0
3
6
9
12
15
18
21
24
Months since TKI commenced
Diagram of clone size
BCR-ABL1 positive clone 100%
New BCR-ABL1 + KD mutation positive clone 3%
BCR-ABL1 + KD mutation positive clone 90%

Appendix E

The hidden pathogenesis of CML: Is BCR-ABL1 the first event?

Shanmuganathan N, Branford S. The Hidden Pathogenesis of CML: Is BCR-ABL1 the First Event? *Current Hematologic Malignancy Reports*. 2019;14(6):501-506.

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Abstract

Purpose of review: Identification of the *BCR-ABL1* fusion oncogene in patients diagnosed with chronic myeloid leukemia (CML) led to the development of targeted therapy responsible for the dramatic survival benefits observed in the past two decades. However, despite these revolutionary findings, there remains marked disparity in patient outcomes. Why do some patients present in de novo or evolve to the more aggressive stages of CML? Why can select patients successfully discontinue therapy whereas others fail to meet molecular milestones?

Recent findings: BCR-ABL1 kinase mutations are only identified in approximately 50% of patients with poor responses and disease progression, suggesting the presence of alternative resistance mechanisms. Numerous institutions have identified the presence of additional genomic events in addition to *BCR-ABL1* with the increasing availability of next generation sequencing.

Summary: We explore the potential pathways and events that may cooperate with *BCR-ABL1* to answer these questions but also challenge the fundamental tenet that *BCR-ABL1* is always the sole event initiating CML.

Introduction

For decades, it has been postulated that cancer development is a multi-step evolution[1] with Knudson formalizing the hypothesis that cancer is at minimum, a "two-hit" process based on his work in pediatric retinoblastoma.[2] However, CML has defied this long-standing principle with only the Philadelphia (Ph) chromosome consistently identified in patients. It has long been the prototype of a disease with a clear genetically based diagnosis due to the unassailable presence of the oncogene, *BCR-ABL1*, formed by the translocation and fusion of *ABL1* from chromosome 9 to the *BCR* gene on chromosome 22.[3] The development of the *BCR-ABL1* fusion gene has always been considered to be the inciting event in the pathogenesis of CML with its acquisition in a pluripotent stem cell conferring a marked survival advantage to its progeny via hyper-proliferative and anti-apoptotic mechanisms, resulting in effacement of normal hematopoiesis.[3] The identification of this single mutation is also the only molecular requirement for the establishment of the diagnosis of CML,[4,5] unlike other haematological malignancies such as acute myeloid leukemia[6] or myelodysplastic syndrome which have a far more heterogenous molecular profile. Adding to the burden of evidence regarding the importance of *BCR-ABL1* in the pathogenesis of CML is the marked efficacy of the currently available targeted molecular therapies, the tyrosine kinase inhibitors (TKIs) – imatinib, nilotinib, dasatinib, bosutinib and ponatinib, which can induce significant cytogenetic and molecular remissions resulting in dramatic survival benefits[7-10] for CML patients compared to historical controls. Conversely, despite that only a single mutational event need be identified for patients to manifest CML, responses and outcomes are heterogeneous. While many patients are able to attain and maintain deep molecular responses, a proportion of patients either present or progress to the more aggressive accelerated phase (AP) or blast crisis (BC) CML, despite appropriate TKI therapy.[11-14] Other patients are able to successfully discontinue TKI under appropriate medical supervision,[15-17] clearly demonstrating the diverse clinical landscape associated

with CML. The *BCR-ABL1* oncogene is clearly sufficient to initiate chronic phase CML but resistance and evolution to the more aggressive stages requires accumulation of additional events.

BCR-ABL1 must occur in the pluripotent stem cell to cause disease

Transfection of murine stem cells with the *BCR-ABL1* fusion gene has resulted in the rapid development of a myeloproliferative neoplasm very similar to CML, leading to almost definitive confirmation that *BCR-ABL1* is the mandatory event required to trigger leukemic evolution.[18,19] However Fialkow's establishment of multiple B-lymphoid cell lines from a CML-affected patient with co-existing heterozygous expression of glucose-6-phosphate dehydrogenase revealed that only a fraction of resultant daughter cells expressed *BCR-ABL1*.[20] These experiments and observations indicate that the *BCR-ABL1* alone is sufficient to provide a survival advantage for leukemic cells but an additional event is likely required for transmission to the descendants of the mutated leukemic stem cell (LSC).[20] *BCR-ABL1* has also been detected in healthy individuals with neither clinical nor laboratory evidence of CML.[21-23] With limited follow-up, these patients do not develop CML, likely as these events are generally detected in terminally differentiated leukocytes as opposed to the LSC.[23] The absence of BCR-ABL1 in the pluripotent stem cell explains the lack of proliferative potential, corroborating that this genetic event must develop in the LSC for CML to develop.

Disease progression requires additional events

BCR-ABL1 dependent resistance

TKI failure still occurs with progression to AP/BC occurring in 4-7% despite potent BCR-ABL1 inhibition.[11-13,24] Intrinsically, *BCR-ABL1* promotes genetic instability, more so with the co-occurrence of deletions of the derivative chromosome 9,[25] providing the perfect environment for the further gain of additional mutations.[14,26,27] Kinase domain (KD) mutations, currently the best understood resistance mechanism, are an adverse prognostic factor, conferring an increased risk of transformation to AP/BC CML while predicting for an inferior progression free survival (PFS) and overall survival (OS).[28] The risk of KD mutation development with upfront second-generation TKI therapy is lower compared with imatinib therapy although the prognostic implication is uncertain.[12,13] The most common *BCR-ABL1* mutation, T315I, confers resistance against all first and second-generation TKIs, and results in inferior OS and PFS, even in comparison to the other KD mutations.[28] Harbouring multiple KD mutations is also associated with a poorer outcome.[29] IC_{50} (50% inhibitory concentration) values are published for the most frequently detected KD mutations, enabling appropriate sequential therapy selection.[30] Inadvertent selection of a TKI with reported resistance against the identified mutation is usually associated with therapy failure.[31] However, KD mutations are not always identified in TKI resistant patients, suggesting the presence of alternative resistance pathways. Furthermore, despite appropriately selected therapy based on identified KD mutations, not all patients respond.

At diagnosis, additional chromosomal abnormalities are observed in ~5% of chronic phase (CP) patients but more frequently in AP/BC. Historically, major route abnormalities (trisomy 8, isochromosome 17q, a second Philadelphia chromosome, trisomy 19) at diagnosis predicted for poorer survival and molecular outcomes[32,33] although a recent publication questions this.[34] Increased *BCR-ABL1* expression (via a second Philadelphia chromosome, *BCR-ABL1* gene amplification or increased oncogene transcription), although uncommon, has also been associated with TKI resistance.[35] Efflux and influx transporters have been implicated in resistance mechanisms in many malignancies and *in vitro*, overexpression of ABCB1 and ABCG2 is associated with a reduction in intracellular TKI concentrations, potentially contributing to clinical TKI resistance.[36] Influx transporters such as OCT-1 mediate imatinib entry into cells and patients with low OCT-1 activity have been shown to have inferior molecular and survival outcomes, increasing the risk of mutation development and disease progression.[37]

Potential *BCR-ABL1* independent resistance mechanisms

While the mechanisms underlying disease progression are poorly understood, for some patients it is likely dependent on the cooperation of BCR-ABL1 with other dysregulated genes and signalling pathways (including STAT3, PI3K, SRC) implicated in malignancy.[38] The inherent genetic instability associated with unrestrained BCR-ABL1 activity has been demonstrated to trigger the development of point mutations, insertions and deletions.[14,27,38] The largest published investigation to date into the genomic profile of CML patients revealed that at the time of diagnosis, 54% of patients who eventually progressed to AP/BC had additional detectable cancer gene variants, which also predicted for earlier onset of BC.[39] The most frequently mutated cancer associated genes were *ASXL1*, *IKZF1* and *RUNX1*.[39] Patients with optimal outcomes, defined as eventual major molecular response, were less likely to acquire additional genomic events, occurring in 16% of the tested cohort.[39]

The mutational landscape observed in patients evolving to more aggressive disease is heterogenous, with no individual signature profile observed. As expected, almost 60% of patients will have identified KD mutations at the time of progression.[39] At the time of BC, all patients sequenced had at least 1 variant detected in known cancer genes which were frequently mutated at diagnosis in other CP patients.[39] Achievement of basic cytogenetic responses may also be prevented by the acquisition of these variants at diagnosis as 75% of poor outcome patients with failure to achieve at least a major cytogenetic response (6 out of 8 patients) had additional mutations in cancer genes, whereas this was only observed in 17% (1 out of 6 patients) achieving a complete cytogenetic response.[39]

BC can be myeloid, lymphoid or undifferentiated/mixed phenotype, supporting the hypothesis that *BCR-ABL1* develops in the pluripotent stem cell.[40] There are some detectable differences in mutation profile depending on whether patients were either in lymphoid or myeloid blast crisis.[39] KD mutations involving *BCR-ABL1* were more often observed in patients with lymphoid as opposed to myeloid blast crisis.[39,41] Mutations within the tumor suppressor gene, *TP53*, have been implicated in the progression to

myeloid BC in almost 25% of patients,[42-44] although a clear correlation with *TP53* has not been consistently demonstrable.[39,45] However stabilization of *TP53* in BC has been shown to restore apoptotic regulation, terminating primitive granulocytic proliferation.[46] Gene fusions, where one of the gene partners is a cancer-associated gene, and *ASXL1* mutations also accompany myeloid BC.[39,44,47,48] *CDKN2A*, a gene frequently involved in the pathogenesis of acute lymphoblastic leukemia (ALL), regulates *TP53* and *RB1* activity.[49] Deletions involving the *CDKN2A* locus have been demonstrated in patients with BC, predominantly lymphoid.[50] Co-deletions in *IKZF1* (IKAROS), another gene implicated in the development of ALL,[51] are also frequently observed in patients progressing to lymphoid BC.[39,48,52]

One of the most frequently mutated genes in CML is *ASXL1*,[39] which is also implicated as an adverse prognostic marker in other haematological malignancies including acute myeloid leukemia and myelodysplastic syndromes.[6,53-55] At the time of diagnosis, mutated *ASXL1* has been identified in approximately 10% of CML patients.[27,39,45] However not all patients had an adverse outcome with only 56-66% progressing to blast crisis.[27,39] The median time to progression to BC was also prolonged compared with patients without *ASXL1* mutations.[39] At the time of BC, the *ASXL1* mutations identified at diagnosis were frequently not detected, although other cancer gene variants were.[39] For patients progressing to BC with *ASXL1* mutations identified only at the time of progression, these variants generally co-existed in conjunction with other genomic events.[39,48] Surprisingly, occasional *ASXL1*-mutated patients actually had optimal outcomes with achievement of major molecular response.[39,56] Subsequent testing on remission samples demonstrated disappearance of mutated *ASXL1* in some cases, suggesting the variant was present in a leukemic clone that was eliminated or reduced with TKI therapy.[27,39] Additional studies are required to establish the clinical relevance of mutated *ASXL1* at diagnosis. Acquisition following initial diagnosis is more likely to align with an adverse outcome and due to the co-occurrence of additional events, perhaps indicating underlying genetic instability of the patient.[39,48]

A putative mechanism for the differentiation block accompanying myeloid blast crisis is diminished expression of CEBPα, a key transcription factor regulating granulocytic differentiation.[57,58] Lower levels of CEBPα have been identified in patients demonstrating imatinib-resistance, especially in patients progressing to AP or BC CML.[59] Reduced expression of *CEBPα* is found in KCL-22, a BC CML cell line, while restoration of *CEBPα* expression results in terminal granulocytic differention.[60] Physiological regulation of *CEBPα* in stem cells is performed by a +37 kilobyte (kb) enhancer, activity of which can be perturbed by the fusion oncoproteins *RUNX1-RUNX1T1* and *CBFB-MYH11*.[57] Identification of these fusions in myeloid BC or patients failing therapy may illuminate a *BCR-ABL1*-independent mechanism of progression.[39,61] Hence, in conjunction with other studies evaluating the genomic landscape of CML,[14,48,62,63] the presence of additional mutations is perhaps a hallmark of the risk of evolution to AP/BC.

Evidence of preleukemic variants

With increasing ability to identify variants due to technological and bioinformatic advances, the identification of clonal mutations in the absence of clinical disease is a recent topic of interest. These variants are frequently detected in healthy older individuals and are termed clonal hematopoiesis of indeterminate potential (CHIP). The presence of CHIP predicts for a slight increase in risk of development of myeloid neoplasms but frequently, these patients never manifest any evidence of malignancy.[1] The most frequently implicated CHIP mutations are *DNMT3A*, *TET2* and *ASXL1*, which are often identified in myeloid malignancies.[1] CHIP mutations are not disease specific and are often detected in patients with existing myeloid malignancies, obscuring the significance of the identified variants. Persistence of CHIP mutations despite successful curative therapy, suggests clearance of the dominant disease initiating clone while the less pathogenic sub-clones persevere.[1] Due to the potential of these driver mutations to persist at high levels despite appropriate therapy, they are excluded from minimal residual disease analysis in acute myeloid leukemia, obscuring the prognostic implications of these mutations.[64] While the CHIP mutations in myeloid malignancies generally connote an adverse outcome, perhaps their first contribution to malignancy is to prime sub-clones for the acquisition of additional disease-causing events.[1]

Persistence of leukemic clones despite appropriate *BCR-ABL1* inhibition has been identified in CML.[14] Kim *et al.* found persistence of *TET2* mutations on diagnostic and follow up samples of responsive patients with minimal variance in allele burden, attributing the presence of *TET2* to a Ph-negative preleukemic clone.[27,45] Likewise, Schmidt *et al.* demonstrated *DNMT3A* persistence in both diagnostic and follow-up remission samples, again present from a preleukemic clone.[65] Conversely, *DNMT3A* and *TET2* have been identified in remission samples alone, implying evolution within Ph-negative clones.[39,65] Other patients had mutations, frequently the CHIP-associated variants, identified at diagnosis that were not detected in remission samples, likely involving the Ph-positive clone.[27,65] The presence of the preleukemic variants may be a manifestation of aging but we postulate that in select patients the presence of *ASXL1*, *DNMT3A, TET2* and other CHIP mutations could possibly indicate a favourable milieu for development of further mutations, including *BCR-ABL1*.

Conclusion

The BCR-ABl1 fusion gene is sufficient to manifest CP CML. However, disease progression and resistance likely occur due to the accumulation of additional events including kinase domain mutations, additional chromosomal abnormalities and variants in known cancer genes. Inadequate disease control, either through intrinsic resistance mechanisms or non-compliance, promotes genetic instability required for these additional events to develop. By highlighting these alternative pathways, more appropriate targeted therapy can follow, perhaps enhancing the 'cure' rate of CML with more patients able to discontinue TKI therapy instead of anticipating decades of treatment with associated toxicities. The detection of mutated cancer-associated genes at diagnosis indicates that these mutations either occurred soon after the acquisition of the *BCR-ABL1* fusion or in some cases, pre-dated the acquisition of the fusion. With increasing use of next-generating sequencing techniques, the clinical relevance of

mutated cancer genes detectable at the time of CML diagnosis should be resolved over the coming years. Important questions remain to be answered, not least of which is whether additional mutations at diagnosis impact the chance of achieving treatment free remission.

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** Of major importance

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Appendix F

Asciminib for chronic myeloid leukaemia: Next Questions

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Abstract

Recent approval of asciminib, a novel "Specifically Targeting the ABL Myristoyl Pocket" (STAMP) *BCR::ABL1* inhibitor, for treatment of chronic myeloid leukemia patients who have either failed ≥2 lines of therapy or have the T315I mutation has provided clinicians with a wider selection of potentially effective treatment options. Asciminib has the attractive twin attributes of high potency directed against *BCR::ABL1* and good tolerability based on its limited off-target effects. However, it is unclear exactly where asciminib will be positioned amongst the other available tyrosine kinase inhibitors (TKIs), especially ponatinib which is also available for the same indications. There are many questions that are yet to be answered with regards to the optimal use of asciminib which include its role in the first- and second-line settings, combination therapy with other TKIs, and effectiveness in advanced phase CML. In this review, we discuss the available data on asciminib while exploring a number of clinical trials in progress. Finally, we provide our opinion, based on the current data, about where asciminib is most likely to be the optimal choice, which will hopefully assist clinicians with therapy selection.

1. Introduction

The therapeutic landscape of chronic myeloid leukaemia (CML) has dramatically shifted in the past two decades,[1] transforming the disease from a primary indication for an allogeneic stem cell transplant to essentially a chronic illness. The altered paradigm is solely due to the development of tyrosine kinase inhibitors (TKIs) that target the disease-defining *BCR::ABL1* fusion[2] with the first foray into targeted therapy being imatinib, the first-generation TKI.[3] With the introduction of imatinib, and the subsequent development of more potent TKIs including the second-generation agents (nilotinib,[4] dasatinib[5] and bosutinib[6]) and the third-generation TKI (ponatinib),[7] the 10-overall survival for patients diagnosed with chronic phase CML exceeds 80%,[8] almost matching age-matched healthy population comparators.[9] However following the initial survival benefits observed with imatinib, the more potent TKIs did not demonstrate substantive gains in overall survival when used frontline,[10,11] the benefits likely tempered by the toxicity associated with the second-generation TKIs. Furthermore, while many chronic-phase CML patients will achieve deep molecular responses, there remains a small proportion of patients who progress to the more advanced stages of accelerated phase (AP) or blast phase (BP) CML[3] which remain therapeutic challenges. The critical determinants of resistance and progression while on TKI therapy are poorly understood but development of point mutations in the kinase domain of *BCR::ABL1* remains the best recognised mechanism of resistance, influencing sensitivity to TKI therapy.[12] Therefore identifying novel pathways to treat CML are imperative to address the toxicity burden associated with conventional TKI therapy, bypass known resistance mechanisms and treat disease progression. To address these unmet needs asciminib, which is the first in class "Specifically Targeting the ABL Myristoyl Pocket" (STAMP) inhibitor, was developed. In this manuscript, we review the published data and speculate about where asciminib might fit into the current and future therapeutic repertoire.

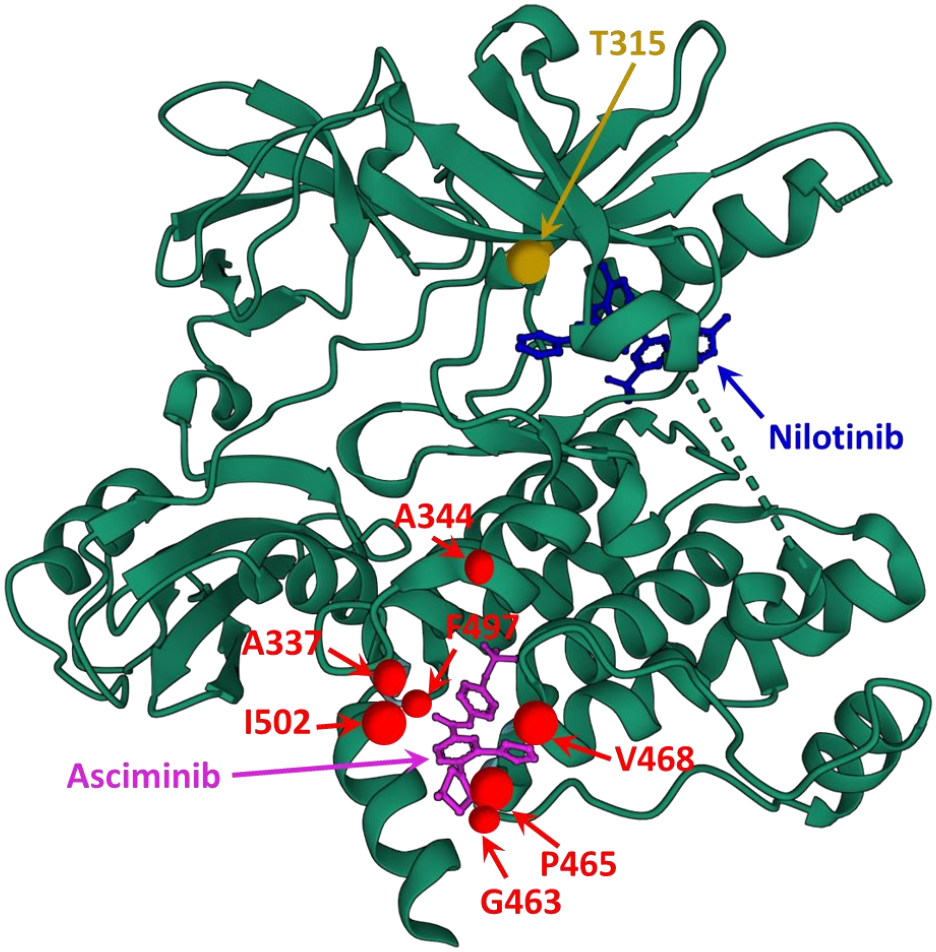
2. Mechanism of action of asciminib

With the formation of BCR::ABL1 oncoprotein, the regulatory N-terminal cap on ABL1 is lost, leading to constitutive activation of the tyrosine kinase.[2] The conventional TKIs act as an ATP-mimic and occupy the ATP-binding pocket located in the ABL1 catalytic domain (Figure 1).[2] This inhibits binding of ATP to ABL1, preventing tyrosine residues on substrate proteins from being phosphorylated and the subsequent conformational change which is necessary for downstream signalling,[13] impeding the unrestricted activity of the BCR::ABL1 fusion oncoprotein.[6] However, while conventional TKIs were designed to primarily target BCR::ABL1, they are not specific, binding to other tyrosine kinases including c-KIT, PDGFR, SRC family members and BTK.[14] These off-target effects likely contribute to the various toxicity profiles unique to each TKI.

Asciminib binds to the BCR::ABL1 myristoyl pocket, completely distinct from the conventional TKIs which bind to the catalytic domain of BCR::ABL1.[15] The allosteric binding of asciminib to BCR::ABL1 induces a conformational change that mimics the structural changes induced by the myristate binding to the N-terminal cap of ABL1, the physiological regulator of ABL1 activity.[15] Crystallography confirmed the marked similarities between ABL1 bound to either asciminib or myristate.[15] In contrast to conventional TKIs, asciminib has fewer off-target effects due to the limited number of

tyrosine kinases containing myristoyl-binding sites analogous to ABL1,[15] likely contributing to the improved safety profile evident with asciminib therapy. Activity against the closely related ABL2 kinase[16] may account for some of the off-target effects such as lipase elevation and pancreatitis.

Figure 1: Structure of the ABL1 kinase with site of binding of asciminib and other TKIs indicated in addition to the sites of ascminib-specific mutations.
Myristoyl site indicated by the binding of asciminib, indicated in pink, whereas the catalytic site domain, the active site for all other conventional TKIs, is indicated by the presence of nilotinib (blue). The site of *ABL1* kinase domain mutations are illustrated as well. T315 is highlighted in yellow, representing the site of kinase domain mutations, whereas the location of asciminib-specific mutations, shown in red, are clearly distinct. Image adapted from the RCSB PDB[17] (rcsb.org) of PDB ID 5MO4 (Wylie AA, Schoepfer J, Jahnke W, Cowan-Jacob SW, Loo A, Furet P, et al. The allosteric inhibitor ABL001 enables dual targeting of *BCR–ABL1*. *Nature*. 2017;543:733)



3. Toxicity profile of asciminib compared with conventional TKIs

Imatinib has a relatively benign safety profile with the primary toxicities manifesting as superficial oedema and gastrointestinal events such as nausea and diarrhoea.[3] However with increasing levels of potency, the severity of the potential complications related to TKI therapy increases.

3.1 Cardiovascular toxicity

The more potent TKIs are associated with significant cardiovascular risk, most importantly arterial occlusive events. This was first observed with nilotinib with the 5-year follow up of the ENESTnd study reporting that 300mg twice daily of nilotinib had a 7.5% risk of cardiovascular events compared with only 2.1% in the imatinib-treated cohort.[10] The cardiovascular risk is dose dependent with the same study demonstrating that the 5-year risk of cardiovascular events with 400mg twice daily of nilotinib was 13.4%.[10] Concerningly, the cardiovascular events continue over time with the 10-year cardiovascular risk increasing to 16.5% and 23.5% respectively with nilotinib 300mg and 400mg twice daily doses whereas the risk with imatinib remained far lower at 3.6%.[18] Furthermore, the nilotinib toxicity profile also includes the risk factors which contribute to the higher risk of cardiovascular events, hypertension, hyperlipidaemia and impaired glucose metabolism.[10,19,20] While the 5-year incidence of cardiovascular events associated with dasatinib therapy (4%)[11] in the DASISION trial appeared to be less than the rates observed with nilotinib, other studies would suggest that the rates are similar to nilotinib.[21,22] Data from the BFORE study suggests that the cardiovascular event risk may be lower with bosutinib treatment compared with the other second-generation TKIs.[6]

The highest risk of arterial occlusive events lies with ponatinib. Based on cardiovascular toxicity observed in the early studies, ponatinib was transiently withdrawal from the market before being re-marketed with a black box warning for iatrogenic cardiac risk.[23] The 5-year follow up of the PACE study suggested the rate of arterial occlusive events was up to 31% in chronic phase CML patients treated with ponatinib.[24] The term arterial occlusive events was an umbrella term that encompassed all cardiovascular, cerebrovascular and peripheral vascular disease. However post-hoc analysis of pooled data from three clinical trials, specifically the phase I study, PACE and EPIC clinical trials, the ponatinib dose intensity was strongly associated with the risk of cardiovascular and arteriovenous events. For each 15mg decrease of ponatinib, the risk of an arterial occlusive event fell by 33%.[25] A prior history of ischaemic heart disease or older age were also associated with higher risk of arterial occlusive event development. Data from the OPTIC study supported this observation where patients commenced either 45, 30 or 15mg of ponatinib with the view to de-escalate to 15mg daily once a *BCR::ABL1*^{IS} of ≤1% was achieved.[26] Only 6% of the total cohort (n=282) developed arterial occlusive events with the event rate being 9.6%, 5.3%, and 3.2% for each dosing cohort respectively.[26] Moreover the rate of these events decreased over time in the 45mg cohort, from 7.6 events per 100 person-years in the first year of therapy to 5.9 events in the second year and falling to 0 events for years 3-5.[26] This corresponded with the median dose intensity of ponatinib falling to 15mg from the second year of study enrolment.[26] The 45mg

cohort was also more likely to achieve a *BCR::ABL1*^{IS} of ≤1% by 12 months (51.6%), enabling earlier dose reduction compared with the 30mg treatment arm (35.5%).[26]

While the long-term toxicity data for asciminib requires time to mature, early clinical trial data is available. The phase I study reported hypertension as the only cardiovascular adverse event, observed in 19% of patients.[27] Initial reports from the phase 3 ASCEMBL study, comparing asciminib to bosutinib in the third-line setting, reported a 48-week frequency of arterial occlusive events with asciminib 40mg twice daily as 4.5% compared with 1.3% with bosutinib 500mg daily.[28] However, of the events observed with asciminib, two were fatalities with one patient succumbing to ischaemic colitis following therapy failure with asciminib and switch to ponatinib and the second dying from an ischaemic stroke.[29] Notably, both patients had preceding cardiovascular risk factors and were treated with second-generation TKIs prior to asciminib therapy which arguably contributed to their cardiovascular risk. While at present, the risk of cardiovascular events with 40mg twice daily of asciminib appears to be less than nilotinib and ponatinib, this requires careful assessment over the long term, particularly in the frontline setting where prior TKI exposure is not a factor.

3.2 Pulmonary toxicity

The most significant toxicity related to dasatinib therapy is the development of pleural effusion, the risk cumulatively increasing over time. The incidence of pleural effusions within the first year of therapy with dasatinib 100mg daily was reported to be 8%, the annual risk similar for each subsequent year of therapy.[11] By 5 years, the incidence reported in the DASISION study was 28% in the dasatinib arm compared to only 0.8% of imatinib-treated patients.[11] More concerningly, dasatinib treatment carried a 5% risk of developing pulmonary hypertension based on the 5-year DASISION data whereas the risk was substantially lower with imatinib therapy.[11] Furthermore, while initial reports suggested that dasatinib-induced pulmonary hypertension was a reversible phenomenon once the offending TKI was ceased,[30] longer-term follow up suggests that significant changes in pulmonary vasculature may persist in up to a third of cases.[31,32] In comparison, the risk of pleural effusion appears to be less with asciminib therapy with 6% of patients reporting a pleural effusion in the phase I study on the latest follow up (median 14 months).[27] The incidence of grade 3/4 pleural effusion events were even less at approximately 3%.[27] However further detail about these events is yet to be released, specifically whether these patients had a prior history of dasatinib-related pleural effusions. There are currently no reports of pleural effusion nor pulmonary hypertension with asciminib monotherapy in the phase 3 ASCEMBL trial.[29]

3.3 Lipase elevation and pancreatitis

TKI therapy has also been associated with pancreatic enzyme elevation and in turn pancreatitis. The exact mechanism of the pancreatic dysfunction is postulated to be mediated by off-target effects of c-ABL inhibition by TKI therapy,[33] especially nilotinib and ponatinib. Grade 3/4 lipase elevations were observed in 9-10% of patients treated with nilotinib at the 5-year follow up of the ENESTnd study.[10] In comparison, only 4.3% of imatinib treated patients developed grade 3/4 lipase elevations by 5 years of

therapy.[10] The incidence of clinical pancreatitis however was only observed in 2-3% of nilotinib-treated patients whereas <1% of the imatinib cohort had reported pancreatitis.[10] The rates of pancreatitis are higher with ponatinib therapy with up to 7% of chronic phase CML patients developing pancreatitis by 5 years.[24] Pancreatitis has been infrequently reported with dasatinib and bosutinib therapy although up to 13.4% of patients treated with bosutinib in the BFORE study were reported to have grade 3/4 increase in serum lipase by 5 years.[34]

The most frequent non-haematological adverse event observed in asciminib therapy in the phase I study was lipase elevation which was reported in almost 27% of patients with only 10% being grade 3/4 events.[27] The incidence of lipase elevation was lower in ASCEMBL with only 3.8% of patients treated with asciminib having lipase elevation compared with 5.3% with bosutinib.[29] Clinical episodes of pancreatitis were noted in 3% of patients, most of these patients had documented episodes of pancreatitis with previous TKI therapy.[27] Also, all patients had been treated with doses of asciminib higher than the final recommended treatment dose of 40mg twice daily.[27] Importantly in the phase 3 study, where patients were treated with 40mg twice daily of asciminib, there were no reports of pancreatitis by 24 weeks.[29]

3.4 Other toxicities

The most frequently observed adverse events associated with asciminib therapy are haematological toxicities, predominantly thrombocytopenia and neutropenia. In the phase 3 study, almost 30% of patients in the asciminib arm developed thrombocytopenia with 21.8% reported as grade 3 or 4 events by 24 weeks.[29] By the same data cut off, only 9.2% of the bosutinib cohort had grade 3/4 thrombocytopenia.[29] Although difficult to compare to a third-line study, the upfront DASISION study had rates of grade 3/4 thrombocytopenia were 22% with 100mg of dasatinib compared with 14% with imatinib therapy by 5 years. Grade 3/4 thrombocytopaenia was only observed in 10.4% of patients following 5 years of nilotinib 300mg twice daily on ENESTnd. When evaluating the rates of grade 3/4 neutropenia, the results were similar between asciminib (17.9%) and bosutinib (14.5%) in the phase 3 ASCEMBL study. Dasatinib had a higher incidence of grade 3/4 neutropenia, reported as 29%, as did imatinib (24%) from the 5-year follow up of DASISION. Nilotinib was less likely to contribute to grade 3/4 neutropenia, reported at 12.2% from ENESTnd.

One of the other concerns associated with TKI therapy is the incidence of gastrointestinal toxicity such as diarrhoea, which can be very debilitating. Asciminib is infrequently associated with diarrhoea, with no cases reported in the phase 3 study by the 24-week data cut off.[29] In comparison, up to 10.5% of patients treated in the bosutinib arm of the ASCEMBL study developed grade 3/4 diarrhoea. Diarrhoea is infrequently reported with dasatinib therapy although there are rare cases of dasatinib-induced colitis.[35] Only 1.1% of patients treated with 300mg twice daily of nilotinib compared with 3.6% of imatinib-treated patients developed grade 3/4 diarrhoea by 5 years of the ENESTnd study. For patients with a history of gastrointestinal events or with concerns regarding gastrointestinal toxicity, asciminib may be the preferred agents.

4. What we know regarding the efficacy of asciminib

4.1 Pre-clinical data

In vitro models utilizing *BCR::ABL1* transformed Ba/F3 cells confirmed asciminib as having anti-proliferative effects on the target cells.[15] In fact, asciminib had marked inhibitory activity (based on IC50 values) against a multitude of *BCR::ABL1*-positive cell lines which included KCL22, a BP-CML cell line.[15] The transcript type (i.e. p210 or p190) had no effect on asciminib activity against the relevant cell lines.[15] Xenograft modelling derived from KCL22 and Ph-positive acute lymphoblastic leukaemia patients confirmed that appropriate concentrations of asciminib were able to regress and prevent cell growth with higher doses able to cause regression of the clonal population.[15]

4.2 Asciminib in the third line setting

4.2.1 Phase I data

The phase I dose escalation study included 141 CP-CML patients who were either intolerant or resistant to at least two prior conventional TKIs.[27] In addition, patients with T315I were eligible for study enrolment following at least one TKI if no other effective treatment was available. Additional arms were also available for patient enrolment which included relapsed/refractory CP/AP CML patients to be initiated on combination therapy with asciminib and either imatinib, nilotinib or dasatinib and relapsed/refractory BP CML or Ph-positive acute lymphoblastic leukaemia. However, data from these cohorts has not yet been published. At study enrolment, patients deemed resistant had haematologic, cytogenetic or molecular evidence of active CML.[27] The primary objective was to identify the maximum tolerated dose while also assessing the safety, pharmacokinetics and efficacy of asciminib. The study also involved dose-escalation and expansion phases. The twice daily schedule investigated seven dose levels (10mg, 20mg, 40mg, 80mg, 15omg, 160mg and 200mg) while once daily dosing evaluated three dose levels (80mg, 120mg and 200mg).[27] The higher doses were associated with more grade ≥3 dose limiting toxicities. Irrespective of the dosing schedule, the peak blood concentrations and the area under the curve was proportional to the administered asciminib dose. Preclinical xenograft models demonstrated that either 40mg twice daily or 80mg daily exceeded the 90% minimum inhibitory concentration.[27] This led to pharmacological modelling predicting that 40mg twice daily would maintain the 90% inhibitory concentration observed in the xenograft model, assuming the T315I kinase domain mutation was not present.[27]

A complete haematological response was observed in 92% of patients who entered the study in haematological relapse.[27] These patients did not harbour the T315I mutation. Complete cytogenetic remission (CCyR) was achieved in 54% of patients who entered the study without CCyR at a median time of 24 weeks.[27] At 6 and 12 months, 37% and 48% of evaluable patients achieved a major molecular response (MMR; *BCR::ABL1*^{IS}≤0.1%) respectively.[27] Patients with lower *BCR::ABL1* values at study entry (i.e. *BCR::ABL1*^{IS} <1%) were more likely to achieve MMR.[27] Of the 14 patients deemed either resistant or having unacceptable toxicity to ponatinib, 8 patients (57%) achieved/maintained MMR by 12 months. Deep molecular responses (MR4,

BCR::ABL1^{IS}≤0.01%) were achieved by 20% of patients by 12 months of asciminib monotherapy.[27]

Asciminib was discontinued in 17/141 patients due to progressive disease but only 4 patients had a documented initial response to asciminib (defined as ≥1-log reduction in *BCR::ABL1*^{IS} transcripts).[27] Two patients had documented myristoyl-site mutations at the time of progression. Disease progression was attributed to asciminib non-compliance in one patient while another with a documented E279K mutation at study enrolment had haematological and cytogenetic progression with failure to achieve MMR. Thirteen patients did not demonstrate any response to asciminib, defined as <1-log reduction in *BCR::ABL1*^{IS} transcripts,[29] and were withdrawn from the study. Eight of these patients harboured baseline kinase domain mutations, 6 with T315I mutation and 2 with other kinase domain mutations.[36]

4.2.2 Phase 3 data (ASCEMBL) study

Data from the phase 3 ASCEMBL study was recently published which directly compared asciminib to the second-generation TKI, bosutinib.[29] CP-CML patients who had been treated with ≥2 conventional TKIs who experienced either treatment failure[36] or intolerance were eligible for enrolment. *BCR::ABL1*^{IS} transcripts at the time of screening needed to be ≥1% and patients underwent a 2:1 randomization for either asciminib 40mg twice daily (n=157) or bosutinib 500mg once daily (n=76) stratified by their cytogenetic response at baseline (major cytogenetic response or not). A later protocol amendment permitted enrolment of patients with intolerance and *BCR::ABL1*^{IS} >0.1%.[36] The presence of either T315I or V299L deemed patients ineligible for the study because bosutinib would not be an effective option for these patients. Baseline mutation assessment identified 30 patients with kinase domain mutations which included 4 patients with the T315I and 1 patient with V299L mutations.[9] Given the study entry requirements, these patients were discontinued from the study. The rate of MMR achievement at 24 weeks of therapy was 25.5% with asciminib and 13.2% with bosutinib.[29] This increased to 29.3% and 13.2% by 48 weeks in the asciminib and bosutinib arms respectively.[28] Moreover, for patients that were not in CCyR at study entry, the 48-week rate of CCyR (equating to a *BCR::ABL1*^{IS} level of ≤1%) was 50.8% with asciminib compared with 33.7% in the bosutinib arm.[28] The 48-week rate of MR4 was also higher with asciminib (14%) compared with bosutinib (6.6%).[28] By the 48-week data cutoff, treatment failure, as defined by the ELN,[36] was 48.4% in the asciminib arm compared with (80.3%)[28] for those treated with bosutinib. This included therapy discontinuation for any reason. Asciminib was also better tolerated with fewer grade ≥3 adverse events compared to the bosutinib arm (54.5% vs. 67.1%) with markedly fewer patients ceasing asciminib due to toxicity (7.1% vs. 25%).[28]

4.2.4 Combination therapy

The distinct mechanism by which asciminib inhibits BCR::ABL1 function has made dual inhibition with conventional TKIs a promising therapeutic possibility. This has been assessed in the Phase I trial to determine whether the combination might improve molecular outcomes. *In vitro* data demonstrated that the addition of asciminib

enhanced BCR::ABL1 inhibition when combined with imatinib, nilotinib or dasatinib.[15] Neither myristoyl-site mutations nor kinase domain mutations emerged *in vitro* when asciminib was combined with nilotinib. Another study demonstrated that the combination of asciminib and ponatinib *in vitro* was able to inhibit leukaemic growth of T315I-containing compound mutants, that is where there are ≥2 mutations on a single *BCR::ABL1* molecule, in murine models.[37] The phase I study had treatment arms which combined lower doses of asciminib with either imatinib, nilotinib or dasatinib but this data is yet to be formally published.

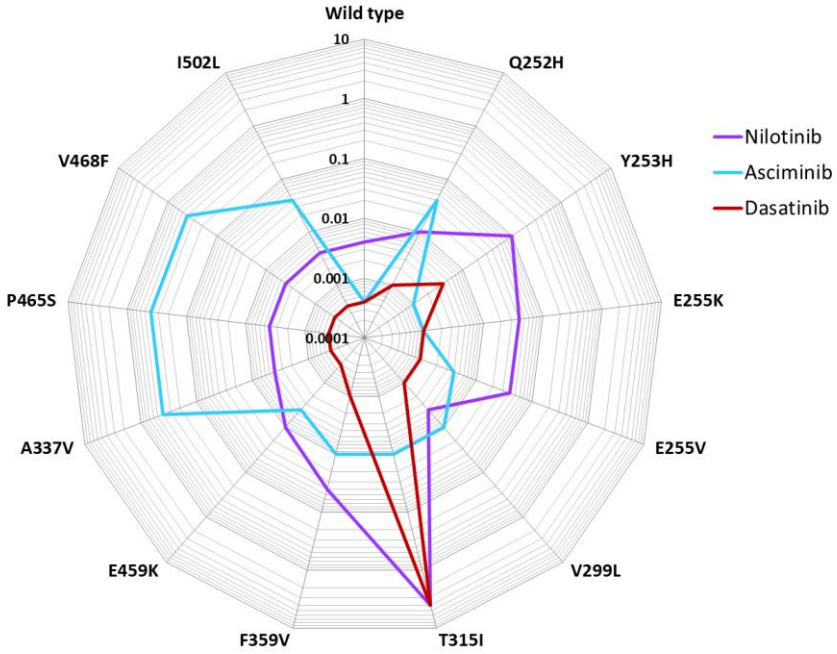
4.2.5 Asciminib and kinase domain mutations

As asciminib has a binding site distant from the kinase domain where all other conventional TKIs are active, it was hypothesized that asciminib would have activity against kinase domain mutations including T315I, which is the pan-TKI resistant kinase domain mutation only responsive to ponatinib. *In vitro* testing demonstrated that asciminib was active, to a variable degree, against all kinase domain mutations including T315I in engineered Ba/F3 cells containing the relevant kinase domain mutations.[15] *In vitro* investigation of asciminib-resistant cells identified point mutations in the myristoyl site (Table 1, Figure 1).[15] Functional validation utilizing engineered *BCR::ABL1*-expressing Ba/F3 cells containing individual myristoyl-site mutations as well as the kinase domain mutations were performed.[15] Asciminib was active against all kinase domain mutations including T315I however it had minimal activity against myristoyl-site mutations (Figure 2).[15]

Table 1: Myristoyl-site mutations that have emerged in the in vitro setting and on clinical trials

In vitro	**Emergence on clinical trials**
A337V	G109D
A344P	Y115N
P465S	A337T
F497L	G463D
	P465S
	V468F
	I502L

Figure 2: Kinase domain mutation sensitivity spectrum, specifically related to asciminib and catalytic domain TKIs, nilotinib and dasatinib.
Proliferation IC50 profiles in Ba/F3 *BCR::ABL1*-positive mutant cell lines based on in vitro data of asciminib, nilotinib and dasatinib. Image adapted from the data extracted from Wylie AA, Schoepfer J, Jahnke W, Cowan-Jacob SW, Loo A, Furet P, et al. The allosteric inhibitor ABL001 enables dual targeting of *BCR–ABL1*. *Nature*. 2017;543:733



From a clinical perspective, there were a total of 8 patients in the phase I study who had kinase domain mutations other than T315I at study entry, of which 5 (62%) achieved MMR following 12 months of asciminib monotherapy.[27] A total of 28 patients harboured T315I, of which 16 patients lacked hematological remission at study entry. Fourteen patients (88%) of those without haematological remission at study entry achieved this.[27] CCyR was attained by 41% of those without CCyR at baseline while MMR was realised in 24% of patients.[27] By 12 months, 50% of patients had demonstrated an improvement in their molecular response category.[27] However the doses required to achieve the same molecular responses as observed with patients without the T315I mutation appeared to be far higher. Early reports from the T315I-mutated arm of the phase I study, where patients were commenced on escalating asciminib doses, demonstrated a favourable safety profile for T315I mutated patients treated with 200mg twice daily asciminib, irrespective of prior ponatinib treatment.[38] Eight of the 28 patients enrolled with the T315I mutation achieved MMR by 24 weeks.[38] It is noteworthy that of the 13 patients who failed to demonstrate any response to asciminib in the phase I study, KD mutations were identified in 8 patients with 6/8 being T315I.[27] In the phase 3 study, 17 patients had kinase domain mutations (excluding T315I and V299L) with 6 patients (35%) achieving the primary end point of MMR by 24 weeks confirming asciminib's activity against these mutations. However, the F359 residue may confer a degree of asciminib resistance based on three patients who progressed on the phase I asciminib study as well as in vitro data.[37]

Myristoyl-site mutations (Table 1) were specifically assessed in the phase 1 study by means of bidirectional Sanger sequencing and NGS in evaluable patients with either molecular disease progression, loss of complete cytogenetic response or at 12 months of asciminib therapy.[27] In the 20 patients experiencing disease progression, 2 had identifiable novel myristoyl-pocket mutations.[33] Only 2 out of the 66 patients who completed 12 months of asciminib monotherapy without evidence of progressive disease had incidental detection of myristoyl-site mutations.[32] An AP-CML patient enrolled in the Phase I study with known V299L had early progression to BC-CML with

emergence of the novel V468F and I502L mutations.[14,15] Whether developing myristoyl-site mutations is always associated with resistance development is still not clear, but specific mutations with a sufficient allelic burden likely contribute to disease resistance.

4.2.6 Advanced stages of CML and Ph+ ALL

The phase I study only enrolled 9 patients with AP-CML with 7 patients demonstrating CHR with asciminib.[27] MMR was achieved in a single patient.[27] The phase I study also had a treatment arm for relapsed/refractory Ph+ ALL patients to be treated with dose escalation of asciminib but the outcome from this patient cohort is yet to be published. There was another phase I study (NCT03595917) investigating the combination of asciminib, dasatinib with prednisolone in Ph+ ALL in either the relapsed/refractory or upfront therapy in unfit patients.[39] Asciminib was dosed at either 40mg, 80mg and 160mg in combination with 140mg of dasatinib and prednisolone with the primary objective of evaluation for maximum tolerated dose followed by defining the depth and durability of responses.[39] The primary toxicity was asymptomatic amylase and lipase with preliminary results demonstrating all evaluable patients achieved at least a hematological remission.[39] Further results from this trial are awaited.

4.2.7 Ponatinib or asciminib?

Based on preliminary data, asciminib certainly is an excellent choice in the resistant and intolerant setting following at least 2 lines of TKI therapy. In comparison to bosutinib, asciminib-treated patients were more likely to achieve MMR by 48 weeks and also had fewer toxicities.[28] The phase 1 and ASCEMBL data enabled the FDA to grant accelerated approval for asciminib in either the third-line setting or for T315I mutated patients. However, the option of ponatinib already exists for these two patient groups, so clinicians have a choice to make which cannot be informed by any trial which compares asciminib to ponatinib. How can clinicians determine the best approach for their patient who is eligible for either ponatinib or asciminib? This choice has become even more difficult since the results of the OPTIC trial were published[26] and a recent spotlight on asciminib explores this specific issue.[40] Asciminib has excellent tolerability in comparison to conventional TKIs at the standard dose of 40mg twice daily, with an early signal suggesting lower rates of cardiovascular toxicity compared with the second- and third-generation TKIs. It also has demonstrated efficacy against the T315I mutation which has long been a clear-cut indication for ponatinib. However, data from OPTIC demonstrates that the risk of ponatinib-associated arteriopathy is proportional to the dose administered and that the rate of arterial occlusive events was substantially less with dose modified ponatinib[26] compared with the earlier published data from the PACE study.[24] Intention-to-treat analysis demonstrated that the exposure-adjusted risk of an arterial occlusive event in the 45mg ponatinib cohort was 7.6% per 100-person years[26] during the first year of treatment, although the majority of patients had early de-escalation to 15mg by 12 months due to achievement of *BCR::ABL1*^{IS} of ≤1%. Furthermore, the risk of arterial occlusive events in this cohort fell further in the second year, due to the marked dose modification that occurred within the first year of therapy as the median dose intensity of ponatinib was 15mg by 12 months.[26] In comparison, the 48 week rate of arterial occlusive events observed with asciminib was 4.5%.[28] The

ongoing risk associated with asciminib will only become evident with longer follow-up, but it is unclear whether the risk of cardiovascular events will plateau or continue to rise with cumulative dosing. In the T315I setting however, early limited data from the asciminib studies suggest that higher drug doses are required to achieve molecular response, which are potentially associated with higher rates of toxicity including cardiovascular events and pancreatitis.[27]

Comparing the efficacy of both asciminib and ponatinib is difficult without a head-to-head study but if we contrast the 48-week data from ASCEMBL[28] to 12-month results from OPTIC,[26] some conclusions may be drawn. CCyR was similar across both studies, being achieved by 49% of patients without T315I mutations in the 45mg ponatinib cohort[26] compared to 42% of asciminib-treated patients in ASCEMBL.[28] The cumulative incidence of MMR in the asciminib arm was 29.3% in ASCEMBL[28] compared with 35% for the 45mg ponatinib group of OPTIC.[26] However, the two study populations are slightly different. Almost all patients enrolled in the OPTIC study experienced resistance to the prior line of therapy[41] whereas only 61% of the asciminib cohort had lack of efficacy to the prior TKI.[29] Furthermore, of patients without a major cytogenetic response at ASCEMBL study entry, only 17% achieved MMR at 24 weeks compared to 46% who were in a major cytogenetic response.[29] Similar data from OPTIC is not as yet reported but if we look to the PACE study, 41% of CP-CML patients with resistance to the prior 2G-TKI achieved MMR by 12 months on ponatinib.[24]

Based on this data, there may be specific clinical scenarios where one drug should be favoured over the other.[42] Asciminib should be the preferred agent in the third line setting in intolerant patients without frank TKI resistance whereas ponatinib may be more suited to patients with pan-TKI resistance, especially in patients who have never demonstrated molecular response, with the view to de-escalate dosing once a *BCR::ABL1* ^{IS} of ≤1% is achieved. Either agent has demonstrable activity against the T315I mutation although higher doses of asciminib are required to achieve response which in turn is associated with increased toxicity. Similarly, either agent would be efficacious for non-T315I kinase domain mutations with the possible exception of mutations at the F359 residue which will require extra consideration due to the preliminary data observed with asciminib.[37] Until more data is available, advanced stages of CML or Ph+ ALL would be better managed with ponatinib. However, the addition of asciminib to ponatinib in this setting has merit and should be investigated.

4.3 Upfront therapy

While there is clear evidence of the utility of asciminib in later lines of therapy, the next question is whether asciminib can be utilised in earlier lines of therapy, including the first-line setting. There are now several clinical trials recruiting newly diagnosed CP-CML patients for first line therapy with asciminib. The phase 2 Australian CML13 study (ASCEND, ACTRN12620000851965) is enrolling 100 newly diagnosed CP-CML patients to commence treatment with 40mg twice daily of asciminib with the view to escalate asciminib dosing or add a second TKI (imatinib, nilotinib or dasatinib) if ELN defined milestones[1] are not achieved. The German CMLXI study (FAsciNation, NCT03906292), also recruiting, is investigating the potential of adding on asciminib following 12 weeks

of conventional first-line TKI options. Novartis have also initiated a randomised phase 3 study (ASC4First; NCT04971226) comparing 80mg of asciminib daily to investigator selected TKI, aiming to recruit almost 400 patients.[43] While data in the upfront setting is currently lacking, these clinical trials will hopefully determine safety and efficacy in a cohort who have not already experienced toxicity and/or resistance to prior TKI therapy. Other questions that will hopefully be answered by these frontline studies include whether asciminib has a more favourable toxicity profile compared to second-generation TKIs, especially with regards to cardiovascular events. Moreover what is the rate, timing, and stability of deep molecular response (DMR), equating to an MR4.5 response (*BCR::ABL1*^{IS} ≤0.0032%), of frontline asciminib, which future treatment-free remission (TFR) attempts are completely dependent upon.

4.4 Other possible roles for asciminib in chronic phase CML

Enhancing DMR achievement with asciminib is the focus of another clinical trial which has a similar study design to the ENESTcmr study[44]. The ASC4MORE (NCT03578367) study compares nilotinib switch to the addition of asciminib to imatinib in patients yet to reach DMR on imatinib monotherapy.[45] The primary objectives are safety and rate of DMR of combination therapy (imatinib/asciminib) compared with nilotinib. Increasing the rate of DMR achievement will maximize the number of patients who will be eligible for an attempt at TFR. On a similar note, asciminib is being investigated as consolidative therapy as an add on to other TKIs to maximise the success of TFR attempts, either in the first or second attempt.

5. Future directions and questions

Early data from clinical trials demonstrate that asciminib is a promising therapy in CML in the resistant and intolerant setting. It appears to have a relatively benign toxicity profile although longer term data is needed, especially with regard to the cardiovascular toxicity signal. Its utility in the first line setting is being investigated and data regarding combination therapy or efficacy in Ph+ ALL is still to be published. The most important question to address currently is where asciminib might be preferable to ponatinib in the third line setting and in cases where a second-generation TKI has failed as first-line therapy. Both asciminib and dose-modified ponatinib are excellent options but it is likely that one or other will be a better option in specific settings. For instance, the “pan-intolerant” patient would likely be a better candidate for asciminib whereas the “pan-resistant” patient with no evidence of molecular response to prior TKIs may be better managed with ponatinib initially. Finally, will asciminib have a major role as an “add-on” drug where an ATP-competitive TKI has not achieved a sufficient improvement in molecular response, or would simply switching to asciminib be a better option? As more clinical trial data and experience matures (Table 2), the answers to these critical questions will become clearer.

Table 2: Future directions and questions to be answered

Clinical setting	Clinical trials (if available)	Question to be answered
First-line	Australian CML13 (ASCEND, ACTRN12620000851965)	Efficacy of asciminib in the firstline setting with option to

		dose intensify for failure to achieve ELN-defined molecular milestones
	German CMLXI study (FAsciNation, NCT03906292)	Efficacy and safety of the addition of asciminib to conventional firstline TKIs
	Novartis-led study (ASC4First; NCT04971226)	Comparison of 80mg daily of asciminib to investigator selected TKI
Second-line	-	Can asciminib rescue patients with second-generation TKI resistance or will dose-modified ponatinib ponatinib be preferred
Third-line	Phase 1 (NCT02081378)	Dose finding study of asciminib monotherapy in CML following resistance or intolerance to ≥2 prior TKIs
	ASCEMBL (CABL001A2301, NCT03106779)	Comparison of asciminib to bosutinib in CP-CML patients with resistance or intolerance to ≥2 prior TKIs
Improve rate of DMR achievement	Novartis-led study (ASC4MORE, CABL001E2201, NCT03578367)	Comparison of the combination of imatinib and asciminib versus nilotinib switch in patients who have failure to reach DMR on firstline imatinib
Improve rates of TFR	HJKC3-0003 (NCT04838041)	Efficacy of combination therapy of imatinib and asciminib on rate of second TFR in patients who have previously failed an attempt to discontinue imatinib
Combination therapy with asciminib	Phase 1 (NCT02081378)	Safety and efficacy of the addition of asciminib to either imatinib, nilotinib or dasatinib in CP-CML patients with resistance or intolerance to ≥2 prior TKIs
T315I mutations	Phase 1 (NCT02081378)	Safety and efficacy of asciminib 200mg twice daily in T315I mutated patients
Paediatric CML	Phase 1 (NCT04925479)	Efficacy and pharmacokinetic profile of asciminib in CP-CML paediatric patients treated with ≥1 prior TKIs

Ph+ ALL	Phase 1 (NCT02081378)	Safety and efficacy of asciminib 200mg twice daily in Ph+ ALL resistance or intolerance to ≥2 prior TKIs

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Author contribution

NS and TPH wrote, reviewed and proofread the manuscript.

Data Availability Statement

Data sharing is not applicable to this review article as no new data were created or analyzed in this study.

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Appendix G

Title: Management of TKI resistant chronic phase CML

Hughes TP, Shanmuganathan N: Management of TKI-resistant chronic phase CML. *Hematology* 2022, 2022(1):129-137.

Shortened title: TKI Resistant CP-CML

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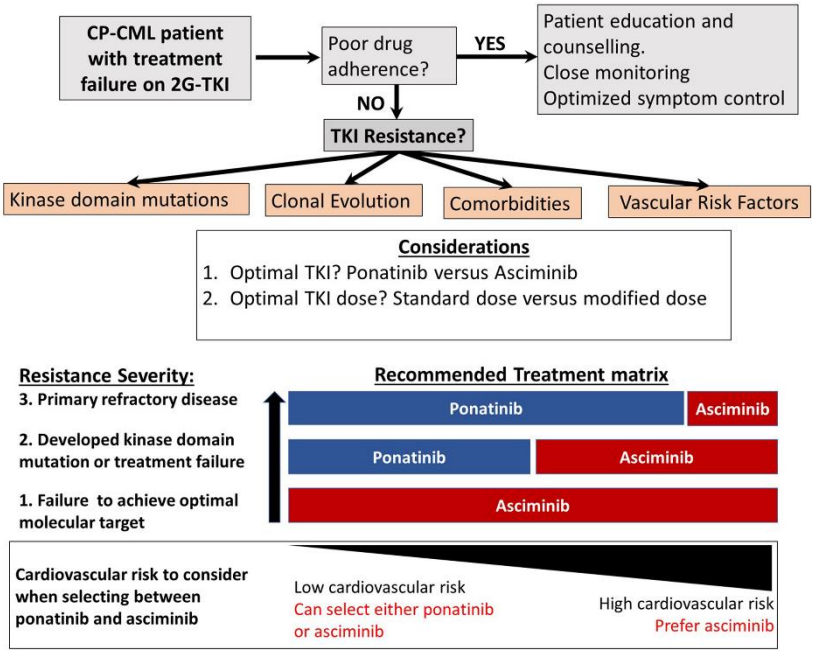
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Visual abstract



Learning Objectives:

- Determine the appropriate assessments on CP-CML patients being considered for a change in therapeutic approach because of TKI resistance.
- Recommend the optimal TKI and the dosing regimen for a CP-CML patient with TKI resistance

Abstract

Chronic phase CML (CP-CML) patients who are resistant to ≥2 tyrosine kinase inhibitors (TKIs) have limited therapeutic options and are at significant risk for progression to the blast phase. Ponatinib has been the drug of choice in this setting for the past decade but when given at full dose (45 mg/day), the risk of serious vascular occlusive events is substantial. Lower doses mitigate this risk but also reduce the efficacy. Emerging data suggests that a high dose of ponatinib is important to achieve response, but a lower dose is usually sufficient to maintain response, introducing a safer therapeutic pathway for many patients. The recent development and approval of the novel allosteric *ABL1* inhibitor, asciminib, for CP-CML patients with resistant disease provides another potentially safe and effective option in this setting. These recent therapeutic advances mean that for most resistant CP-CML patients who have failed ≥2 TKIs, there are two excellent options available for consideration - dose modified ponatinib and asciminib. Patients harbouring the T315I mutation are also candidates for either ponatinib or asciminib, but in this setting higher doses are critical to success. Lacking randomised comparisons of ponatinib and asciminib, the best choice for each clinical circumstance is often difficult to determine. Here we review the emerging evidence from recent trials and make some tentative suggestions about which drug is preferable and at what dose in different clinical settings using case studies to illustrate the key issues to consider.

Clinical Cases: What disease and patient assessments are indicated for these 3 patients with resistant chronic phase CML and what is the best therapeutic approach in each case?

Case 1: A 32 year old woman with CP-CML and a high risk ELTS score received frontline dasatinib 100 mg/day for 4 months, then switched therapy after confirmed EMR failure (>10% *BCR::ABL1*^{IS}). After 4 months of nilotinib 400 mg twice daily there was no improvement in her molecular response.

Case 2: A 55 year old woman with CP-CML and a low risk ELTS score was started on nilotinib and achieved an MMR (<0.1% *BCR::ABL1*^{IS}) by 12 months. After 18 months, she sequentially switched to bosutinib, then dasatinib, both switches for intolerance issues, but gradually lost MR2 (>1% *BCR::ABL1*^{IS}).

Case 3: A 72 year old man with an intermediate risk ELTS score failed to achieve MR2 by 12 months on imatinib then received dasatinib 100 mg/day for 6 months with an initial transient improvement in response (0.5% *BCR::ABL1*^{IS}) but then rapidly lost molecular response (15% *BCR::ABL1*^{IS}).

Introduction

For most patients with chronic phase chronic myeloid leukemia (CP-CML) who have achieved and maintained an optimal molecular response, the ongoing management is fairly straightforward. Ensuring optimal molecular targets are met and maintained, managing drug-related side effects, monitoring drug adherence, and paying careful attention to minimise drug-induced organ toxicities are the main priorities.[1,2] At the extremes of response, management can be more complex. Patients achieving and maintaining deep molecular responses (DMR) over several years are considered candidates for treatment-free remission (TFR) although embarking on a TFR attempt requires extensive discussion and patient education with a careful review of eligibility. Furthermore, once therapy has been ceased, frequent monitoring is essential, as well as a high degree of vigilance looking for molecular relapse.[3-6] At the opposite end of the response spectrum, treatment failure to at least 2 TKIs or to just 1 second-generation TKI (2G-TKI) requires expert management, still aiming to maximise optimal responses but to also prevent disease progression and TKI-induced toxicity. Treatment failure to ≥1 2G-TKIs infrequently can be salvaged by switching to another 2G-TKI,[7-10] usually in cases where specific *ABL1* kinase domain (KD) mutations have emerged. The clinical development of ponatinib has provided an effective option for many of these resistant

patients. The PACE study demonstrated its effectiveness in the setting of resistant disease, both in the case of resistance associated with a KD mutation, including the gatekeeper T315I mutation, and in cases without clear identifiable resistance mechanisms.[11] The key limitation with ponatinib therapy that emerged in the PACE[11] and other studies[12] was the very high rate of arterial occlusive events (AOEs) observed, which necessitated premature termination of some ponatinib-based clinical trials and a transient withdrawal from the market. In the CP-CML arm of the PACE study the reported rate of AOEs was 31%.[11]

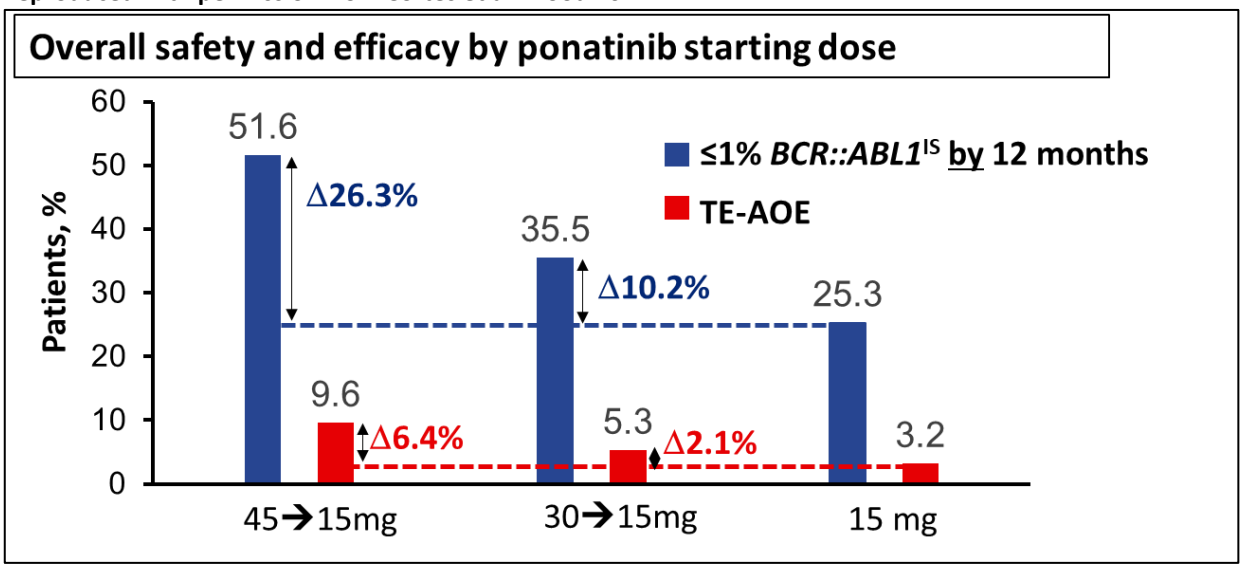
The therapeutic challenges related to managing these TKI-resistant CP-CML patients has become more complex recently because we now have safer and arguably better therapeutic options to consider. The recent demonstration from the OPTIC trial of a less toxic but still efficacious dosing regimen for ponatinib[13] has changed the risk-benefit equation, making ponatinib a more attractive option in this setting. Equally promising has been the approval of the allosteric *ABL1* inhibitor asciminib which offers good tolerability and high efficacy in similar high-risk settings.[14,15] Clinicians now have several options for patients with 2G-TKI resistance, but it is often not clear which is the best drug choice and the optimal dose regimen. The prospects of good disease control and the risks of toxicity with each option need to be carefully assessed. To provide some clarity about how to make the best decision for a particular patient, we explore the emerging findings from recent trials for resistant patients. We also present and discuss several clinical cases to illustrate the value of considering all patient- and drug-specific parameters before selecting the optimal therapeutic approach for each individual patient.

The OPTIC trial

Modelling based on the PACE[11] and other studies suggested the risk of AOEs was significantly lower at lower doses,[16] leading to the approach adopted for the OPTIC trial.[13] In OPTIC, patients were randomised to receive either 45 mg, 30 mg or 15 mg daily as a starting dose.[13] In the case of patients in the higher dosing cohorts, the dose was

reduced to 15 mg/day once MR2 *(BCR::ABL1*^{IS} <1%) was achieved.[13] As predicted, the dosing strategy of 45 mg/day with dose reduction proved to be significantly less toxic in terms of arterial occlusive events (AOEs) compared to the PACE data where doses were often maintained at 45 mg/day throughout.[13] Remarkably this approach using high doses initially and de-escalated doses in maintenance also proved to be highly efficacious, and significantly superior to the other two arms with the rate of MR2 at 12-months being 52% in the 45mg cohort vs 36% in the 30 mg cohort and 25% in the 15 mg cohort.[13] Furthermore, the OPTIC study provides further insights into the delicate balance of efficacy and safety that needs to be considered when selecting the initial dose (Figure 1). In resistant cases with the T315I mutation, the advantage of starting at 45 mg/day over 30 mg/day is significant with MR2 rates by 12 months were 60% and 25% respectively.[13] There was also an advantage with the 45 mg starting dose compared to the 30 mg cohort in patients with a non-T315I KD mutation (56% vs 40% MR2 by 12 months).[13] In patients with resistance or intolerance without a KD mutation the advantage was less marked (46% v 38%).[12]

Figure 1: Overall safety and efficacy of ponatinib based on starting dose. The analysis is a descriptive clinical summary of the data to illustrate the relationship between the efficacy and AOE rate. Reproduced with permission from Cortes et al. Blood 2021[13]



TE-AOE: treatment emergent arterial occlusive events

The higher efficacy achieved with higher starting doses needs to be weighed against the higher toxicity. In patients with recent AOEs on a prior TKI even a brief exposure to full dose ponatinib may prove very hazardous. Treatment emergent AOEs per 100 patient years in OPTIC were 3.2 for the 15 mg cohort, 5.3 for the 30 mg cohort and 9.6 for the

45 mg cohort.[13] To distinguish this dosing strategy from the conventional strategy of maintaining the starting dose if it is well tolerated, we will refer to this dosing regimen as ponatinib 45 mg (OPTIC dosing) and ponatinib 30 mg (OPTIC dosing) for clarity.

The ASCEMBL trial

The ASCEMBL trial randomized CP-CML patients who were intolerant or resistant to ≥2 TKIs to either asciminib or bosutinib in a 2:1 ratio.[15] Asciminib dosing was set at 40mg twice daily based on the tolerability assessments of a number of dosing levels in the phase I study and pharmacokinetic modelling demonstrating that either 40mg twice daily or 80mg daily of asciminib exceeded the 90% threshold for mean inhibitory concentrations.[14] It demonstrated a clearly superior rate of major molecular response (MMR; *BCR::ABL1*^{IS} <0.1%) by 6 months for asciminib, (26% v 13%).[15] However, MR2 may be a more realistic target in this multi-resistant setting. The difference was significant here as well by 12 months, 42% v 19% had achieved MR2.[9] Another major difference between the two arms was the rate of discontinuation. In the most recent update,[17] 43% of patients on the asciminib arm discontinued therapy including 24% for lack of efficacy and 6% for adverse events (median follow up 19 months). In contrast, 78% were no longer taking bosutinib with 36% stopping due to lack of efficacy and 24% because of an adverse event.[17] There were 7 patients on the asciminib arm who had AOEs at the most recent analysis which represents a rate of 3.4 per 100 patient years.[17] All 7 patients had received nilotinib previously and 3 had received ponatinib.[17] Nonetheless, the observed rate of AOEs was higher with asciminib compared with bosutinib. ASCEMBL demonstrated clear superiority of asciminib over bosutinib in the resistant and intolerant third-line setting.

Comparing ponatinib and asciminib trial data

The best way to determine whether ponatinib or asciminib is superior as a third-line option in different clinical settings would be by conducting head-to-head randomised studies. However, as these have not been initiated at this stage, we present our opinions

based on the data available from the Phase I asciminib trial, the ASCEMBL trial and the OPTIC trial to formulate an initial assessment regarding which drug may be preferential in specific cases (Table 1). OPTIC recruited a higher proportion of patients with KD mutations and a higher proportion of patients who failed to achieve any molecular response to their prior TKI.[13] In comparison, ASCEMBL had a higher proportion of intolerant patients compared to OPTIC. Overall response rates look similar comparing the 45 mg cohort of OPTIC[13] to the asciminib arm of ASCEMBL.[17] In patients without T315I, the 12-month rates of MR2 were similar; 42% for asciminib and 49% for the ponatinib 45 mg cohort.[13,15] However response rates appear to be more variable according to the baseline response in the asciminib cohort. Notably only 17% of the patients who were not in a major cytogenetic response (MCyR) at baseline achieved MMR at 6 months, compared to 40% in those patients who were in MCyR at baseline (Figure 2).[15] Moreover, the current accepted standard dose of asciminib of 40mg twice daily may be inadequate to combat highly resistant CML as evidenced by the higher doses required in T315I-mutated patients.[14] Comparable data from OPTIC is not available but in the 45 mg ponatinib cohort, the MR2 rates were 50% by 12 months irrespective of prior response on historical TKI therapy.[13] On this (tentative) basis, ponatinib may be a better choice in highly resistant cases and asciminib, with its superior safety profile, may be preferable in patients with less profound resistance.

Table 1: Comparison of asciminib (ASCEMBL)[15,17] to ponatinib (OPTIC,[13] 45 mg dose cohort)

	Asciminib 40 mg twice daily Arm of ASCEMBL study[15,17]	**Ponatinib 45 mg daily Dose adjusted from OPTIC trial[13]**	**Comment**
Number of patients	157	92	
Eligibility	ELN2013[24] treatment failure (61%) or intolerance (38%)	Intolerant or resistant to > 2 TKIs and not in MR2	
Exclusion criteria	Within 6 months prior to starting study treatment of myocardial infarction, angina pectoris, coronary artery bypass graft	Any history of myocardial infarction, unstable angina, cerebrovascular accident, or transient ischemic	

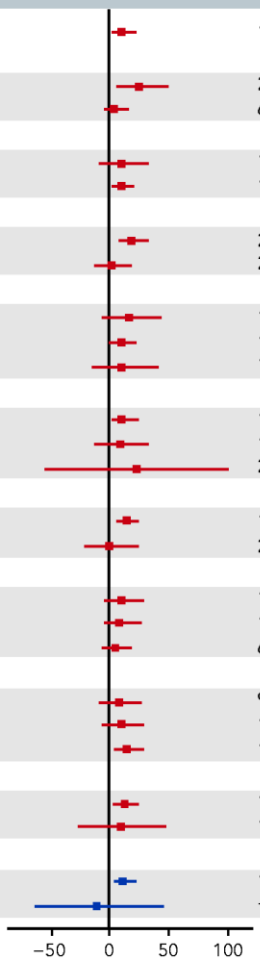
	or clinically significant cardiac arrhythmias	attack, peripheral vascular disease, NYHA class III or IV congestive heart failure or reduced left ventricular ejection fraction within 6 months prior to enrolment, clinically significant cardiac arrhythmias within 6 months	
BASELINE FEATURES			
≥3 Prior TKI lines	48%	53%	
Resistant to last TKI	61%	98%	Disparity in favour of ponatinib in highly resistant cases
Intolerant patients	38%	2%	Disparity in favour of asciminib in intolerant cases
MCyR at baseline	29%	35%	
CCyR at baseline	10%	0%	
KD mutations at baseline (non-T315I)	13%	44%	Lower rate of patients with kinase domain mutations in ASCEMBL
>10% *BCR::ABL1*	62%	NA	
Cardiac risk factors	Not specified	29% had one, 5% had >1	
RESULTS			
Follow up	19 months	32 months	
Still on study therapy	57%	47%	
Discontinuation due to toxicity	6%	17%	Consistent with better tolerance to asciminib than ponatinib
Discontinuation due to lack of efficacy	24%	19%	
MR2 by 12 months	42%	44% (49% in non-T315I group)	
MR2 by 12 months in patients in CHR	NA	27/54 (50%)	Note high response rate on

or worse on prior TKI			ponatinib in highly resistant cohort
MR2 by 12 months in patients who had a better response than CHR to prior TKI	NA	14/28 (50%)	
MMR by 12 months	29%	NA – 34% "overall rate with latest follow up"	
MMR by 6 months in pt in MCyR at baseline	40%	NA	
MMR by 6 months in patients NOT in MCyR at baseline	17%	NA	Suggestion from this data that asciminib response may be more dependent on the level of response to prior therapy than is the case for ponatinib
AOEs per 100 patient years	3.4	7.6 (year 1) 5.9 (year 2)	NB in the ponatinib cohort receiving 30 mg initially AOE rates/100 pt years were 3 (year 1) and 2 (year 2)

AOE: Arterial occlusive events, CCyR: Complete cytogenetic response, CHR: complete hematologic response, KD: Kinase domain, MCyR: Major cytogenetic response, MMR: Major molecular remission, MR2: *BCR::ABL1* ≤1%^{IS}, NA: not available, TKI: tyrosine kinase inhibitor

Figure 2: Subgroup analysis of ASCEMBL study: Risk difference with 95% confidence intervals for MR3 achievement by 24 weeks of either asciminib or bosutinib. Reproduced with permission from Rea Blood 2021[15]

Subgroup	Asciminib n/N (%)	Bosutinib n/N (%)	Risk Difference (95% CI)
All Patients	40/157 (25.5)	10/76 (13.2)	12.3 (2.1 to 22.5)
Strata Based on Randomization Data			
Major cytogenetic response	21/46 (45.7)	4/22 (18.2)	27.5 (5.9 to 49.1)
No major cytogenetic response	19/111 (17.1)	6/54 (11.1)	6.0 (−4.9 to 16.9)
Strata Based on CRF Data			
Major cytogenetic response	23/57 (40.4)	7/25 (28.0)	12.4 (−9.4 to 34.1)
No major cytogenetic response	17/100 (17.0)	3/51 (5.9)	11.1 (1.3 to 20.9)
Sex			
Female	22/75 (29.3)	4/45 (8.9)	20.4 (7.2 to 33.7)
Male	18/82 (22.0)	6/31 (19.4)	2.6 (−13.9 to 19.1)
Race			
Asian	6/22 (27.3)	1/11 (9.1)	18.2 (−7.0 to 43.4)
White	30/118 (25.4)	8/56 (14.3)	11.1 (−0.9 to 23.2)
Others	4/17 (23.5)	1/9 (11.1)	12.4 (−16.4 to 41.2)
Age Category			
18 to <65 years	33/128 (25.8)	8/61 (13.1)	12.7 (1.3 to 24.0)
≥65 years	7/29 (24.1)	2/15 (13.3)	10.8 (−12.4 to 34.0)
≥75 years	3/4 (75.0)	1/2 (50.0)	25.0 (−56.3 to 100.0)
Reason for Discontinuation of the Last Prior TKI			
Lack of efficacy	20/95 (21.1)	3/54 (5.6)	15.5 (5.3 to 25.7)
Intolerance	20/59 (33.9)	7/22 (31.8)	2.1 (−20.8 to 25.0)
Number of Prior TKI Therapies			
2	27/89 (30.3)	6/33 (18.2)	12.2 (−4.1 to 28.4)
3	12/53 (22.6)	4/33 (12.1)	10.5 (−5.3 to 26.4)
≥4	1/15 (6.7)	0/10 (0.0)	6.7 (−6.0 to 19.3)
Line of Therapy of Randomized Treatment			
3	24/82 (29.3)	6/30 (20.0)	9.3 (−8.1 to 26.6)
4	11/44 (25.0)	4/29 (13.8)	11.2 (−6.7 to 29.1)
≥5	5/31 (16.1)	0/17 (0.0)	16.1 (3.2 to 29.1)
BCR-ABL1* Mutation at Baseline			
Unmutated	31/125 (24.8)	7/63 (11.1)	13.7 (2.8 to 24.5)
Mutated	6/17 (35.3)	2/8 (25.0)	10.3 (−27.3 to 47.9)
***BCR-ABL1*^{IS} Transcript Level at Baseline**			
≥1%	34/142 (23.9)	8/72 (11.1)	12.8 (2.7 to 22.9)
<1%	6/15 (40.0)	2/4 (50.0)	−10.0 (−64.9 to 44.9)



CRF, case report form.
*Patients with T315I and V299L *BCR-ABL1* mutations or a nonevaluable mutation assessment were excluded from the subgroup analysis.

Another important comparison is the risk of vascular events with each agent. There are many variables that make this comparison problematic. This includes different eligibility criteria regarding cardiac risk factors and previous cardiovascular history, different duration of follow up, and different criteria for determining a treatment emergent AOE. Acknowledging these limitations, there would appear to be an advantage for asciminib, but this will need careful assessment with ongoing data from these and other studies. Rates of AOEs per 100 patient years were 9.6 for the ponatinib 45 mg cohort in OPTIC compared to 3.4 for asciminib in the ASCEMBL trial.

Optimal therapy for the patients with T315I mutation

Both ponatinib and asciminib are active against the T315I mutation, but in both cases higher doses are important for efficacy. In the OPTIC trial, 15/25 (60%) patients with T315I receiving the 45 mg initial dose achieved MR2 by 12 months, compared to 5/20 (25%) who started at 30 mg/day.[13] Asciminib efficacy against the T315I mutant was assessed in the Phase I trial but response rates were low until doses above 150 mg twice daily were given.[14] For that reason an expanded cohort of 48 CP- and 4 AP-CML patients with the T315I mutation were given asciminib 200 mg twice daily.[18] At this dose, molecular responses were frequent and toxicity appeared similar to standard dose asciminib (Table 2). With a median follow up of 17 months, 35/52 (67%) remained on therapy and 23 out of 49 (47%) who were not in MMR at baseline achieved MMR by 24 months.[18] This is impressive, especially considering that 28/52 (54%) had prior therapy with ponatinib and had developed either intolerance or resistance. In ponatinib-naïve patients, the rate of MMR was 57% by 6 months, compared to 29% in ponatinib resistant/intolerant patients.[18]

Table 2: Comparing baseline variables and outcomes for CP-CML patients receiving asciminib 200 mg twice daily (Phase I study)[14,18] or dose modified ponatinib (OPTIC)[13]

	Asciminib 200 mg twice daily[18]	**Ponatinib 45 mg (OPTIC)[13]**	**Ponatinib 30 mg (OPTIC)[13]**	**Comments**
No. of patients	52	94	94	
Follow up	17 months	32 months	32 months	
Molecular status at baseline	*BCR::ABL1*^{is} 54% >10% 25% 1-10% 15% <1%			
Prior ponatinib therapy	28/52 (54%)	NA	NA	
RESULTS				
Discontinued therapy	17/52 (33%)	44/94 (47%) For all pt on 45 mg, not just T315I cohort	53/95 (56%) For all pt on 30 mg, not just T315I cohort	Asciminib appears to be better tolerated
MMR rate at 12 months	12/21 (57%) in ponatinib naïve cohort	NA	NA	
MMR rate at 24 months	14/21 (66%) in ponatinib naïve cohort	NA	NA	
MR2 at 12 months	NA	15/25 (60%)	5/20 (25%)	
MR2 at 24 months	NA			

AOEs	2/52 (4%)	9/94 (9.6%)	5/94 (5.3%)	AOE risk with asciminib may be similar to ponatinib 30 mg (OPTIC dosing)

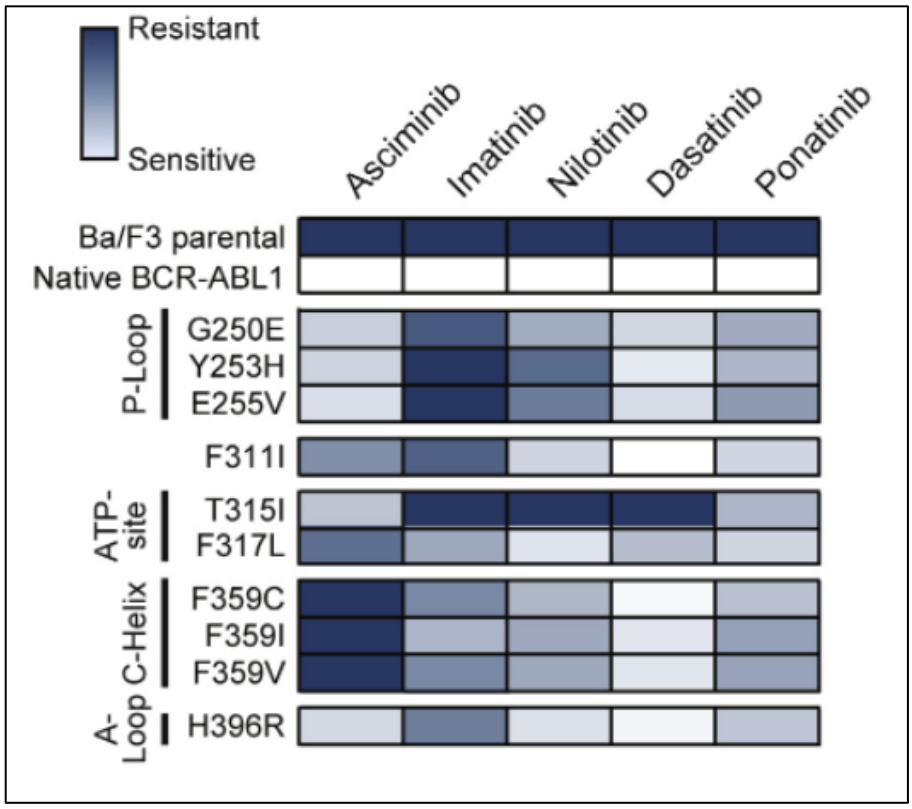
AOE: Arterial occlusive events, CCyR: Complete cytogenetic response, CHR: complete hematologic response, KD: Kinase domain, MCyR: Major cytogenetic response, MMR: Major molecular remission, MR2: *BCR::ABL1* ≤1%^{IS}, NA: not available, TKI: tyrosine kinase inhibitor

Thus, both drugs are highly active when used at the correct dose for patients with the T315I mutation. When patients on ponatinib 45 mg/day de-escalated dosing as per the OPTIC protocol, the overall rate of loss of response exceeded 30% (actual rate was not specified in the OPTIC paper).[13] Therefore, a risk assessment should be undertaken before dose de-escalating in the setting of ponatinib therapy for patients with the T315I mutation. In support of asciminib as first choice in the T315I setting, tolerance appears superior to ponatinib 45 mg (OPTIC dosing), while efficacy appears similar (Table 2).[18] In support of ponatinib as first choice, we have more data and longer follow up to reassure us that responses are stable and durable in T315I patients. Since there is no clear 'winner" the choice should be individualised by weighing up the disease-related factors and the cardiovascular risk.

Optimal therapy in the case of other KD mutations

Both ponatinib[19] and asciminib are active against most ATP-site KD mutations,[20] although sensitivity is generally lower than it is for unmutated *BCR::ABL1*. KD mutations of concern for asciminib include F359V/C/I which either emerged or persisted without evidence of molecular response in several cases in ASCEMBL and the Phase I study.[15,21] This was predicted to be a partially resistant mutation in in-vitro studies.[21] More data is needed to determine asciminib activity against the F317L mutation which appears to induce a degree of asciminib resistance based on *in vitro* studies (Figure 3).[21] However, in all presented scenarios, higher doses of asciminib may be required for optimal efficacy, analogous to the higher doses necessary to combat the T315I mutation.

Figure 3: Heat map summary of IC50 values for TKIs against a panel of Ba/F3 cell lines expressing KD mutations. Reproduced with permission from Eide et al. Cancer Cell 2019[21]



Any role for 2G-TKI in this setting?

In most cases the optimal choice of next-line therapy will be between ponatinib and asciminib. There are specific cases where a 2G-TKI may still have a role. This would include cases where resistance is driven by a mutation which is well covered by another 2G-TKI. For instance, the development of a V299L or F317L mutations are highly sensitive to nilotinib and bosutinib and the F359V/I/C mutations are highly sensitive to both dasatinib and bosutinib (Figure 3).[2]

Additionally, in some countries, eligibility rules prevent access to sequential therapy with either asciminib or ponatinib in the setting of 2G-TKI failure without developing resistance or intolerance to an alternative 2G-TKI. In this setting, a 3-6 month trial is generally sufficient to to determine if the newly commenced 2G-TKI is likely to be effective and salvage the situation. It is rare for patients who have no improvement in

molecular response after 6 months of a salvage therapy to eventually achieve MR2 or better with longer term exposure.

The intolerant patient

The focus of this chapter is TKI resistance, but it is worth commenting on the challenging patient who is intolerant to ≥2 TKIs. In this setting, the efficacy and tolerability of asciminib looks very favourable based on the Phase I asciminib study[14] and in most cases, ponatinib would not be the primary drug of choice due to its higher rates of intolerance and toxicity. Whether lower doses of ponatinib could achieve comparable response and tolerance to asciminib in these patients, where the predominant challenge is intolerance, remains to be established.

Future options

Targeting two distinct regions on *ABL1*, the ATP- and myristate- binding sites, with asciminib and conventional TKIs may also be a future option to combat resistance with the phase I clinical trial yet to formally report outcomes for the relevant treatment arms that investigated combination therapy.[20] Furthermore, murine models have demonstrated that combining asciminib with ponatinib can inhibit the growth of T315I-containing compound mutations.[21] A number of novel *BCR::ABL1* targeting agents are under investigation for resistant CML. One such drug is vodobatinib, a novel third-generation TKI with limited off target activity.[22] The phase I dose escalation study was recently reported with promising safety and efficacy results generated in both ponatinib-naïve and ponatinib-treated patients.[22] Furthermore, cardiovascular toxicity did not appear to be a prominent feature at this early stage which may distinguish vodobatinib from the other available agents for resistant CML. Similarly, another third-generation TKI, olverembatinib, has also had early phase I results reported in first- and second-generation resistant CP- and AP-CML.[23] Efficacy was observed irrespective of the study entry response, predominantly in T315I-mutated patients.[23] Responses were less impressive in the absence of T315I. While longer term follow up in larger datasets are required, these agents may offer future options for this resistant patient cohort.

Case 1: A 32 year old woman with CP-CML and a high risk ELTS score received frontline dasatinib 100 mg/day for 4 months, then switched to nilotinib 400 mg twice daily for 4 months after confirmed EMR failure. After a total of 8 months, her *BCR::ABL1*^{IS} remained >10%, having never fallen below 10%.

Further assessments: Cytogenetics at 8 months did not show evidence of clonal evolution but 18/20 metaphases were Ph+. No KD mutation was identified at 4 or 8 months. She had no cardiovascular risk factors or significant comorbidities.

Options:

1. **Continue nilotinib and review at 12 months**
2. **Switch to bosutinib**
3. **Switch to ponatinib 45 mg/day (OPTIC dosing)**
4. **Switch to asciminib**
5. **Proceed to an allograft**

Discussion of options:

1. **Continue nilotinib:** Unless there were significant interruptions to therapy due to toxicity issues or poor adherence which had resolved or been overcome, there is very little prospect that the molecular response would improve by continuing the current therapy. In this setting, the risk of disease progression is high. One important consideration is the need to identify those patients who will not achieve response with any TKI as soon as possible so that an allograft can still be scheduled in the chronic phase where there is still a good prospect of cure.
2. **Switch to bosutinib:** The likelihood of a response if a third 2G-TKI is given is very low. In ASCEMBL, only 19% achieved MR2 by 12 months on the bosutinib arm, and the response rate was even poorer in patients who were not in a MCyR at baseline, as in this case.
3. **Switch to ponatinib 45 mg/day (OPTIC dosing):** Given the complete lack of response after 8 months of potent TKI therapy, this may be the best option. In OPTIC, 50% of patients who were not in MCyR at baseline who received 45 mg/day of ponatinb achieved MR2 by 12 months. If this patient is given ponatinib and doesn't achieve MR2 after a further 6 months, the prospects of achieving this target with ongoing ponatinib is low, hence a switch to asciminib or proceeding to an allograft would be appropriate.
4. **Switch to asciminib:** As discussed earlier, responses to asciminib are probably inferior to ponatinib 45 mg/day (OPTIC dosing) in patients with little or no response to prior therapy. The exception would be in cases where the risk of a vascular event was high, where asciminib (or lower dose ponatinib) may be preferred.
5. **Proceed to an allograft:** While an allograft is not directly recommended at this stage, it would be prudent to tissue type the patient and identify a allogeneic donor in case of failure to respond to third-line therapy. In the event of persistent therapy failure, the patient can proceed to an allogeneic stem cell transplant promptly without delay.

BEST OPTION: Ponatinib 45 mg daily with dose reduction to 15 mg daily if MR2 achieved

Case 2: A 55 year old woman with CP-CML and a low risk ELTS score was started on nilotinib and responds well, achieving MMR by 12 months. After 18 months, she developed peripheral vascular disease and switched to bosutinib and then dasatinib due to further toxicity, but sequentially lost MMR and MR2 after 3 years.

Further assessments: There are no KD mutations or evidence of clonal evolution on marrow examination. There were substantial interruptions secondary to adverse events as well as periods of poor drug adherence. She has peripheral vascular disease and well controlled hypertension.

Options:

1. **Switch to imatinib**
2. **Switch to ponatinib 45 mg/day with dose reduction once MR2 achieved**
3. **Switch to ponatinib 30 mg/day with dose reduction once MR2 achieved**
4. **Switch to asciminib 40 mg twice daily**

Discussion of options:

1. **Switch to imatinib:** It is possible that her loss of response is more related to extensive interruptions to therapy and poor adherence rather than drug resistance so imatinib may still be an effective drug in this setting. However, given her intolerance to all 3 2G-TKIs, imatinib may not be well tolerated and her adherence may be poor once again.
2. **Switch to ponatinib 45 mg/day (OPTIC dosing) with dose reduction once MR2 achieved:** While she has lost MR2 it is still very possible that she still has TKI sensitive disease so she would likely have an excellent response to full dose ponatinib. However, the risk of AOEs is still significant at this dose especially given her history and other options may be equally or more efficacious without the same level of risk.
3. **Switch to ponatinib 30 mg/day (OPTIC dosing) with dose reduction once MR2 achieved:** This dose provides a lower risk of AOEs (5.3 events per 100 patient years compared to 9.6 for the 45 mg ponatinib cohort in OPTIC). However, the rate of molecular response is lower than with the higher dose of ponatinib. In patients with no KD mutations, 46% and 38% achieved MR2 by 12 months in the ponatinib 45 mg and 30 mg dose cohort respectively. This may not be a major difference as progression-free and overall survival were not substantially inferior with the modified dose. This would be an acceptable option except for the fact that the patient has a history of peripheral vascular disease.
4. **Switch to asciminib 40 mg twice daily:** This would be a relatively safe and effective option in this setting. Patients in MCyR in ASCEMBL who received asciminib had a 40% rate of MMR by 6 months. The risk of AOEs (per 100 patients years) in this cohort was 3.4.[17] Given her consistent tolerability issues with all 3 2G-TKIs, asciminib would be the option most likely to be well tolerated out of these options.

Best Option: Asciminib 40 mg twice daily and consider a switch to ponatinib if MR2 not achieved after 6-12 months. Imatinib may be another option but given the loss of responses on previous therapy, asciminib would be preferentially recommended.

Case 3: A 72 year old man with intermediate risk ELTS failed to achieve MR2 by 12 months on imatinib. He then received dasatinib 100 mg/day for 6 months with an initial improvement in response (best response 0.5% *BCR::ABL1^{IS}*) but then lost molecular MR2 (15% *BCR::ABL1^{IS}*)

Further assessments: He had no mutation detected when switching to dasatinib but when he lost MR2 there was evidence of emergence of the F317L mutation. From a cardiovascular risk perspective, he had a single coronary stent inserted 10 years ago and has poorly controlled hypertension.

Options:

5. **Switch to bosutinib,**
6. **Switch to nilotinib,**
7. **Switch to ponatinib 45 mg/day (OPTIC dosing),**
8. **Switch to ponatinib 30 mg/day (OPTIC dosing) or**
9. **Switch to asciminib 40 mg twice daily**

Discussion of options

1. **Switch to bosutinib:** The F317L mutation would appear to be sensitive to bosutinib but in this patient there was also evidence of resistance to imatinib which was not driven by a KD mutation. Bosutinib may still be a reasonable choice in this setting – especially given its lower risk of AOEs. However, the experience in ASCEMBL would suggest that it may be poorly tolerated.
2. **Switch to nilotinib:** The F317L mutation is sensitive to nilotinib but the risk of vascular events is probably too high, especially since a higher dose is likely to be needed (400 mg twice daily).
3. **Switch to ponatinib 45 mg/day (OPTIC dosing):** Patients in the OPTIC study with a non-T315I mutation who received the higher initial dose of ponatinib had an rate of MR2 by 12 months of 56%. The main concern would be the AOE risk given the patient's history and poorly controlled hypertension. This patient would not have been eligible for the OPTIC trial so the rate of AOEs would likely be significantly higher than the rate observed on this arm of the OPTIC study.
4. **Switch to ponatinib 30 mg/day (OPTIC dosing):** Given the concern about the risk of AOEs in this patient, even this modified dose represents a significant risk. The rate of AOEs per 100 patient years was 5.3 for this dose in OPTIC, but this patient would not have been eligible for OPTIC, so the AOE-risk is probably higher.
5. **Switch to asciminib 40 mg twice daily:** There is very limited data regarding the sensitivity of the F317L mutation to asciminib. In-vitro studies suggest a level of resistance which is greater than that seen with the T315I mutation. This mutation was only found in 2 patients at baseline in the asciminib arm of ASCEMBL, neither had a molecular response.[15] Along with the F359C/I/V mutation, we need more clinical data before we can determine whether asciminib is effective in this setting, and if so, at what dose.

BEST OPTION: Bosutinib may be safest option with a switch to ponatinib at modified dose if no molecular response after 3-6 months.

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Appendix H

Asciminib for chronic myeloid leukaemia: Next Questions

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Abstract

Recent approval of asciminib, a novel “Specifically Targeting the ABL Myristoyl Pocket” (STAMP) *BCR::ABL1* inhibitor, for treatment of chronic myeloid leukemia patients who have either failed ≥2 lines of therapy or have the T315I mutation has provided clinicians with a wider selection of potentially effective treatment options. Asciminib has the attractive twin attributes of high potency directed against *BCR::ABL1* and good tolerability based on its limited off-target effects. However, it is unclear exactly where asciminib will be positioned amongst the other available tyrosine kinase inhibitors (TKIs), especially ponatinib which is also available for the same indications. There are many questions that are yet to be answered with regards to the optimal use of asciminib which include its role in the first- and second-line settings, combination therapy with other TKIs, and effectiveness in advanced phase CML. In this review, we discuss the available data on asciminib while exploring a number of clinical trials in progress. Finally, we provide our opinion, based on the current data, about where asciminib is most likely to be the optimal choice, which will hopefully assist clinicians with therapy selection.

1. Introduction

The therapeutic landscape of chronic myeloid leukaemia (CML) has dramatically shifted in the past two decades,[1] transforming the disease from a primary indication for an allogeneic stem cell transplant to essentially a chronic illness. The altered paradigm is solely due to the development of tyrosine kinase inhibitors (TKIs) that target the disease-defining *BCR::ABL1* fusion[2] with the first foray into targeted therapy being imatinib, the first-generation TKI.[3] With the introduction of imatinib, and the subsequent development of more potent TKIs including the second-generation agents (nilotinib,[4] dasatinib[5] and bosutinib[6]) and the third-generation TKI (ponatinib),[7] the 10-overall survival for patients diagnosed with chronic phase CML exceeds 80%,[8] almost matching age-matched healthy population comparators.[9] However following the initial survival benefits observed with imatinib, the more potent TKIs did not demonstrate substantive gains in overall survival when used frontline,[10,11] the benefits likely tempered by the toxicity associated with the second-generation TKIs. Furthermore, while many chronic-phase CML patients will achieve deep molecular responses, there remains a small proportion of patients who progress to the more advanced stages of accelerated phase (AP) or blast phase (BP) CML[3] which remain therapeutic challenges. The critical determinants of resistance and progression while on TKI therapy are poorly understood but development of point mutations in the kinase domain of *BCR::ABL1* remains the best recognised mechanism of resistance, influencing sensitivity to TKI therapy.[12] Therefore identifying novel pathways to treat CML are imperative to address the toxicity burden associated with conventional TKI therapy, bypass known resistance mechanisms and treat disease progression. To address these unmet needs asciminib, which is the first in class "Specifically Targeting the ABL Myristoyl Pocket" (STAMP) inhibitor, was developed. In this manuscript, we review the published data and speculate about where asciminib might fit into the current and future therapeutic repertoire.

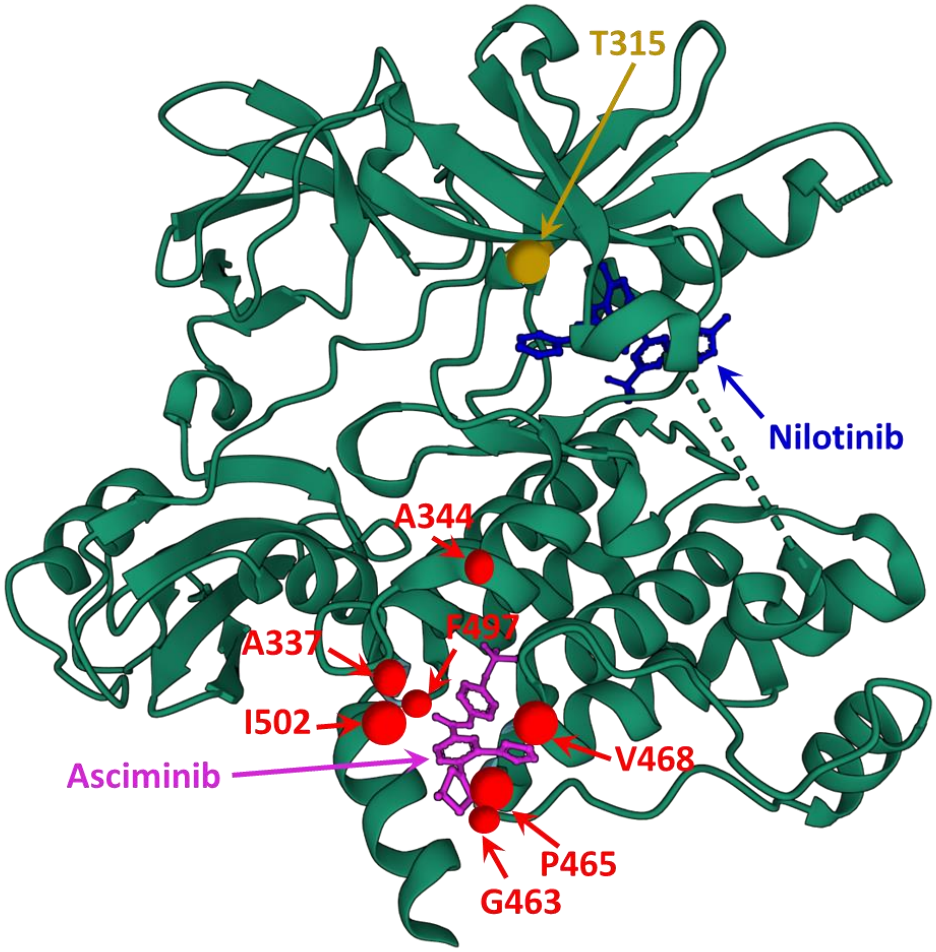
2. Mechanism of action of asciminib

With the formation of BCR::ABL1 oncoprotein, the regulatory N-terminal cap on ABL1 is lost, leading to constitutive activation of the tyrosine kinase.[2] The conventional TKIs act as an ATP-mimic and occupy the ATP-binding pocket located in the ABL1 catalytic domain (Figure 1).[2] This inhibits binding of ATP to ABL1, preventing tyrosine residues on substrate proteins from being phosphorylated and the subsequent conformational change which is necessary for downstream signalling,[13] impeding the unrestricted activity of the BCR::ABL1 fusion oncoprotein.[6] However, while conventional TKIs were designed to primarily target BCR::ABL1, they are not specific, binding to other tyrosine kinases including c-KIT, PDGFR, SRC family members and BTK.[14] These off-target effects likely contribute to the various toxicity profiles unique to each TKI.

Asciminib binds to the BCR::ABL1 myristoyl pocket, completely distinct from the conventional TKIs which bind to the catalytic domain of BCR::ABL1.[15] The allosteric binding of asciminib to BCR::ABL1 induces a conformational change that mimics the structural changes induced by the myristate binding to the N-terminal cap of ABL1, the physiological regulator of ABL1 activity.[15] Crystallography confirmed the marked similarities between ABL1 bound to either asciminib or myristate.[15] In contrast to conventional TKIs, asciminib has fewer off-target effects due to the limited number of

tyrosine kinases containing myristoyl-binding sites analogous to ABL1,[15] likely contributing to the improved safety profile evident with asciminib therapy. Activity against the closely related ABL2 kinase[16] may account for some of the off-target effects such as lipase elevation and pancreatitis.

Figure 1: Structure of the ABL1 kinase with site of binding of asciminib and other TKIs indicated in addition to the sites of ascminib-specific mutations.
Myristoyl site indicated by the binding of asciminib, indicated in pink, whereas the catalytic site domain, the active site for all other conventional TKIs, is indicated by the presence of nilotinib (blue). The site of *ABL1* kinase domain mutations are illustrated as well. T315 is highlighted in yellow, representing the site of kinase domain mutations, whereas the location of asciminib-specific mutations, shown in red, are clearly distinct. Image adapted from the RCSB PDB[17] (rcsb.org) of PDB ID 5MO4 (Wylie AA, Schoepfer J, Jahnke W, Cowan-Jacob SW, Loo A, Furet P, et al. The allosteric inhibitor ABL001 enables dual targeting of *BCR–ABL1*. *Nature*. 2017;543:733)



3. Toxicity profile of asciminib compared with conventional TKIs

Imatinib has a relatively benign safety profile with the primary toxicities manifesting as superficial oedema and gastrointestinal events such as nausea and diarrhoea.[3] However with increasing levels of potency, the severity of the potential complications related to TKI therapy increases.

3.1 Cardiovascular toxicity

The more potent TKIs are associated with significant cardiovascular risk, most importantly arterial occlusive events. This was first observed with nilotinib with the 5-year follow up of the ENESTnd study reporting that 300mg twice daily of nilotinib had a 7.5% risk of cardiovascular events compared with only 2.1% in the imatinib-treated cohort.[10] The cardiovascular risk is dose dependent with the same study demonstrating that the 5-year risk of cardiovascular events with 400mg twice daily of nilotinib was 13.4%.[10] Concerningly, the cardiovascular events continue over time with the 10-year cardiovascular risk increasing to 16.5% and 23.5% respectively with nilotinib 300mg and 400mg twice daily doses whereas the risk with imatinib remained far lower at 3.6%.[18] Furthermore, the nilotinib toxicity profile also includes the risk factors which contribute to the higher risk of cardiovascular events, hypertension, hyperlipidaemia and impaired glucose metabolism.[10,19,20] While the 5-year incidence of cardiovascular events associated with dasatinib therapy (4%)[11] in the DASISION trial appeared to be less than the rates observed with nilotinib, other studies would suggest that the rates are similar to nilotinib.[21,22] Data from the BFORE study suggests that the cardiovascular event risk may be lower with bosutinib treatment compared with the other second-generation TKIs.[6]

The highest risk of arterial occlusive events lies with ponatinib. Based on cardiovascular toxicity observed in the early studies, ponatinib was transiently withdrawal from the market before being re-marketed with a black box warning for iatrogenic cardiac risk.[23] The 5-year follow up of the PACE study suggested the rate of arterial occlusive events was up to 31% in chronic phase CML patients treated with ponatinib.[24] The term arterial occlusive events was an umbrella term that encompassed all cardiovascular, cerebrovascular and peripheral vascular disease. However post-hoc analysis of pooled data from three clinical trials, specifically the phase I study, PACE and EPIC clinical trials, the ponatinib dose intensity was strongly associated with the risk of cardiovascular and arteriovenous events. For each 15mg decrease of ponatinib, the risk of an arterial occlusive event fell by 33%.[25] A prior history of ischaemic heart disease or older age were also associated with higher risk of arterial occlusive event development. Data from the OPTIC study supported this observation where patients commenced either 45, 30 or 15mg of ponatinib with the view to de-escalate to 15mg daily once a *BCR::ABL1*^{IS} of ≤1% was achieved.[26] Only 6% of the total cohort (n=282) developed arterial occlusive events with the event rate being 9.6%, 5.3%, and 3.2% for each dosing cohort respectively.[26] Moreover the rate of these events decreased over time in the 45mg cohort, from 7.6 events per 100 person-years in the first year of therapy to 5.9 events in the second year and falling to 0 events for years 3-5.[26] This corresponded with the median dose intensity of ponatinib falling to 15mg from the second year of study enrolment.[26] The 45mg

cohort was also more likely to achieve a *BCR::ABL1*^{IS} of ≤1% by 12 months (51.6%), enabling earlier dose reduction compared with the 30mg treatment arm (35.5%).[26]

While the long-term toxicity data for asciminib requires time to mature, early clinical trial data is available. The phase I study reported hypertension as the only cardiovascular adverse event, observed in 19% of patients.[27] Initial reports from the phase 3 ASCEMBL study, comparing asciminib to bosutinib in the third-line setting, reported a 48-week frequency of arterial occlusive events with asciminib 40mg twice daily as 4.5% compared with 1.3% with bosutinib 500mg daily.[28] However, of the events observed with asciminib, two were fatalities with one patient succumbing to ischaemic colitis following therapy failure with asciminib and switch to ponatinib and the second dying from an ischaemic stroke.[29] Notably, both patients had preceding cardiovascular risk factors and were treated with second-generation TKIs prior to asciminib therapy which arguably contributed to their cardiovascular risk. While at present, the risk of cardiovascular events with 40mg twice daily of asciminib appears to be less than nilotinib and ponatinib, this requires careful assessment over the long term, particularly in the frontline setting where prior TKI exposure is not a factor.

3.2 Pulmonary toxicity

The most significant toxicity related to dasatinib therapy is the development of pleural effusion, the risk cumulatively increasing over time. The incidence of pleural effusions within the first year of therapy with dasatinib 100mg daily was reported to be 8%, the annual risk similar for each subsequent year of therapy.[11] By 5 years, the incidence reported in the DASISION study was 28% in the dasatinib arm compared to only 0.8% of imatinib-treated patients.[11] More concerningly, dasatinib treatment carried a 5% risk of developing pulmonary hypertension based on the 5-year DASISION data whereas the risk was substantially lower with imatinib therapy.[11] Furthermore, while initial reports suggested that dasatinib-induced pulmonary hypertension was a reversible phenomenon once the offending TKI was ceased,[30] longer-term follow up suggests that significant changes in pulmonary vasculature may persist in up to a third of cases.[31,32] In comparison, the risk of pleural effusion appears to be less with asciminib therapy with 6% of patients reporting a pleural effusion in the phase I study on the latest follow up (median 14 months).[27] The incidence of grade 3/4 pleural effusion events were even less at approximately 3%.[27] However further detail about these events is yet to be released, specifically whether these patients had a prior history of dasatinib-related pleural effusions. There are currently no reports of pleural effusion nor pulmonary hypertension with asciminib monotherapy in the phase 3 ASCEMBL trial.[29]

3.3 Lipase elevation and pancreatitis

TKI therapy has also been associated with pancreatic enzyme elevation and in turn pancreatitis. The exact mechanism of the pancreatic dysfunction is postulated to be mediated by off-target effects of c-ABL inhibition by TKI therapy,[33] especially nilotinib and ponatinib. Grade 3/4 lipase elevations were observed in 9-10% of patients treated with nilotinib at the 5-year follow up of the ENESTnd study.[10] In comparison, only 4.3% of imatinib treated patients developed grade 3/4 lipase elevations by 5 years of

therapy.[10] The incidence of clinical pancreatitis however was only observed in 2-3% of nilotinib-treated patients whereas <1% of the imatinib cohort had reported pancreatitis.[10] The rates of pancreatitis are higher with ponatinib therapy with up to 7% of chronic phase CML patients developing pancreatitis by 5 years.[24] Pancreatitis has been infrequently reported with dasatinib and bosutinib therapy although up to 13.4% of patients treated with bosutinib in the BFORE study were reported to have grade 3/4 increase in serum lipase by 5 years.[34]

The most frequent non-haematological adverse event observed in asciminib therapy in the phase I study was lipase elevation which was reported in almost 27% of patients with only 10% being grade 3/4 events.[27] The incidence of lipase elevation was lower in ASCEMBL with only 3.8% of patients treated with asciminib having lipase elevation compared with 5.3% with bosutinib.[29] Clinical episodes of pancreatitis were noted in 3% of patients, most of these patients had documented episodes of pancreatitis with previous TKI therapy.[27] Also, all patients had been treated with doses of asciminib higher than the final recommended treatment dose of 40mg twice daily.[27] Importantly in the phase 3 study, where patients were treated with 40mg twice daily of asciminib, there were no reports of pancreatitis by 24 weeks.[29]

3.4 Other toxicities

The most frequently observed adverse events associated with asciminib therapy are haematological toxicities, predominantly thrombocytopenia and neutropenia. In the phase 3 study, almost 30% of patients in the asciminib arm developed thrombocytopenia with 21.8% reported as grade 3 or 4 events by 24 weeks.[29] By the same data cut off, only 9.2% of the bosutinib cohort had grade 3/4 thrombocytopenia.[29] Although difficult to compare to a third-line study, the upfront DASISION study had rates of grade 3/4 thrombocytopenia were 22% with 100mg of dasatinib compared with 14% with imatinib therapy by 5 years. Grade 3/4 thrombocytopaenia was only observed in 10.4% of patients following 5 years of nilotinib 300mg twice daily on ENESTnd. When evaluating the rates of grade 3/4 neutropenia, the results were similar between asciminib (17.9%) and bosutinib (14.5%) in the phase 3 ASCEMBL study. Dasatinib had a higher incidence of grade 3/4 neutropenia, reported as 29%, as did imatinib (24%) from the 5-year follow up of DASISION. Nilotinib was less likely to contribute to grade 3/4 neutropenia, reported at 12.2% from ENESTnd.

One of the other concerns associated with TKI therapy is the incidence of gastrointestinal toxicity such as diarrhoea, which can be very debilitating. Asciminib is infrequently associated with diarrhoea, with no cases reported in the phase 3 study by the 24-week data cut off.[29] In comparison, up to 10.5% of patients treated in the bosutinib arm of the ASCEMBL study developed grade 3/4 diarrhoea. Diarrhoea is infrequently reported with dasatinib therapy although there are rare cases of dasatinib-induced colitis.[35] Only 1.1% of patients treated with 300mg twice daily of nilotinib compared with 3.6% of imatinib-treated patients developed grade 3/4 diarrhoea by 5 years of the ENESTnd study. For patients with a history of gastrointestinal events or with concerns regarding gastrointestinal toxicity, asciminib may be the preferred agents.

4. What we know regarding the efficacy of asciminib

4.1 Pre-clinical data

In vitro models utilizing *BCR::ABL1* transformed Ba/F3 cells confirmed asciminib as having anti-proliferative effects on the target cells.[15] In fact, asciminib had marked inhibitory activity (based on IC50 values) against a multitude of *BCR::ABL1*-positive cell lines which included KCL22, a BP-CML cell line.[15] The transcript type (i.e. p210 or p190) had no effect on asciminib activity against the relevant cell lines.[15] Xenograft modelling derived from KCL22 and Ph-positive acute lymphoblastic leukaemia patients confirmed that appropriate concentrations of asciminib were able to regress and prevent cell growth with higher doses able to cause regression of the clonal population.[15]

4.2 Asciminib in the third line setting

4.2.1 Phase I data

The phase I dose escalation study included 141 CP-CML patients who were either intolerant or resistant to at least two prior conventional TKIs.[27] In addition, patients with T315I were eligible for study enrolment following at least one TKI if no other effective treatment was available. Additional arms were also available for patient enrolment which included relapsed/refractory CP/AP CML patients to be initiated on combination therapy with asciminib and either imatinib, nilotinib or dasatinib and relapsed/refractory BP CML or Ph-positive acute lymphoblastic leukaemia. However, data from these cohorts has not yet been published. At study enrolment, patients deemed resistant had haematologic, cytogenetic or molecular evidence of active CML.[27] The primary objective was to identify the maximum tolerated dose while also assessing the safety, pharmacokinetics and efficacy of asciminib. The study also involved dose-escalation and expansion phases. The twice daily schedule investigated seven dose levels (10mg, 20mg, 40mg, 80mg, 15omg, 160mg and 200mg) while once daily dosing evaluated three dose levels (80mg, 120mg and 200mg).[27] The higher doses were associated with more grade ≥3 dose limiting toxicities. Irrespective of the dosing schedule, the peak blood concentrations and the area under the curve was proportional to the administered asciminib dose. Preclinical xenograft models demonstrated that either 40mg twice daily or 80mg daily exceeded the 90% minimum inhibitory concentration.[27] This led to pharmacological modelling predicting that 40mg twice daily would maintain the 90% inhibitory concentration observed in the xenograft model, assuming the T315I kinase domain mutation was not present.[27]

A complete haematological response was observed in 92% of patients who entered the study in haematological relapse.[27] These patients did not harbour the T315I mutation. Complete cytogenetic remission (CCyR) was achieved in 54% of patients who entered the study without CCyR at a median time of 24 weeks.[27] At 6 and 12 months, 37% and 48% of evaluable patients achieved a major molecular response (MMR; *BCR::ABL1*^{IS}≤0.1%) respectively.[27] Patients with lower *BCR::ABL1* values at study entry (i.e. *BCR::ABL1*^{IS} <1%) were more likely to achieve MMR.[27] Of the 14 patients deemed either resistant or having unacceptable toxicity to ponatinib, 8 patients (57%) achieved/maintained MMR by 12 months. Deep molecular responses (MR4,

BCR::ABL1^{IS}≤0.01%) were achieved by 20% of patients by 12 months of asciminib monotherapy.[27]

Asciminib was discontinued in 17/141 patients due to progressive disease but only 4 patients had a documented initial response to asciminib (defined as ≥1-log reduction in *BCR::ABL1*^{IS} transcripts).[27] Two patients had documented myristoyl-site mutations at the time of progression. Disease progression was attributed to asciminib non-compliance in one patient while another with a documented E279K mutation at study enrolment had haematological and cytogenetic progression with failure to achieve MMR. Thirteen patients did not demonstrate any response to asciminib, defined as <1-log reduction in *BCR::ABL1*^{IS} transcripts,[29] and were withdrawn from the study. Eight of these patients harboured baseline kinase domain mutations, 6 with T315I mutation and 2 with other kinase domain mutations.[36]

4.2.2 Phase 3 data (ASCEMBL) study

Data from the phase 3 ASCEMBL study was recently published which directly compared asciminib to the second-generation TKI, bosutinib.[29] CP-CML patients who had been treated with ≥2 conventional TKIs who experienced either treatment failure[36] or intolerance were eligible for enrolment. *BCR::ABL1*^{IS} transcripts at the time of screening needed to be ≥1% and patients underwent a 2:1 randomization for either asciminib 40mg twice daily (n=157) or bosutinib 500mg once daily (n=76) stratified by their cytogenetic response at baseline (major cytogenetic response or not). A later protocol amendment permitted enrolment of patients with intolerance and *BCR::ABL1*^{IS} >0.1%.[36] The presence of either T315I or V299L deemed patients ineligible for the study because bosutinib would not be an effective option for these patients. Baseline mutation assessment identified 30 patients with kinase domain mutations which included 4 patients with the T315I and 1 patient with V299L mutations.[9] Given the study entry requirements, these patients were discontinued from the study. The rate of MMR achievement at 24 weeks of therapy was 25.5% with asciminib and 13.2% with bosutinib.[29] This increased to 29.3% and 13.2% by 48 weeks in the asciminib and bosutinib arms respectively.[28] Moreover, for patients that were not in CCyR at study entry, the 48-week rate of CCyR (equating to a *BCR::ABL1*^{IS} level of ≤1%) was 50.8% with asciminib compared with 33.7% in the bosutinib arm.[28] The 48-week rate of MR4 was also higher with asciminib (14%) compared with bosutinib (6.6%).[28] By the 48-week data cutoff, treatment failure, as defined by the ELN,[36] was 48.4% in the asciminib arm compared with (80.3%)[28] for those treated with bosutinib. This included therapy discontinuation for any reason. Asciminib was also better tolerated with fewer grade ≥3 adverse events compared to the bosutinib arm (54.5% vs. 67.1%) with markedly fewer patients ceasing asciminib due to toxicity (7.1% vs. 25%).[28]

4.2.4 Combination therapy

The distinct mechanism by which asciminib inhibits BCR::ABL1 function has made dual inhibition with conventional TKIs a promising therapeutic possibility. This has been assessed in the Phase I trial to determine whether the combination might improve molecular outcomes. *In vitro* data demonstrated that the addition of asciminib

enhanced BCR::ABL1 inhibition when combined with imatinib, nilotinib or dasatinib.[15] Neither myristoyl-site mutations nor kinase domain mutations emerged *in vitro* when asciminib was combined with nilotinib. Another study demonstrated that the combination of asciminib and ponatinib *in vitro* was able to inhibit leukaemic growth of T315I-containing compound mutants, that is where there are ≥2 mutations on a single *BCR::ABL1* molecule, in murine models.[37] The phase I study had treatment arms which combined lower doses of asciminib with either imatinib, nilotinib or dasatinib but this data is yet to be formally published.

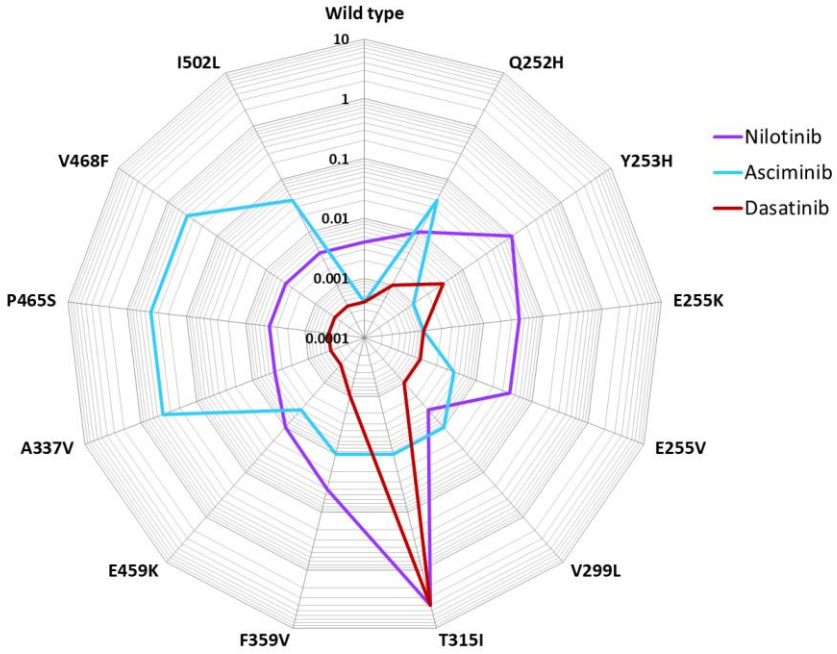
4.2.5 Asciminib and kinase domain mutations

As asciminib has a binding site distant from the kinase domain where all other conventional TKIs are active, it was hypothesized that asciminib would have activity against kinase domain mutations including T315I, which is the pan-TKI resistant kinase domain mutation only responsive to ponatinib. *In vitro* testing demonstrated that asciminib was active, to a variable degree, against all kinase domain mutations including T315I in engineered Ba/F3 cells containing the relevant kinase domain mutations.[15] *In vitro* investigation of asciminib-resistant cells identified point mutations in the myristoyl site (Table 1, Figure 1).[15] Functional validation utilizing engineered *BCR::ABL1*-expressing Ba/F3 cells containing individual myristoyl-site mutations as well as the kinase domain mutations were performed.[15] Asciminib was active against all kinase domain mutations including T315I however it had minimal activity against myristoyl-site mutations (Figure 2).[15]

Table 1: Myristoyl-site mutations that have emerged in the in vitro setting and on clinical trials

In vitro	**Emergence on clinical trials**
A337V	G109D
A344P	Y115N
P465S	A337T
F497L	G463D
	P465S
	V468F
	I502L

Figure 2: Kinase domain mutation sensitivity spectrum, specifically related to asciminib and catalytic domain TKIs, nilotinib and dasatinib.
Proliferation IC50 profiles in Ba/F3 *BCR::ABL1*-positive mutant cell lines based on in vitro data of asciminib, nilotinib and dasatinib. Image adapted from the data extracted from Wylie AA, Schoepfer J, Jahnke W, Cowan-Jacob SW, Loo A, Furet P, et al. The allosteric inhibitor ABL001 enables dual targeting of *BCR–ABL1*. *Nature*. 2017;543:733



From a clinical perspective, there were a total of 8 patients in the phase I study who had kinase domain mutations other than T315I at study entry, of which 5 (62%) achieved MMR following 12 months of asciminib monotherapy.[27] A total of 28 patients harboured T315I, of which 16 patients lacked hematological remission at study entry. Fourteen patients (88%) of those without haematological remission at study entry achieved this.[27] CCyR was attained by 41% of those without CCyR at baseline while MMR was realised in 24% of patients.[27] By 12 months, 50% of patients had demonstrated an improvement in their molecular response category.[27] However the doses required to achieve the same molecular responses as observed with patients without the T315I mutation appeared to be far higher. Early reports from the T315I-mutated arm of the phase I study, where patients were commenced on escalating asciminib doses, demonstrated a favourable safety profile for T315I mutated patients treated with 200mg twice daily asciminib, irrespective of prior ponatinib treatment.[38] Eight of the 28 patients enrolled with the T315I mutation achieved MMR by 24 weeks.[38] It is noteworthy that of the 13 patients who failed to demonstrate any response to asciminib in the phase I study, KD mutations were identified in 8 patients with 6/8 being T315I.[27] In the phase 3 study, 17 patients had kinase domain mutations (excluding T315I and V299L) with 6 patients (35%) achieving the primary end point of MMR by 24 weeks confirming asciminib's activity against these mutations. However, the F359 residue may confer a degree of asciminib resistance based on three patients who progressed on the phase I asciminib study as well as in vitro data.[37]

Myristoyl-site mutations (Table 1) were specifically assessed in the phase 1 study by means of bidirectional Sanger sequencing and NGS in evaluable patients with either molecular disease progression, loss of complete cytogenetic response or at 12 months of asciminib therapy.[27] In the 20 patients experiencing disease progression, 2 had identifiable novel myristoyl-pocket mutations.[33] Only 2 out of the 66 patients who completed 12 months of asciminib monotherapy without evidence of progressive disease had incidental detection of myristoyl-site mutations.[32] An AP-CML patient enrolled in the Phase I study with known V299L had early progression to BC-CML with

emergence of the novel V468F and I502L mutations.[14,15] Whether developing myristoyl-site mutations is always associated with resistance development is still not clear, but specific mutations with a sufficient allelic burden likely contribute to disease resistance.

4.2.6 Advanced stages of CML and Ph+ ALL

The phase I study only enrolled 9 patients with AP-CML with 7 patients demonstrating CHR with asciminib.[27] MMR was achieved in a single patient.[27] The phase I study also had a treatment arm for relapsed/refractory Ph+ ALL patients to be treated with dose escalation of asciminib but the outcome from this patient cohort is yet to be published. There was another phase I study (NCT03595917) investigating the combination of asciminib, dasatinib with prednisolone in Ph+ ALL in either the relapsed/refractory or upfront therapy in unfit patients.[39] Asciminib was dosed at either 40mg, 80mg and 160mg in combination with 140mg of dasatinib and prednisolone with the primary objective of evaluation for maximum tolerated dose followed by defining the depth and durability of responses.[39] The primary toxicity was asymptomatic amylase and lipase with preliminary results demonstrating all evaluable patients achieved at least a hematological remission.[39] Further results from this trial are awaited.

4.2.7 Ponatinib or asciminib?

Based on preliminary data, asciminib certainly is an excellent choice in the resistant and intolerant setting following at least 2 lines of TKI therapy. In comparison to bosutinib, asciminib-treated patients were more likely to achieve MMR by 48 weeks and also had fewer toxicities.[28] The phase 1 and ASCEMBL data enabled the FDA to grant accelerated approval for asciminib in either the third-line setting or for T315I mutated patients. However, the option of ponatinib already exists for these two patient groups, so clinicians have a choice to make which cannot be informed by any trial which compares asciminib to ponatinib. How can clinicians determine the best approach for their patient who is eligible for either ponatinib or asciminib? This choice has become even more difficult since the results of the OPTIC trial were published[26] and a recent spotlight on asciminib explores this specific issue.[40] Asciminib has excellent tolerability in comparison to conventional TKIs at the standard dose of 40mg twice daily, with an early signal suggesting lower rates of cardiovascular toxicity compared with the second- and third-generation TKIs. It also has demonstrated efficacy against the T315I mutation which has long been a clear-cut indication for ponatinib. However, data from OPTIC demonstrates that the risk of ponatinib-associated arteriopathy is proportional to the dose administered and that the rate of arterial occlusive events was substantially less with dose modified ponatinib[26] compared with the earlier published data from the PACE study.[24] Intention-to-treat analysis demonstrated that the exposure-adjusted risk of an arterial occlusive event in the 45mg ponatinib cohort was 7.6% per 100-person years[26] during the first year of treatment, although the majority of patients had early de-escalation to 15mg by 12 months due to achievement of *BCR::ABL1*^{IS} of ≤1%. Furthermore, the risk of arterial occlusive events in this cohort fell further in the second year, due to the marked dose modification that occurred within the first year of therapy as the median dose intensity of ponatinib was 15mg by 12 months.[26] In comparison, the 48 week rate of arterial occlusive events observed with asciminib was 4.5%.[28] The

ongoing risk associated with asciminib will only become evident with longer follow-up, but it is unclear whether the risk of cardiovascular events will plateau or continue to rise with cumulative dosing. In the T315I setting however, early limited data from the asciminib studies suggest that higher drug doses are required to achieve molecular response, which are potentially associated with higher rates of toxicity including cardiovascular events and pancreatitis.[27]

Comparing the efficacy of both asciminib and ponatinib is difficult without a head-to-head study but if we contrast the 48-week data from ASCEMBL[28] to 12-month results from OPTIC,[26] some conclusions may be drawn. CCyR was similar across both studies, being achieved by 49% of patients without T315I mutations in the 45mg ponatinib cohort[26] compared to 42% of asciminib-treated patients in ASCEMBL.[28] The cumulative incidence of MMR in the asciminib arm was 29.3% in ASCEMBL[28] compared with 35% for the 45mg ponatinib group of OPTIC.[26] However, the two study populations are slightly different. Almost all patients enrolled in the OPTIC study experienced resistance to the prior line of therapy[41] whereas only 61% of the asciminib cohort had lack of efficacy to the prior TKI.[29] Furthermore, of patients without a major cytogenetic response at ASCEMBL study entry, only 17% achieved MMR at 24 weeks compared to 46% who were in a major cytogenetic response.[29] Similar data from OPTIC is not as yet reported but if we look to the PACE study, 41% of CP-CML patients with resistance to the prior 2G-TKI achieved MMR by 12 months on ponatinib.[24]

Based on this data, there may be specific clinical scenarios where one drug should be favoured over the other.[42] Asciminib should be the preferred agent in the third line setting in intolerant patients without frank TKI resistance whereas ponatinib may be more suited to patients with pan-TKI resistance, especially in patients who have never demonstrated molecular response, with the view to de-escalate dosing once a *BCR::ABL1* ^{IS} of ≤1% is achieved. Either agent has demonstrable activity against the T315I mutation although higher doses of asciminib are required to achieve response which in turn is associated with increased toxicity. Similarly, either agent would be efficacious for non-T315I kinase domain mutations with the possible exception of mutations at the F359 residue which will require extra consideration due to the preliminary data observed with asciminib.[37] Until more data is available, advanced stages of CML or Ph+ ALL would be better managed with ponatinib. However, the addition of asciminib to ponatinib in this setting has merit and should be investigated.

4.3 Upfront therapy

While there is clear evidence of the utility of asciminib in later lines of therapy, the next question is whether asciminib can be utilised in earlier lines of therapy, including the first-line setting. There are now several clinical trials recruiting newly diagnosed CP-CML patients for first line therapy with asciminib. The phase 2 Australian CML13 study (ASCEND, ACTRN12620000851965) is enrolling 100 newly diagnosed CP-CML patients to commence treatment with 40mg twice daily of asciminib with the view to escalate asciminib dosing or add a second TKI (imatinib, nilotinib or dasatinib) if ELN defined milestones[1] are not achieved. The German CMLXI study (FAsciNation, NCT03906292), also recruiting, is investigating the potential of adding on asciminib following 12 weeks

of conventional first-line TKI options. Novartis have also initiated a randomised phase 3 study (ASC4First; NCT04971226) comparing 80mg of asciminib daily to investigator selected TKI, aiming to recruit almost 400 patients.[43] While data in the upfront setting is currently lacking, these clinical trials will hopefully determine safety and efficacy in a cohort who have not already experienced toxicity and/or resistance to prior TKI therapy. Other questions that will hopefully be answered by these frontline studies include whether asciminib has a more favourable toxicity profile compared to second-generation TKIs, especially with regards to cardiovascular events. Moreover what is the rate, timing, and stability of deep molecular response (DMR), equating to an MR4.5 response (*BCR::ABL1*^{IS} ≤0.0032%), of frontline asciminib, which future treatment-free remission (TFR) attempts are completely dependent upon.

4.4 Other possible roles for asciminib in chronic phase CML

Enhancing DMR achievement with asciminib is the focus of another clinical trial which has a similar study design to the ENESTcmr study[44]. The ASC4MORE (NCT03578367) study compares nilotinib switch to the addition of asciminib to imatinib in patients yet to reach DMR on imatinib monotherapy.[45] The primary objectives are safety and rate of DMR of combination therapy (imatinib/asciminib) compared with nilotinib. Increasing the rate of DMR achievement will maximize the number of patients who will be eligible for an attempt at TFR. On a similar note, asciminib is being investigated as consolidative therapy as an add on to other TKIs to maximise the success of TFR attempts, either in the first or second attempt.

5. Future directions and questions

Early data from clinical trials demonstrate that asciminib is a promising therapy in CML in the resistant and intolerant setting. It appears to have a relatively benign toxicity profile although longer term data is needed, especially with regard to the cardiovascular toxicity signal. Its utility in the first line setting is being investigated and data regarding combination therapy or efficacy in Ph+ ALL is still to be published. The most important question to address currently is where asciminib might be preferable to ponatinib in the third line setting and in cases where a second-generation TKI has failed as first-line therapy. Both asciminib and dose-modified ponatinib are excellent options but it is likely that one or other will be a better option in specific settings. For instance, the “pan-intolerant” patient would likely be a better candidate for asciminib whereas the “pan-resistant” patient with no evidence of molecular response to prior TKIs may be better managed with ponatinib initially. Finally, will asciminib have a major role as an “add-on” drug where an ATP-competitive TKI has not achieved a sufficient improvement in molecular response, or would simply switching to asciminib be a better option? As more clinical trial data and experience matures (Table 2), the answers to these critical questions will become clearer.

Table 2: Future directions and questions to be answered

Clinical setting	Clinical trials (if available)	Question to be answered
First-line	Australian CML13 (ASCEND, ACTRN12620000851965)	Efficacy of asciminib in the firstline setting with option to

		dose intensify for failure to achieve ELN-defined molecular milestones
	German CMLXI study (FAsciNation, NCT03906292)	Efficacy and safety of the addition of asciminib to conventional firstline TKIs
	Novartis-led study (ASC4First; NCT04971226)	Comparison of 80mg daily of asciminib to investigator selected TKI
Second-line	-	Can asciminib rescue patients with second-generation TKI resistance or will dose-modified ponatinib ponatinib be preferred
Third-line	Phase 1 (NCT02081378)	Dose finding study of asciminib monotherapy in CML following resistance or intolerance to ≥2 prior TKIs
	ASCEMBL (CABL001A2301, NCT03106779)	Comparison of asciminib to bosutinib in CP-CML patients with resistance or intolerance to ≥2 prior TKIs
Improve rate of DMR achievement	Novartis-led study (ASC4MORE, CABL001E2201, NCT03578367)	Comparison of the combination of imatinib and asciminib versus nilotinib switch in patients who have failure to reach DMR on firstline imatinib
Improve rates of TFR	HJKC3-0003 (NCT04838041)	Efficacy of combination therapy of imatinib and asciminib on rate of second TFR in patients who have previously failed an attempt to discontinue imatinib
Combination therapy with asciminib	Phase 1 (NCT02081378)	Safety and efficacy of the addition of asciminib to either imatinib, nilotinib or dasatinib in CP-CML patients with resistance or intolerance to ≥2 prior TKIs
T315I mutations	Phase 1 (NCT02081378)	Safety and efficacy of asciminib 200mg twice daily in T315I mutated patients
Paediatric CML	Phase 1 (NCT04925479)	Efficacy and pharmacokinetic profile of asciminib in CP-CML paediatric patients treated with ≥1 prior TKIs

Ph+ ALL	Phase 1 (NCT02081378)	Safety and efficacy of asciminib 200mg twice daily in Ph+ ALL resistance or intolerance to ≥2 prior TKIs

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Author contribution

NS and TPH wrote, reviewed and proofread the manuscript.

Data Availability Statement

Data sharing is not applicable to this review article as no new data were created or analyzed in this study.

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APPENDIX I
LOW-LEVEL BCR-ABL KINASE DOMAIN MUTATION DETECTION

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1. PURPOSE / BACKGROUND

BCR-ABL1 kinase domain mutations are the most common known mechanism of resistance to tyrosine kinase inhibitors (TKI) for patients with chronic myeloid leukaemia. Some mutations may be present below the level of detection by standard Sanger Sequencing. Our studies have demonstrated that low-level *BCR-ABL1* mutations are predictive of treatment outcome when detected in patients with TKI failure who subsequently switched to nilotinib or dasatinib. 84% of low-level mutants that conferred resistance to the subsequent TKI rapidly emerged with treatment failure. Furthermore, patients with multiple mutations at second-line TKI initiation had a poor response to subsequent therapy, irrespective of the resistance profile of the mutations. These patients also had a high rate of acquiring new resistant

mutations on nilotinib/dasatinib therapy. In contrast, our recent work demonstrated that multiple mutations detected prior to ponatinib initiation did not confer a poor outcome after TKI failure for most patients. The exception were patients with a T315I mutation plus other subclonal mutations. These patients had substantially inferior responses. The data indicate that the detection of low-level mutations after TKI resistance offers critical information to guide therapy selection. The Mass Spectrometry method allows detection of low-level *BCR-ABL1* kinase domain mutation at an average sensitivity of 0.2% mutant. The assay has now been modified to incorporate additional mutations including those observed with the novel TKI, asciminib.

This document was originally prepared by: Susan Branford
This document has been modified by: Naranie Shanmuganathan

2. PERIODIC REVIEW REQUIREMENT (RISK ASSESSMENT)

See PRC-EXE-249 for risk assessment methodology.
Risk Rating: Low
Review Period: 12 months

3. WH&S CONSIDERATIONS

3.1. Safe Work Practices

3.1.1. Procedures should be conducted in accordance with safe work practices.
3.1.2. Minimum PPE and Precautions in PRC-EXE-369 Biosafety Manual Mandatory Precautions and Personal Protection must be followed for laboratory and Specimen Collection procedures.
3.1.3. The adoption of sound ergonomic work practices and safe manual handling techniques is mandatory.

3.2. Specific Hazards

3.2.1. Physical: NA
3.2.2. Chemical: NA
3.2.3. Biological: NA

4. PROCEDURE

4.1. Reagents

4.1.1. iPLEX® Pro, PCR reagent and SpectroCHIP kit
Agena, #10217
EXT-INS-2404
Materials provided
PCR reagent set

- $MgCl_2$
- 10X PCR buffer
- dNTP mix
- PCR enzyme

iPlex reagent set

- iPLEX Pro enzyme
- iPLEX termination mix (not used)
- iPLEX buffer plus
- SAP buffer
- SAP buffer
- 3-Pt calibrant

SpectroCHIPs x10
Clean resin
Store reagents at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186)
Store 3-Pt calibrant in ≤-10°C (asset number 48126) in front of 0-18-N
Store SpectroCHIPs at room temperature in compactus (0-24-N)

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4.1.2. MassArray Clean Resin, 28g
Agena, #08040
Store at room temperature in lab area

4.1.3. Individual iPLEX® Pro terminators A/C/G/T
Agena, #17936
Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186)

4.1.4. Mutant and wild type Plasmids
Stored in cold room (0-26-N)

4.1.5. gBlocks (see Appendix 1)
IDT, https://sg.idtdna.com
Ordered at 1000ng (1727fmol)
See 5.1.1 for storage and reconstitution

4.1.6. Primers (see Appendix 2 and 3)
IDT, https://sg.idtdna.com
Order PCR primers as 25 nmole scale, UEP as 250 nmole scale
Order PCR primers and UEP primers in separate DNA plates.
Both desalted purification.
Ship dry and reconstitute to 100 uM for PCR primers and 500 uM for UEPs with water.
Get primers normalized to the yield of the lowest primer when ordering from IDT.
Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186)

4.1.7. Salmon sperm DNA (50ng/ul)
Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186)

4.1.8. 1:10 Bromophenol Blue (3.13mg/ml)
Stored at room temperature in dye box outside office 0-18-N next to UV hood in post PCR area

4.1.9. 60x Xylene cyanol dye (3.2mg/ml)
Stored at room temperature in dye box outside office 0-18-N next to UV hood in post PCR area

4.1.10. Standard diluent
Reagents: 1mmol/L Tris-HCl pH 8.0, 0.1 mmol/L EDTA, 50ug/ml Salmon sperm DNA
To make up to 100ml, combine: 100uL 1M Tris-HCl pH 8.0
3.7mg EDTA
0.5ml 10mg/ml Salmon sperm DNA
Store at 2-8°C in DNA room (0-33-N)

4.1.11. Molecular grade water – should be the only water used throughout the whole assay

4.2. Consumables

4.2.1. 4titude adhesive PCR seals
Integrated Sciences, #4ti-0500 (100 sheets)

4.2.2. 384 well plates: Thermo Fast 384 PCR plate fully skirted clear (50 plates/box)
ThermoFisherScientific. # TF-0384

4.2.3. Combitips advanced ® 0.1ml (100 pieces)
Eppendorf, #0030089405

4.2.4. One Touch Tips 10ul barrier tip
Sorenson Bioscience Inc, #10320

5. MASS SPECTROMETRY

5.1. Preparation for assay

5.1.1. Prepare standards.

Wells 1-4: mutant plasmid mixture, wild type plasmid and water. These are mixtures of plasmids, mutant *BCR-ABL1* plasmids diluted in wild type *BCR-ABL1* plasmid. They were prepared in pGEM-T-easy and contain the e14a2 *BCR-ABL1* kinase domain amplified using Long1-F and Long1-R primers (PRC-GMO-342). The mutant plasmids have been diluted in wild type plasmid

as detailed in the JCO 2011 paper[1] 2%, 0.5%, 0.2%, 0.05%, such that approximately $3.2x10^5$ copies of *BCR-ABL1*/2ul.

If need to prepare new controls, use Long1-F and Long1-R primers (PRC-GMO-342) (2ul plasmid plus 2ul Hela cDNA.

- Purify the PCR product using the ExoProStar method outlined in PRC-GMO-342 and dilute 1 in 3 to mimic the amount of PCR product recovered in patient samples.
- The plasmid mixes are stored in a box marked v11.3 plasmids in the cold room (0-26-N).

Wells 5-8: mutant gBlocks, wild type gBlock and standard diluent. gBlocks are double-stranded DNA fragments with the required mutations diluted in wild type gBlock to the required mutant percentage. The gBlock design is illustrated in Appendix 1.

The instruction for gBlocks reconstitution can be found at the IDT website and is detailed below: https://sg.idtdna.com/pages/education/decoded/article/tips-for-working-with-gblocks-gene-fragments

- To reconstitute the gBlocks
  - Pulse-spin the tube for 5 seconds
  - Add 100uL molecular grade water for a final concentration of 10ng/uL
  - Vortex and incubate at 50˚C for 20 minutes.
  - Briefly vortex and centrifuge. Determine the concentration using the Qubit HS kit PRC-GMO-358 (repeat quantification at least twice).
- The estimated copy number for each gBlock is 1.041806×10^{10} copies/uL.
  - Add 3.1uL of stock solution to 1mL of standard diluent containing salmon sperm as a carrier.
  - Perform a further 1:100 dilution with the standard diluent) to reduce the copy number of the gBlock to a closer level to the plasmid concentrations in wells 1-4 ($3.2x10^5$ copies of total *BCR-ABL1* per 2 µL) but to also minimize off-target amplification.
- Create a dilution series for gBlocks 1, 2 & 3 (dilute in wild type gBlock) to obtain the required mutant percentages (2%, 1.5, 1%, 0.5%). The working solution of the gBlocks is estimated to have 3.2×10^5 copies/uL.
  - Frozen aliquots of the gBlock dilutions are stored in a box labelled “gBlock dilutions” in the -20°C freezer (asset number 48126) in front of 0-18-N
- Amplify the fragments using the specifically designed primers (Appendix 2) following the long PCR protocol in PRC-GMO-342 and purify the PCR product using the ExoProStar method outlined in PRC-GMO-342 prior to use in the mass spectrometry assay.

5.1.2. *BCR-ABL1* negative patients for negative cluster control

Select cDNA samples from patients being screened for CML that have been proven to be *BCR-ABL1* negative through gold standard *BCR-ABL1* quantitative PCR testing (PRC-GMO-353)

- Amplify the cDNA using the specifically designed primers (Appendix 2) following the long PCR protocol in PRC-GMO-342 and purify the PCR product using the ExoProStar method outlined in PRC-GMO-342 prior to use in the mass spectrometry assay.

5.1.3. Prepare PCR primer pools for each well according to the volumes listed in FOR-5447 (Appendix 4).

- Pools contain ~500nM of each mutation PCR primer (stocks at 100uM each).
- Reconstitute as indicated in the PCR shipment then
  1. Add the appropriate amount of each primer to indicated volume of molecular grade water according to the “PCR primers for W1-8” tab.
  2. Label with type of assay and well number, date and initials.

3. To ensure that the PCR primer mix works, can perform first step of mass spectrometry assay and perform gel to ensure that PCR products are generated
4. Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186) for multiple use.

5.1.4. Prepare UEP mixes for each well as indicated in FOR-5446 UEP pools W1-4 low-level *BCR-ABL1* and FOR-5448 UEP pools W5-8 low level *BCR-ABL1* (Appendix 5).

- Reconstitute primers as indicated to a make the UEP concentration to 500nM.
- Optimization step (will need to be performed prior to use of newly ordered extension primer or when UEP mix needs to be remade).
  1. Enter volume of final UEP mix (orange square) (Usually 1000ul)
  2. Pool displayed volumes of UEP stock (green squares) and add molecular grade water to make up final UEP volume
  3. Take aliquot and fire on MALDI-TOF (2ul UEP mix + 25ul water)
  4. Adjust UEP mix according to primer adjustment report and spectrum results (see Appendix 6)
  5. Pool the UEP mixes together (2uL of each UEP mix + 19ul water) and fire on MALDI-TOF and adjust to ~ intensity 30
  6. Once satisfied with UEP mix, label with type of assay and well number, date and initials.
  7. Store at ≤10°C in PCR room (1-218-H) freezer (asset number 49186) for multiple use.
- Note there is redundancy built into the assay with the A337V included in both wells 7 and 8. The F497L is also included in both wells 7 and 8 but does not work efficiently in well 7 and so only analyse in well 8. The assay is validated with both primer sets and therefore include both mutation sets when reordering primers.

5.1.5. Prepare oligo spikes FOR-5449 (Appendix 7)

1. Enter volume of final UEP mix + 10% (orange square)
2. Add displayed volume of oligo QC stock to 3 separate tubes (pink square)
3. Add displayed volume of molecular grade water to each tube (yellow square)
4. Pool 0.5ul of each oligo spike in one tube (0.5ul of the 3 oligo spikes + 25.5ul water) and run on mass spectrometry to ensure the intensities match and adjust as required
5. Make up enough volume for 10 wells to get average reading
6. Adjust oligo spikes according to the primer adjustment report and spectrum results (Appendix 6)
7. Once satisfied with oligo spikes mix, label with type of spike, date and initials.
8. Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186) for multiple use.

5.1.6. Prepare Terminator Mix (see Table 1)

1. Pipette 27ul of each required terminator nucleotide into an 1.5 Eppendorf tube
2. Add Molecular grade water to make a total volume of 300ul
3. Label with type of assay and well number, date and initials and type of terminators (i.e. A,G,T,C)
4. Store at ≤-10°C in PCR room (1-218-H) freezer (asset number 49186) for multiple use

Table 1: Terminator nucleotide mix for each well

Terminator Mixes	27ul of each terminator in 300uL volume	Total number of terminators	Water to be added (ul)
W1	ACG	3	219
W2	CGT	3	219
W3	TC	2	246
W4	TG	2	246
W5	TGC	3	219
W6	TG	2	246
W7	TGA	3	219
W8	AC	2	246

N.B. W2 and W5 have same terminator mix as does W4 and W6.

5.1.7. Prepare mutant 50% mutant KU812 cell line mix (PRC-GMO-342)

1. Vials of wild-type and mutant cell line cells stored in freezer (asset number 535139) in DNA room (0-33-N).
2. Vials of extracted RNA stored in freezer (asset number 535139) in DNA room (0-33-N).
3. Generate cDNA via RT-PCR but run cDNA in parallel to patient samples to serve as a positive control in the mass spectrometry assay.
4. Mix mutant in wild type KU812 cell line in a 1:1 ratio to generate a 50% mutant mix
5. Store at ≤-10°C in PCR room (0-33-N) freezer (asset number 37214) for multiple use

5.1.8. Prepare BV173 mutant cell line (PRC-GMO-342)

1. 50% mutant mix of BV-173 already stored in DNA room in freezer (asset number 371299).

5.1.9. Prepare resin

1. Using a sealable 30ml clean trough, add clean resin to a third of the height of the trough. Fill the remaining two-thirds with molecular grade water. This does not need to be precise. This setup is for multiple uses and can keep adding more resin and water as required.
2. Let the resin settle before use and ensure uniform spread in trough.
3. Store at room temperature in post PCR area outside 0-18-N

5.2. Performing the mass spectrometry chemistry reactions

The samples utilized for the mass spectrometry assay are required to be either a long or a nested PCR product. Mass spectrometry will be performed in duplicate to validate results. Consequently, prepare PCR products of samples in duplicate. On occasion, a third mass spectrometry run may be required for consensus results to be reported. PCR products for the additional run can be generated as needed.

5.2.1. Sample preparation

For all samples to be tested (including patient samples, standards, negative and positive controls)

- Perform RT-PCR to generate cDNA from extracted RNA (PRC-GMO-353)
- Generate long PCR products (PRC-GMO-342) with relevant PCR primers and perform an e-gel to ensure PCR product present.
    - Long2 primers are used for p210 transcript patients if asciminib-specific mutations are being investigated to ensure that the myristoyl site is captured
    - e1a2 primers are to be utilized for patients with an e1a2 transcript

  - Long2 primers are to be used for the positive controls (mutant KU812 and BV173 cell lines)
  - Use the *ABL1* primer (Appendix 2) for *BCR-ABL1* negative patients, Hela and gBlock dilution standards
  - Use Long2 primers for the 100% *BCR-ABL1* wild type plasmid
- Clean up samples using ExoSAPit (PRC-GMO-342)
  - If a band is observed on e-gel, the sample is ready for mass spectrometry
  - If no band is observed on e-gel, perform nested PCR (PRC-GMO-342) using relevant primers and perform e-gel
  - If a band is observed on e-gel, then clean up samples using ExoSAPit (PRC-GMO-342) and the sample is ready for mass spectrometry
  - If no band is observed despite nested PCR, then sample analysis cannot proceed and record the results as failed long and nested PCR. This usually occurs when there are inadequate *BCR-ABL1* transcripts for amplification of the kinase domain
- If samples are anticipated to have a *BCR-ABL1* <1%, omit the e-gel step following the long PCR, clean up with ExoSAPit and proceed straight to a nested PCR.

The final design of the 8 well mass spectrometry assay is listed in Appendix 8.

- For a full 384-plate to be maximized, it is recommended to divide each plate into 4 sections (6 columns wide) (Figure 1)
- This will allow up to 96 samples to be tested in each well, including the 10 controls. Therefore, a maximum of 86 patient samples can be tested.
- Wells 5-8 enable detection of the asciminib-resistant mutations. If these mutations are not required, then only wells 1-4 are used for mutation detection. Wells 1-8 are used when asciminib resistant mutations are analysed
- Two plates will be needed for 86 patient samples to be tested for all 8 wells.
- For efficiency, prepare plates for wells 1-4 and wells 5-8 separately

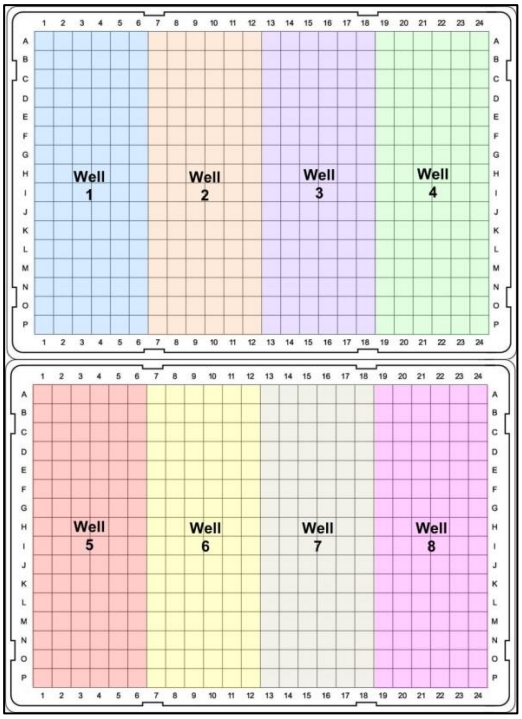
5.2.2. Arrange samples and load into 384-well microtitre plate

1. Use the ‘Plate Setup sheet’ in FOR-5450 to determine which order to add the PCR tubes to a 96 well plate. Setup the tubes in 3 x 96 well plates that have been UV treated so that there are 2 empty columns between each column of tubes, to allow ease of tube opening. Copy the relevant controls (listed at the bottom of the ‘Sample’ tab) into the sample list.

   For W5-8, the plasmid standards and the negative control used for W1-4 will need to be substituted with the gBlock standards. This will be the only sample difference between W1-4 and W5-8.

2. Determine where the samples will be added into the 384 plate (see Figure 1). Mark the plate with a marker pen to locate the wells for addition of the different master mixes. Add sample in 24-well (6x4) blocks to minimize chip wastage. Set up the samples for a whole plate of *BCR-ABL1* analysis with W1 in plate well A1-P6, W2 in A7-P12, W3 in A13-P18 and W4 in A19-P24. Therefore, will have the same sample in A1, A7, A13 and A19. Mark the regions on the plate where the master mixes will be added. If asciminib-specific mutations are to be investigated, then the same arrangement will follow but with W5 in place of W1 etc. Label the side of the plate with the date and initials.

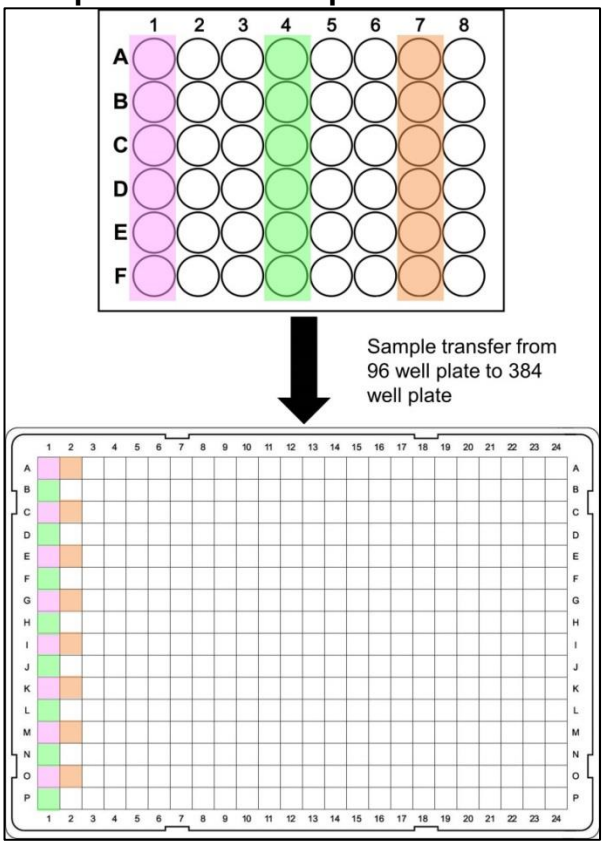
Figure 1: Recommended 384-well plate set up for all 8 wells to be tested on mass spectrometry.



3. In the UV hood, add **1ul** of cDNA sample to each well using the 0.5-10 ul pipette (8 or 12 channel) and the One Touch Tips 10ul barrier tips. Ensure the tips are firmly secured. After aspirating the sample, check the liquid level in the tip before dispensing. The samples are added in the following positions (as shown on the 'plate setup sheet') which is also demonstrated in Figure 2:

96-well plate	384-well plate
A1 – and rest of col 1	A1, A7, A13, A19
A2 – and rest of col 2	B1, B7, B13, B19
A3 – and rest of col 3	A2, A8, A14, A20
A4 – and rest of col 4	B2, B8, B14, B20
etc…	

Figure 2: Sample transfer from 96 well plate to 384 well plate



4. Cover with “4titude Adhesive PCR seals” adhesive film and firmly seal.
5. Centrifuge plate for 30 seconds at 2000rpm
6. **Check by eye that there is sample in each well** and if necessary, add an extra 1ul – make sure that the correct sample is added. Store in the cold room until ready to add PCR master mix.

5.2.3. Perform PCR reaction

1. Use Mass spectrometry assay reagent template form (FOR-5451) and populate the form with the date of plate setup, book and Page #s, Operator initials and Thermal cycler asset No.
2. Enter number of samples + ~20% being tested in the ‘# reactions’ box to calculate the volumes of reaction.
3. Print form and take to PCR room (1-218-H) with an esky of ice for transport.
4. Prepare PCR mixes for each well as indicated by printed setup sheet.
    a. Thaw PCR primer pools, PCR oligo spike, 10x PCR buffer, MgCL2 (25nM), dNTP mix and Salmon sperm DNA.
        i. Vortex and centrifuge briefly before use.
    b. Take out iPLEX PCR enzyme and leave in ice in esky or on cold block in freezer (asset number 49186) located in PCR room (1-218-H).
    c. Label 1.5ml Eppendorf tubes with appropriate well number and add indicated reagents. Tick off reagents as they are added in the appropriate boxes on set up sheet.

d. Vortex the 4 master mixes until thoroughly mixed and transport on ice to the post PCR area outside office 0-18-N.

5. In the UV hood, remove seal from 384 well plate while taking care to ensure no spillage of sample from plate.
6. In the UV hood, **add 4ul of master mix** to each well using either a 0.5-10 ul multistepper or a 0.5-10 ul multichannel pipette (ie eppendorf) using 10 ul tips. Open the box of tips in the UV cabinet and be careful to not touch the tip when removing – i.e. touch the butt only. Check by eye that liquid has been added to each well.
7. Use 4titude PCR seal on plate and seal firmly over surfaces and edges.
8. Mix on the plate mixer using the 384-well settings for 2 minutes (unclip the 96-well block and clip in the 384-well block).
9. Centrifuge plate for 30 seconds at 2000rpm.
10. Cycle in 384 well cycler using the IPLEXPCR program.
    This program is in the folder labelled 'Research' under Leukaemia Unit. The IPLEXPCR program should take ~2.5 hours.
    1. 94^{o}C for 4 minutes
    2. 94^{o}C for 20 seconds
    3. 60^{o}C for 30 seconds
    4. 72^{o}C for 1 minute
    5. *35* cycles steps 2-4
    6. 72^{o}C for 3 minutes
    7. 12^{o}C infinity
11. At the end of cycling, centrifuge the plate for 30 seconds at 2000 rpm.
12. If needed, plate can be stored in the cold room over night before proceeding to the next stage.

5.2.4. Perform SAP (shrimp alkaline phosphatase) reaction to inactivate unused dNTPs

1. If 384 well plate was stored overnight, centrifuge for 30 seconds at 2000 rpm.
2. Take an esky of ice with set up sheet to the PCR room (1-218-H).
3. Prepare PCR mixes for each well as indicated by printed setup sheet.
    a. Thaw SAP oligo spike and SAP buffer
        i. Vortex and centrifuge briefly before use.
    b. Take out SAP enzyme and leave in ice in esky or on cold block in freezer (asset number 49186) located in PCR room (1-218-H).
    c. Label 1.5ml Eppendorf tubes with appropriate well number and add indicated reagents. Tick off reagents as they are added in the appropriate boxes on set up sheet.
    d. Vortex the 4 master mixes and transport on ice to the post PCR area outside office 0-18-N.
    e. Add the recommended volume of Bromophenol Blue Dye in the UV hood and vortex master mixes well. The four master mixes will be exactly the same.
4. In the UV hood, remove seal from 384 well plate while taking care to ensure no spillage of sample from plate.
5. In the UV hood, **add 2ul of master mix** to each well using either a 0.5-10 ul multistepper or a 0.5-10 ul multichannel pipette (ie eppendorf) using 10 uL tips. Multichannel pipette is preferred to minimize contamination. Open the box of tips in the UV cabinet and be careful to not touch the tip when removing – i.e. touch the butt only. Check by eye that liquid has been added to each well as wells with new mix will have a light blue shine to them.
    a. If there are many samples in the run (i.e. a full plate), place the plate in ice while master mix is being added
6. Use 4titude PCR seal on plate and seal firmly over surfaces and edges.

7. Mix on the plate mixer using the 384-well settings for 2 minutes (unclip the 96-well block and clip in the 384-well block).
8. Centrifuge plate for 30 seconds at 2000rpm.
9. Cycle in 384 well cycler using the IPLEXSAP program.
   This program is in the folder labelled 'Research' under Leukaemia Unit. The IPLEXSAP program should take ~40 minutes.
   1. 37ºC for 40 minutes
   2. 85ºC for 5 minutes
   3. 12ºC infinity
10. At the end of cycling, centrifuge the plate for 30 seconds at 2000 rpm.
11. If needed, plate can be stored in the cold room over night before proceeding to the next stage.

5.2.5. Prepare iPLEX reaction (extension)

1. If 384 well plate was stored overnight, centrifuge for 30 seconds at 2000 rpm.
2. Take an esky of ice with set up sheet to the PCR room (1-218-H).
3. Prepare PCR mixes for each well as indicated by printed setup sheet.
   a. Thaw EXT oligo spike, iPLEX buffer plus, iPLEX terminator and UEP primer pool for each well
      ii. Vortex and centrifuge briefly before use.
   f. Take out iPLEX Pro enzyme and leave on ice in esky or on cold block in freezer (asset number 49186) located in PCR room (1-218-H).
   g. Label 1.5ml Eppendorf tubes with appropriate well number and add indicated reagents. Tick off reagents as they are added in the appropriate boxes on set up sheet.
   h. Vortex the 4 master mixes and transport on ice to the post PCR area outside office 0-18-N.
   i. Add the recommended volume of Xylene Cyanol in the UV hood and vortex master mixes well.
4. In the UV hood, remove seal from 384 well plate while taking care to ensure no spillage of sample from plate.
5. In the UV hood, **add 2ul of master mix** to each well using either a 0.5-10 ul multistepper or a 0.5-10 ul multichannel pipette (ie eppendorf) using 10 uL tips. Open the box of tips in the UV cabinet and be careful to not touch the tip when removing – i.e. touch the butt only. Check by eye that liquid has been added to each well as wells with new mix will have a blue tinge to them.
   a. If there are many samples in the run (i.e. a full plate), plate the plate in ice while master mix is being added
6. Use 4titude PCR seal on plate and seal firmly over surfaces and edges.
7. Mix on the plate mixer using the 384-well settings for 2 minutes (unclip the 96-well block and clip in the 384-well block).
8. Centrifuge plate for 30 seconds at 2000rpm.
9. Cycle in 384 well cycler using the SEQIPLEX program.
   This program is in the folder labelled 'Research' under Leukaemia Unit. The SEQIPLEX program should take ~40 minutes.
   1. 94ºC for 30 seconds
   2. 94ºC for 5 seconds
   3. 58ºC for 5 seconds
   4. 80ºC for 5 seconds
   5. 5 cycles steps 3-4
   6. 40 cycles steps 2-5
   7. 72ºC for 3 minutes
   8. 12ºC infinity
10. At the end of cycling, centrifuge the plate for 30 seconds at 2000 rpm.

11. If needed, plate can be stored in the cold room overnight before proceeding to the next stage.

5.2.6. Resin clean up

1. If 384 well plate was stored overnight, centrifuge for 30 seconds at 2000 rpm.
2. Place the trough containing resin and water mix in UV hood in post PCR area outside 0-18-N.
    a. Check that the resin is not pale and dry as it will not remove excess salts from the reaction adequately.
    b. Ensure there is sufficient resin and water for the reaction.
3. In the UV hood, remove seal from 384 well plate while taking care to ensure no spillage of sample from plate.
4. Using a multichannel pipette, add 10ul of resin to each well from the trough, changing pipette tips between each pass.
    a. Do not return excess resin to trough.
5. Using the multichannel pipette, add 10ul of water to each well from the trough, changing pipette tips between each pass.
    a. Do not return excess water to trough.
6. Confirm each well has resin and water added before using 4titude PCR seal on plate and seal firmly over surfaces and edges.
7. Place plate on rotogenie for 15 minutes on lowest speed.
8. Centrifuge plate for 30 seconds at 2000rpm.
9. If needed, plate can be stored in the cold room overnight before proceeding to the next stage

5.2.7. Prepare template for sample name import using FOR-5450

1. Copy column A and B containing the 96 sample names (including the ‘blank’) from ‘Sample Name Import’ tab on FOR-5450
2. Paste in a new excel document and save as a ‘Tab delimited’ (*.txt) file on secure server.
3. Open saved Tab delimited file and delete white space at the end of the document as this will prevent the sample names being appropriately loaded into the Typer 4 software.
4. Save and close file.

5.2.8. Nanodispenser maintenance and checks

1. If 384 well plate was stored overnight, centrifuge for 30 seconds at 2000 rpm.
2. Thaw calibrant (if using a new SpectroCHIP) in a tube rack and collect a SpectroCHIP.
3. Take 384 well plate, calibrant and SpectroCHIP to Nanodispenser (asset number 65491; EXT-INS-0196) in 0-49-N.
    a. Booking sheet adjacent to nanodispenser.
4. Check nanodispenser maintenance before use
    a. Check that waste tank (on ground) is not full – otherwise empty into sink. Check that supply tank (Molecular Grade water) is not empty – can look in the bottom of the spotter or check the instrument status. If necessary fill them – follow instructions on instruction sheet near instrument.
    b. Check if weekly clean performed
        i. If not, load 30ul NaOH 0.1M to a separate empty 384 well plate in wells A1-D6 (24 wells in total)
            1. Plates stored in 0-49-N adjacent to Nanodispenser
            2. NaOH 0.1M solution stored at room temperature in rack adjacent to Nanodispenser
        ii. Load weekly clean plate to Nanodispenser and go to the Maintenance screen from main menu and click on ‘CONDITION

WEEKLY' and select 384 MTP which will commence a 10 minute soak of the 6x4 pins into the NaOH solution.

iii. Once cycle complete, remove and dispose plate.

c. Check if daily clean performed
   i. If not, go to Maintenance screen and tap 'SONICATOR SOLUTION Drain'.
   ii. Once draining finished, tap 'SONICATOR SOLUTION Fill' and remove sonicator drum and fill to the brim with 100% EtOH (kept above sink in 0-49-N).
   iii. Place sonicator drum back in position in Nanodispenser
   iv. Tap 'CLEAN DAILY' which will commence a 30 minute pin clean.
   v. Record in maintenance sheet adjacent to Nanodispenser.
d. Then replace sonicator solution with 50% EtOH (kept above sink in 0-49-N) to prepare to sample transfer into SpectroCHIP.

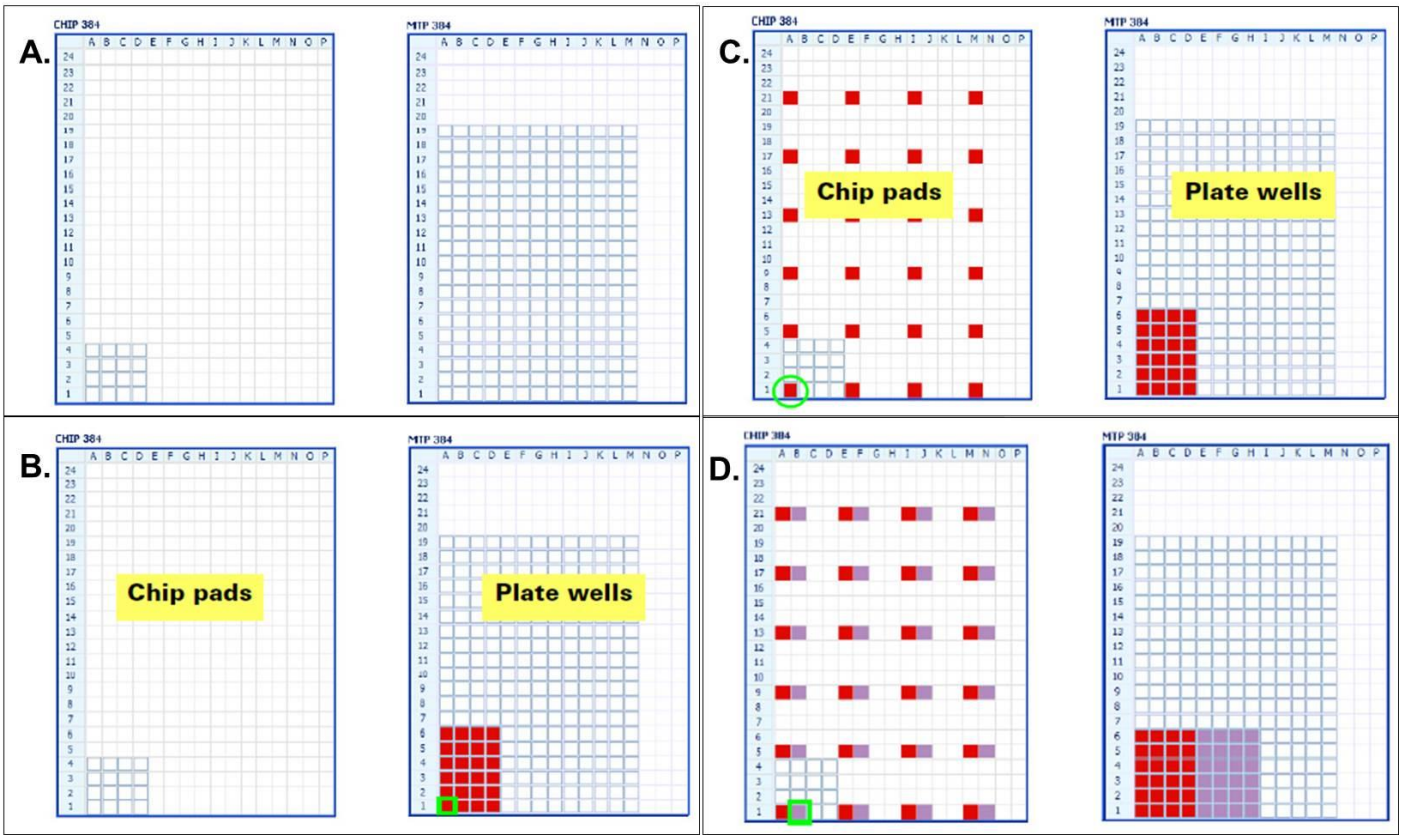
5.3. Transfer the reaction chemistry to the SpectroCHIP

5.3.1. Setup Mapping of plate

1. Click on Mapping from main menu and select 384 MTP before opening saved files.
2. Select 'sLab' and open design which will display both a 384 chip and plate.
3. Remove existing design and program in desired mapping (Figure 3).
4. A full plate loaded onto the SpectroCHIP is demonstrated in Appendix 9.

Figure 3: Directing the Nanodispenser to load the SpectroCHIP with the desired mapping design

(A) Mapping window demonstrates both SpectroCHIP (left) and the 384 microplate (right). (B) Click position A1 on the plate and (C) Tap position A1 on the chip pads. This will direct the aspirate from A1 on the plate to be deposited on A1 on the SpectroCHIP. The pins are spaces so that they land on every fourth pad so that B1 on the plate deposits sample in E1 on the SpectroCHIP etc. (D) Click on E1 on the plate and then tap on B1 on the chip. Continue until the whole plate is covered.



5.3.2. Setup method

1. Click on Methods in the main menu
2. Select setup tab, ensure 'sLab' is already selected and tick volume check
3. Select cleaning tab and check all cleaning steps are ticked
    a. Check cleaning times
        i. Rinse time 3.5 seconds
        ii. Wash time 5 seconds
        iii. Dry time 0.2 seconds
4. Select aspirate/dispense tab
    a. In the operation window
        i. Select analyte and calibrant if using a new chip
        ii. If not, select analyte
    b. Spotting should be set to wet run
    c. Check calibrant settings
        i. Dispense/aspirate 1
        ii. Dispense speed 140 mm/sec
    d. Check aspirate settings
        i. Aspirate time 6 seconds
        ii. Aspirate offset 5 minutes
        iii. Aspirate speed 60 mm/sec
    e. Check dispense settings
        i. Dispense time 0.1 seconds
        ii. Dispense offset 1 mm
        iii. Dispense speed 90 mm/sec
5. Press back and apply
6. If any of the settings required alteration from saved measurements, record and change back to baseline settings once all steps completed.

5.3.3. Load SpectroCHIP and 384 well plate into position (once seal is removed)

1. Position 384 well plate with well A1 in the bottom left-hand corner.
2. Carefully position SpectroCHIP with barcode on the bottom left-hand corner using tweezers. Ensure magnet locks into position.
3. Record SpectroCHIP barcode, date and initials onto the SpectroCHIP packet before loading into Nanodispenser.
    a. Also indicate which areas of the SpectroCHIP have been used on the packet to minimize wastage.
4. If new SpectroCHIP used, load 60ul calibrant into calibrant reservoir.

5.3.4. Perform sample transfer and select transfer on the main menu

    a. Press 'step' to test the transfer process which will only perform a single 6x4 transfer.
        a. Once the barcode has been read and the SpectroCHIP calibrated (if new SpectroCHIP), the sample transfer will begin.
        b. Check the volume dispensed and ensure that a minimum of 8-10nl has been spotted on the SpectroCHIP.
            i. If inadequate volume dispensed, alter the dispense speed by going back to the methods and applying any changes made.
            ii. If <4nl was spotted, can re-spot on the same pad once all samples completed.
            iii. If needed, can repeat 'step' which will test the new settings on the next planned 6x4 transfer.
            iv. Once satisfied with the settings, transfer all by pressing run.
        c. Once the transfer is completed, remove SpectroCHIP with tweezers and transfer to the MassARRAY® Typer 4 (asset number 65482) co-located in 0-49-N (see step 5d).

d. On the Nanodispenser, go back to the maintenance window from the main menu and 'complete cycle' and press the big red stop button when pins in sonicator solution to ensure that the pins do not dry out.
e. Remove 384 well plate, re-seal and store in cold room in case repeat testing is required. Ensure plate is clearly labelled for easy identification later.

5.4. Fire the chip using the MassARRAY® Typer 4

5.4.1. Open the Typer4 software from the computer adjacent to the MassARRAY® Typer 4.
1. Can set this up while samples transferring to the SpectroCHIP on the Nanodispenser.
    a. Username: charles
    b. Password: Darwin

5.4.2. Plate setup
1. The plate will look like the original 384 well plate and not the SpectroCHIP
2. Open the Typer4 Plate Editor software
    a. Make new plate under "BCR-ABL Somatic Mutation V11.2" by right clicking on folder name (Leukaemia Unit) and name the plate with date and initials
    b. Click on 'Assay view' tab
        i. Right click and select all wells for each multiplex
        ii. Right click and allocate relevant assay to wells from the Leukaemia Unit folder
            1. If wells 1-4 being performed, then open "BCR_ABL_mutation_use_this_one_080513"
            2. If wells 5-8 being performed, then open "BCR_ABL_ABL001_20.02.2020 v2"
    c. Click on 'Sample' tab
        i. Change the plate orientation from horizontal to vertical
        ii. Import sample sheet (tab delimited file saved earlier)
        iii. Save the sample list using date, well number and initials
        iv. Allocate the sample names by selecting wells and right clicking 'apply samples to wells'. Have to perform this three more times for the each of the other multiplexes
        v. If the cursor is passed over each well, the position number, sample name and assay details should pop up.
3. Save all changes.

5.4.3. Open the 'CHIPlinker' program to link the chip barcode to the designed plate.
1. If you do not CHIPlink, then the MassARRAY will fire using the last SpectroCHIP that was linked.
    a. Ensure the run type is selected to genotype + area
    b. Name the experiment
    c. Enter the SpectroCHIP barcode
    d. Find the plate that was just saved in the left window and select 'add'
    e. Click 'create'

5.4.4. Load the SpectroCHIP in the MassARRAY® Typer 4
1. Press the central silver button to release the vacuum seal.
2. Wait ~1 minute until the lid can be opened (a click will occur indicating when it is safe to open).
3. Place the SpectroCHIP into the position marked "1" on the left. Ensure that the magnet at the back of the SpectroCHIP has locked into position. Leave the existing SpectroCHIP in position 2.
4. Close the lid and press the silver button again.
5. Wait until the green "ready" light comes on (should take <5 minutes).

5.4.5. Firing the chip
1. Open the MassARRAY4 software.
2. Check that iPLEX.par is being used.

3. Under “automatic run setup” tab, tick chip1 box
4. Check that the ‘turn off HV after run complete’ box is ticked.
5. Switch to the “Automatic Run” tab and press “Start AutoRun” at the top of the screen once the MassARRAY® Typer 4 green light is on.
6. Once the SpectroCHIP position is confirmed and the barcode is read, the chip will fire.
7. The whole SpectroCHIP takes about 30-45 mins to fire.
8. Once finished, check that HV (high voltage) is off and then click “shut down all processes icon” (the red ‘X’ icon).
9. Once this step has been completed, remove SpectroCHIP with tweezers by pressing silver button on MassARRAY® Typer 4 and once door has been released, place SpectroCHIP back in its packet.
    a. Seal the packet with tape to ensure it remains airtight.
    b. The SpectroCHIP can last 2 weeks before requiring disposal.
10. Press the silver button once more to ensure that the vacuum is reset. Otherwise, can take up to a week to reset vacuum later.
11. The data will be automatically transferred to the Agena database which can be accessed from the computer (asset number 202898) in the Leukaemia unit office (0-18-N).

5.5. Analysis of mass spectrometry results

1. Open FOR-5450 and select the ABC Raw Results tab.
2. Open the Typer 4 software and select the Typer Analyser
3. Find the recently run experiment (will have the same name as the details that were entered in the experiment field in CHIPlinker).
4. Select the SpectroCHIP barcode listed
5. Make sure that the Call Cluster Plot is set to ‘Yield’.
6. To order samples so that analysis is easier, in the Plate Data Pane, click Sample ID, then Assay ID.
7. Increase the size of the Details Pane to improve visibility. To be accepted as a valid result, the intensity in either the mutation, control nucleotide or UEP peak needs to be >5.
    a. If the intensity is consistently <5 in a well, then there is likely an issue with the whole well and highlight in results sheet. Discuss with supervisor but will likely need to be repeated.
8. Check the well control assays
    a. This will always be the wildtype nucleotide and should be present in all samples, including the negative controls.
        i. An intensity of >5 is required for the sample to be accepted.
            1. Mark the relevant cell in the ABC raw results file as ‘y’
    b. If not present, the well cannot be accepted and will need to be repeated.
        i. Mark the relevant cell in the ABC raw results file as ‘n’ and highlight in red and cannot analyse the remainder of the well for that sample
9. Check the negative control samples
    a. Should be negative for all mutations except for the control.
    b. For all assays other than the well control, the UEP intensity should be >5 to accept as a true negative result as <5 indicates less sensitivity.
    c. On the call cluster plot, should also have 0-0.2 yield.
    d. Mark the relevant cell in the ABC raw results file as ‘n’ if truly negative
    e. If any of the negative controls are positive, then may still be able to analyse further if alternative negative controls are negative. Discuss with supervisor before proceeding further.
10. Check the plasmid/gBlock standards
    a. At the mutation being examined, the mutant nucleotide intensity should be >5.
        i. If <5, cannot accept the standard.
            1. Mark the relevant cell in the ABC raw results file as ‘n’

b. On the call cluster plot, for the examined sample
    i. If the mutation is called by the software, it can be accepted
        1. Mark the relevant cell in the ABC raw results file as 'y'
    ii. If the mutation is not called by the software but has clear separation from the negative cluster and the intensity on the details pane was also >5, can manually change the call by right clicking on the sample and changing the call.
        1. Mark the relevant cell in the ABC raw results file as 'y'
        2. If any of the negative controls have a higher yield than the standard, then the standard cannot be accepted.
            a. Mark the relevant cell in the ABC raw results file as 'n'
    iii. Ideally need 2/4 standards to be positive to provide a more accurate low-level mutant percentage estimation

11. Check all the samples for a particular mutation by clicking down the Plate Data Pane after selecting the mutation assay. Check for peaks in all the possible allele masses (should be tall and slender with clear separation from the background).
    a. Positive for investigated mutation: mutant nucleotide intensity >5 at the expected mass.
    b. Negative for mutation will have the UEP peak >5 with no mutant nucleotide.
    c. Calling: If a sample has a higher yield than the plasmid sample that was determined to be the 'limit of detection', mark that cell in the results spreadsheet with a 'y' with a comment re mutant estimate – except in the following circumstance:
        i. For assays where the limit of detection standard is close to the negative cluster or the negative cluster is not very compact.
            1. In this case mark samples between the 'limit of detection' standard and the next standard with a '?' on the results spreadsheet.
            2. Also insert a comment with the approximate mutant percentage.
        ii. When calling samples around the limit of detection, look at the level of background in that particular spectrum, if there are a lot of background peaks, then be more conservative when making the call.
            i. To determine what the final call for such samples would be, look at the duplicate mass spectrometry run.
            ii. If the duplicate sample is clearly positive for that mutation, may need a third run for concordance.
            iii. If the duplicate is negative or also a '?', then call it negative.
    d. If the assay has <u>more than 2 possible alleles</u> (i.e. V299L), then check all alleles on the spectrum. First look at the Call Cluster Plot for all possible allele combinations (ie display C vs G, C vs T, G vs T) to evaluate the level of background for each allele. Determine if the background is even between the alleles – i.e. the shape of the negative cluster – is this circular or elongated? Then to analyse the patient samples, set the Call Cluster Plot to display the mutant with the plasmid controls and the other mutant allele. If there are >2 mutant alleles (i.e. for F317I_L_V) will need to look at the plot 3 times. Sometimes the software will not call 2 high peaks for assays like this. Therefore, make sure these assays are checked thoroughly. If some samples have peaks that have not been called, can make a User Call. Can also make a user call if an allele is called by the software but visual analysis discredits the call.
    e. If a peak that is at an unexplained mass is present, but is not present in every well, make a comment on this in the 'comments' column. Can determine the mass of an unexplained peak by zooming in and then hovering over the apex of the peak – the mass will be at the top left corner of the Details Pane. Using this mass, determine if this peak corresponds to another mutation (i.e. E355A). Use the mass of the terminator nucleotides to determine this.

iPLEX Terminator bases	Mass in Daltons
C	247.19
A	271.21
G	287.21
T	327.1

f. If there are peaks at unexplained masses, these may have adducts of K (38Da) or Na (22Da), Na+K (59da) matrix adducts (63, 94, 138/139, 188 (also plus Na)). If these have the mass of a terminator base or dNTP, then this may indicate self-extension or an inefficient SAP reaction. If there has been inefficient SAP reaction then the dNTPs from the PCR may still be present, so a peak will be recovered with the mass of UEP+1dNTP+1terminator. Can determine the mass of this peak by investigating the direction of the assay (in the Assay Design tab of the Results Template file) and the sequence of *BCR-ABL1*. Calculate which dNTP and Terminator would be added and the weight of the resulting product. If this product is present and the expected UEP+1terminator peak is not present, then repeat the assay for that sample. If this happens for 1 sample, it will probably happen for all samples and may be related to an extended storage time of the SAP at room temperature. Note these and other comments in the 'comments' tab of the results template and alert supervisor.

dNTP	Mass in Daltons
T	304.19
A	313.2
C	289.18
G	329.2

g. If peaks corresponding to the wild type allele are observed, this might be contamination of the well with wild type terminator. This is most likely to happen at the edge of the wells section (i.e. in columns 6, 7, 12, 13, 18, 19) from spray of the primer extension master mix or inadequate plate seal. OR this might be due to a background peak that is at that particular weight (ie the Q252H assay). To determine which of these is the case, examine all the assays in the well. If it is associated with contamination of wild type terminator, then all assays will have wild type peaks and repeat of this well is required as the sensitivity will be decreased. If the wild type nucleotide is detected in multiple runs, the contamination may be within the terminator mix itself.

h. If mutations are repeatedly detected in all samples it may indicate contamination and repeat is required.

i. Refer to EXT-INS-2404 MassARRAY® Analyser 4 Users Guide for more information

5.6. Recording and reporting of mass spectrometry results

1. Save a copy of this file with the date the chemistry was performed and the user initials, book and page number. Also save the 'Plate Data' file, the raw data file and the raw excel file with analysis in a folder named 'date, book#pg#, initials' in the Results folder. Save a new folder for each plate analyzed
2. Once data is analysed and the mutations recorded, column FN "Mutations found by Mass spectrometry" will automatically populate. Copy the mutations and the relevant book, page and sample number into the Results spreadsheet. The mutant estimates will need to be manually entered.
3. *BCR-ABL1* mutations are numbered according to reference sequence NM_005157 (Appendix 10) Example for reporting mutations:

   I502L officially reported as

   ABL1 NM_005157.6:c.1504A>C:p.(Ile502Leu):chr9(GRCh37):g.133755535

5.7. *BCR-ABL1* mutations included in the assay

Kinase domain mutation	Nucleotide position change	Nucleotide change+	Reason for inclusion
M244V	730A>G	ATG>GTG	
L248V	742C>G	CTG>GTG	
G250E	749G>A	GGG>GAG	
Q252H	756G>C	CAG>CAC	
Q252H	756G>T	CAG>CAT	
Y253H	757T>C	TAC>CAC	
Y253F	758A>T	TAC>TTC	
E255K	763G>A	GAG>AAG	
E255V	764A>T	GAG>GTG	
D276G	827A>G	GAC>GGC	
E279K	835G>A	GAG>AAG	
V299L	895G>C	GTG>CTG	
V299L	895G>T	GTG>TTG	
F311I*	931T>A	TTC>ATC	Rare imatinib resistant mutation
T315A	943A>G	ACT>GCT	
T315I	944C>T	ACT>ATT	
T315L*	943A>C 944C>T 945T>A	ACT>CTA	P,CL Rare ponatinib resistant mutation[2, 3]
T315M*	944 C>T 945 T>G	ACT>ATG	P,CL Rare ponatinib resistant mutation[2, 3]
F317I	949T>A	TTC>ATC	
F317L	949T>C	TTC>CTC	
F317V	949T>G	TTC>GTC	
F317C	950T>G	TTC>TGC	
F317S	950T>C	TTC>TCC	
F317L	951C>G	TTC>TTG	
F317L	951C>A	TTC>TTA	
A337V*	1010C>T	GCC>GTC	P,CL Asciminib resistant mutation[4, 5]
M351T	1052T>C	ATG>ACG	
E355G	1064A>G	GAG>GGG	
F359I	1075T>A	TTC>ATC	
F359V	1075T>G	TTC>GTC	
F359C	1076T>G	TTC>TGC	
H396P	1187A>C	CAT>CCT	
H396R	1187A>G	CAT>CGT	
E450G*	1349A>G	GAG>GGG	Rare imatinib resistant mutation
E459K	1375G>A	GAG>AAG	
G463S*	1387G>A	GGC>AGC	P Asciminib resistant mutation[6]
C464W*	1392C>G	TGC>TGG	CL Asciminib resistant mutation[7]
P465S*	1393C>T	CCA>TCA	P,CL Asciminib resistant mutation[4]
V468F*	1402G>T	GTC>TTC	P,CL Asciminib resistant mutation[4]
F486S	1457T>C	TTT>TCT	
F497L*	1491C>G	TTC>TTG	CL Asciminib resistant mutation[8]
I502L*	1504A>C	ATC>CTC	P,CL Asciminib resistant mutation[4]

+Nucleotide change indicated in red
*Additional mutations incorporated into the mass spectrometry assay
CL Mutation reported in cell line data
P Reported in patients

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FOR-0076 rev2

6. TRAINING AND COMPETENCY

Training in analysis of results is by correct identification of test run data:

New staff members are to be trained in procedures outlined in TRD-0524 Leukaemia Unit Training Program and TRD-0716 Low-Level BCR-ABL Kinase Domain Mutation Detection.
Refer to "I:\mp-leukaemia_unit\Sequenom\Analysis Training 8.9.11"
Competency is assessed by correct identification of all of the known mutations.

7. APPLICABILITY

This method is used by the Leukaemia Unit, Department of Genetics and Molecular Pathology (Frome Rd), SA Pathology for research purposes.

8. ASSOCIATED DOCUMENTS

In support of this procedure, the following documents apply:

Microsoft access sample database located in I:\mp-common\Database\BCR-ABL

Patient excel files located in I:\mp-leukaemia_unit\CML databases\CML study databases

The following Q-Pulse documents:

FOR-1251 – Long PCR

FOR-1258 – Nested PCR

PRC-GMO-353 Quantitation of BCR-ABL

PRC-GMO-342 – BCR-ABL Kinase domain mutation analysis

PRC-GMO-461 Leukaemia Unit Specimen Receipt

TRD-0524 Leukaemia Unit Training Program

TRD-0716 Low-Level BCR-ABL Kinase Domain Mutation Detection

EXT-INS-0196 MassArray Nanodispenser RS1000 user guide

EXT-INS-2403 iPLEX Pro and Gold Reagents user guide

EXT-INS-2404 MassArray Analyser 4 Users Guide

EXT-INS-2405 MassArray Typer 4 user guide

PRC-GMO-356 Cell culture Techniques for the preparation of quantitative BCR-ABL Quality Controls

PRC-GMO-358 Operation of Qubit spectrophotometer

FOR-5446 UEP pools W1-4 low-level *BCR-ABL1*

FOR-5447 PCR primers low-level *BCR-ABL1* W1-8

FOR-5448 UEP pools W5-8 low-level *BCR-ABL1*

FOR-5449 Oligo spikes low-level *BCR-ABL1*

FOR-5450 Mass spectrometry assay Template

FOR-5451 Mass spectrometry assay reagent template

9. DEFINITIONS

For purposes of this document, unless otherwise stated, the following definitions shall apply:

CML – Chronic Myeloid Leukaemia

TKI – Tyrosine Kinase Inhibitor

AA – Amino Acid

Imatinib (Glivec, Gleevec) – First generation Tyrosine Kinase Inhibitor

Nilotinib (Tasigna) – 2nd Generation Tyrosine Kinase Inhibitor

Dasatinib (Sprycel) – 2nd Generation Tyrosine Kinase Inhibitor

Ponatinib – 3rd Generation Tyrosine Kinase Inhibitor

Asciminib – allosteric inhibitor

10. REFERENCES

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11. APPENDICES

Appendix 1: Details of gBlocks used in wells 5-8 in mass spectrometry assay

	Terminator mix	Mutations	Control
Well 5	TGC	T315M_L, E450G, I502L	Control L323 >G
Well 6	TG	F311I	Control L323 >G
Well 7	TGA	A337V_2, C464W, V468F, F497V	Control G323 >G
Well 8	AC	A337V, G463S, P465S, F497V	Control L323 >C

gBlock-1: Used in well 5

- Mutations tested – F/R indicated the direction of the extension primer
  - E450G (F), I502L (F), T315L (R) - extension primer will detect the 3rd base of amino acid 315, detection of the T315L mutation is dependent on detection of a C nucleotide in the T315A assay in well 2.

Nucleotide sequence for gBlock-1

GGGCGGGGGCCAGTACGGGGAGGTGTA
CGAGGGCGTGTGGAAGAAATACAGCCTGACGGTGGCCGTGAAGACCTTGAAG**GAGGACACCATGGAGGTG**
GAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAACACCCTAACCTG**GTG**CAGCTCCTTGGGG
TCTGCACCCGGGAGCCCCCG**T**TCTATATCATC**CTA**GAGTTCATGACCTACGGGAACCT**C**CTGGACTACCT
GAGGGAGTGCAACCGGCAGGAGGTGAACG**C**CGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCC
ATGGAGTACCTGGAGAAGAAAAACTT**CATCCACAGAGATCTTGCTG**CCCGAAACTGCCTGGTAGGGGAGA
ACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCAGGTTGATGACAGGGGACACCTACACAGCCCATGC
TGGAGCCAAGTTCCCCATCAAATGGACTGCACCCGAGAGCCTGGCCTACAACAAGTTCTCCATCAAGTCC
GACGTCTGGGCATTTGGAGTA**TTGCTTTGGGAAATTGCTACCTATG**GCATGTCCCCTTACCCGGGAATTG
ACCTGTCCCAGGTGTATG**G**GCTGCTAGAGAAGGACTACCGCATGGAGCGCCCAGAA**G**GCTG**CC**CAGAGAA
G**G**TCTATGAACTCATGCGAGCATGTTGGCAGTGGAATCCCTCTGACCGGCCCTCCTTTGCTGAAATCCAC
CAAGCCTTTGAAACAAT**G**TT**C**CAGGAATCCAGT**C**TCTCAGACGAAGTGGAAAAGGAGCTGGGGAAACAAG
GCGTCCGTGGGGC**TGTGAGTACCTTGCTGCAGG**CCCCAGAGCTGCCCACCAAGACGAGGACCTCCAGGAG
AGCTGCAGAGCACAGAGACACCACTGACGTGCCTGAGATGCCTCACTCCAAGGGCCAGGGAGAGAGCGAT

Red nucleotides indicate the mutated nucleotides
The single green coloured nucleotide indicates the location of the well control
Underlined sequences indicate the extension primer locations
Coloured multiple nucleotide sequences are the locations of the fragment amplification primers

gBlock-2: used in well 7 – F/R indicated the direction of the extension primer

- Mutations tested
  - A337V_2 (F), C464W (F), V468F (R), F497V (F), T315M (R)
    Well 5, terminator mix TGC, extension primer will detect 3rd base of 315

Nucleotide sequence for gBlock 2

GGGCGGGGGCCAGTACGGGGAGGTGTA
CGAGGGCGTGTGGAAGAAATACAGCCTGACGGTGGCCGTGAAGACCTTGAAG**GAGGACACCATGGAGGTG**
GAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAACACCCTAACCTGGTGCAGCTCCTTGGGG
TCTGCACCCGGGAGCCCCCG**T**TCTATATCATCA**TG**GAGTTCATGACCTAC**G**GGAACCT**C**CTGGACTACCT
GAGGGAGTGCAACCGGCAGGAGGTGAACG**T**CGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCC
ATGGAGTACCTGGAGAAGAAAAACTT**CATCCACAGAGATCTTGCTG**CCCGAAACTGCCTGGTAGGGGAGA
ACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCAGGTTGATGACAGGGGACACCTACACAGCCCATGC
TGGAGCCAAGTTCCCCATCAAATGGACTGCACCCGAGAGCCTGGCCTACAACAAGTTCTCCATCAAGTCC
GACGTCTGGGCATTTGGAGTA**TTGCTTTGGGAAATTGCTACCTATG**GCATGTCCCCTTACCCGGGAATTG
ACCTGTCCCAGGTGTATG**A**GCTGCTAGAGAAGGACTACCGCATGGAGCGCCCAGAA**G**GCTG**GC**CAGAGAA
G**T** TCTATGAACTCATGCGAGCATGTTGGCAGTGGAATCCCTCTGACCGGCCCTCCTTTGCTGAAATCCAC
CAAGCCTTTGAAACAATGTT**G**CAGGAATCCAGT**A**TCTCAGACGAAGTGGAAAAGGAGCTGGGGAAACAAG
GCGTCCGTGGGGC**TGTGAGTACCTTGCTGCAGG**CCCCAGAGCTGCCCACCAAGACGAGGACCTCCAGGAG
AGCTGCAGAGCACAGAGACACCACTGACGTGCCTGAGATGCCTCACTCCAAGGGCCAGGGAGAGAGCGAT

Red nucleotides indicate the mutated nucleotides
The single green coloured nucleotide indicates the location of the well control
Underlined sequences indicate the extension primer locations
Coloured multiple nucleotide sequences are the locations of the fragment amplification primers

gBlock-3: used in wells 6 and 8

- Mutations tested – F/R indicated the direction of the extension primer
  - A337V (R), G463S (F), P465S (R), F497V (R), F311I (R)

Nucleotide sequence for gBlock-3

GGGCGGGGGCCAGTACGGGGAGGTGTA
CGAGGGCGTGTGGAAGAAATACAGCCTGACGGTGGCCGTGAAGACCTTGAAG**GAGGACACCATGGAGGTG**
GAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAACACCCTAACCTGGTGCAGCTCCTTGGGG
TCTGCACCCGGGAGCCCCCG**A**TCTATATCATCAC**T**GAGTTCATGACCTACGGGAACCT**C**CTGGACTACCT
GAGGGAGTGCAACCGGCAGGAGGTGAACG**T**CGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCC
ATGGAGTACCTGGAGAAGAAAAACTT**CATCCACAGAGATCTTGCTG**CCCGAAACTGCCTGGTAGGGGAGA
ACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCAGGTTGATGACAGGGGACACCTACACAGCCCATGC
TGGAGCCAAGTTCCCCATCAAATGGACTGCACCCGAGAGCCTGGCCTACAACAAGTTCTCCATCAAGTCC
GACGTCTGGGCATTTGGAGTA**TTGCTTTGGGAAATTGCTACCTATG**GCATGTCCCCTTACCCGGGAATTG
ACCTGTCCCAGGTGTATG**A**GCTGCTAGAGAAGGACTACCGCATGGAGCGCCCAGAA**A**GCTG**C**T**T**CAGAGAA
GGTCTATGAACTCATGCGAGCATGTTGGCAGTGGAATCCCTCTGACCGGCCCTCCTTTGCTGAAATCCAC
CAAGCCTTTGAAACAATGTT**G**CAGGAATCCAGT**A**TCTCAGACGAAGTGGAAAAGGAGCTGGGGAAACAAG
GCGTCCGTGGGGC**TGTGAGTACCTTGCTGCAGG**CCCCAGAGCTGCCCACCAAGACGAGGACCTCCAGGAG
AGCTGCAGAGCACAGAGACACCACTGACGTGCCTGAGATGCCTCACTCCAAGGGCCAGGGAGAGAGCGAT

Red nucleotides indicate the mutated nucleotides
The single green coloured nucleotide indicates the location of the well control
Underlined sequences indicate the extension primer locations
Coloured multiple nucleotide sequences are the locations of the fragment amplification primers

Wild type gBlock

Nucleotide sequence for wild type gBlock

GGGCGGGGGCCAGTACGGGGAGGTGTA
CGAGGGCGTGTGGAAGAAATACAGCCTGACGGTGGCCGTGAAGACCTTGAAG**GAGGACACCATGGAGGTG**
GAAGAGTTCTTGAAAGAAGCTGCAGTCATGAAAGAGATCAAACACCCTAACCTGGTGCAGCTCCTTGGGG
TCTGCACCCGGGAGCCCCCG**T**TCTATATCATC**ACT**GAGTTCATGACCTAC**G**GGAACCT**C**CTGGACTACCT
GAGGGAGTGCAACCGGCAGGAGGTGAACG**C**CGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCC
ATGGAGTACCTGGAGAAGAAAAACTTCATCCACAGAGATCTTGCTGCCCGAAACTGCCTGGTAGGGGAGA
ACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCAGGTTGATGACAGGGGACACCTACACAGCCCATGC
TGGAGCCAAGTTCCCCATCAAATGGACTGCACCCGAGAGCCTGGCCTACAACAAGTTCTCCATCAAGTCC
GACGTCTGGGCATTTGGAGTA**TTGCTTTGGGAAATTGCTACCTATG**GCATGTCCCCTTACCCGGGAATTG
ACCTGTCCCAGGTGTATG**A**GCTGCTAGAGAAGGACTACCGCATGGAGCGCCCAGAA**G**GCTG**CC**CAGAGAA
G**G**TCTATGAACTCATGCGAGCATGTTGGCAGTGGAATCCCTCTGACCGGCCCTCCTTTGCTGAAATCCAC
CAAGCCTTTGAAACAATGTT**C**CAGGAATCCAGT**A**TCTCAGACGAAGTGGAAAAGGAGCTGGGGAAACAAG
GCGTCCGTGGGGC**TGTGAGTACCTTGCTGCAGG**CCCCAGAGCTGCCCACCAAGACGAGGACCTCCAGGAG
AGCTGCAGAGCACAGAGACACCACTGACGTGCCTGAGATGCCTCACTCCAAGGGCCAGGGAGAGAGCGAT

Red nucleotides indicate the wild type nucleotides
The single green coloured nucleotides indicates the location of the well control
Underlined sequences indicate the extension primer locations
Coloured multiple nucleotide sequences are the locations of the fragment amplification primers

Appendix 2: ABL1 primers for gBlock amplification

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	29	20	60.07	50.00	2.00	2.00	GAGGGCGTGTGGAAGAAATA
RIGHT PRIMER	901	20	59.61	60.00	2.00	2.00	GTGGTGTCTCTGTGCTCTGC

Amplicon length 863 bp

Reference sequence involving ABL1 (transcript NM_007313)

1261 ggtgtacgagggcgtgtggaagaaatacagcctgacggtggccgtgaagaccttgaagga

1321 ggacaccatggaggtggaagagttcttgaaagaagctgcagtcatgaaagagatcaaaca

1381 ccctaacctggtgcagctccttggggtctgcacccgggagcccccgttctatatcatcac

1441 tgagttcatgacctacgggaacctcctggactacctgagggagtgcaaccggcaggaggt

1501 gaacgccgtggtgctgctgtacatggccactcagatctcgtcagccatggagtacctgga

1561 gaagaaaaacttcatccacagagatcttgctgcccgaaactgcctggtaggggagaacca

1621 cttggtgaaggtagctgattttggcctgagcaggttgatgacaggggacacctacacagc

1681 ccatgctggagccaagttccccatcaaatggactgcacccgagagcctggcctacaacaa

1741 gttctccatcaagtccgacgtctgggcatttggagtattgctttgggaaattgctaccta

1801 tggcatgtccccttacccgggaattgacctgtcccaggtgtatgagctgctagagaagga

1861 ctaccgcatggagcgcccagaaggctgcccagagaaggtctatgaactcatgcgagcatg

1921 ttggcagtggaatccctctgaccggccctcctttgctgaaatccaccaagcctttgaaac

1981 aatgttccaggaatccagtatctcagacgaagtggaaaaggagctggggaaacaaggcgt

2041 ccgtggggctgtgagtaccttgctgcaggccccagagctgcccaccaagacgaggacctc

2101 caggagagctgcagagcacagagacaccactgacgtgcctgagatgcctcactccaaggg

Underlined sequences indicate the primer locations

Appendix 3: Primer design for mass spectrometry

WELL	iPLEX terminator nucleotide mix	Mutation	Mutation nucleotide exchange*	Forward amplification primer†	Reverse amplification primer†	Extension primer direction	Extension primer	Extension primer mass (Daltons)
W1	ACG	E255V	c.764A>T	ACGTTGGATGGGCTGTATTTCTTCCACACG	ACGTTGGATGACATCACCATGAAGCACAAG	R	ACACGCCCTCGTACACC	5331.5
W1	ACG	H396R_P	c.1075T>A/G	ACGTTGGATGTTTGGCCTGAGCAGGTTGAT	ACGTTGGATGGGTGCAGTCCATTTGATGGG	R	GGGAACTTGGCTCCAGCA	5771.8
W1	ACG	F486S	c.1457T>C	ACGTTGGATGAGCATGTTGGCAGTGGAATC	ACGTTGGATGTTGTTTCAAAGGCTTGGTGG	F	CCCTCTGACCGGCCCTCCT	5883.9
W1	ACG	D276G V11.2	c.827A>G	ACGTTGGATGGTGGCCGTGAAGACCTTGA	ACGTTGGATGTCTTTCATGACTGCAGCTTC	R	ACTCTTCCACCTCCATGGTG	6251.08
W1	ACG	Control_R_G_W1	c.1263C	ACGTTGGATGGCCATAGGTAGCAATTTCCC	ACGTTGGATGCTGGCCTACAACAAGTTCTC	R	AATACTCCAAATGCCCAGAC	6310.2
W1	ACG	F317I_L_V	c.949T>A/C/G	ACGTTGGATGAGCCCCCGTTCTATATCATC	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	F	CCCGTTCTATATCATCACTGAG	6892.5
W1	ACG	F359I_V	c.1075T>A/G	ACGTTGGATGTACATGGCCACTCAGATCTC	ACGTTGGATGGTTTCGGGCAGCAAGATCTC	F	GGAGTACCTGGAGAAGAAAAAC	7121.7
W2	CGT	Y253F	c.758A>T	ACGTTGGATGACAAGTGGGAGATGGAACGC	ACGTTGGATGTATTTCTTCCACACGCCCTC	F	GGGCGGGGGCCAGT	4633.1
W2	CGT	Y253H	c.757T>C	ACGTTGGATGACAAGTGGGAGATGGAACGC	ACGTTGGATGTATTTCTTCCACACGCCCTC	R	CTCGTACACCTCCCCGT	5313.5
W2	CGT	F317C_S	c.950T>G	ACGTTGGATGAGCTCCTTGGGGTCTGCAC	ACGTTGGATGTCAGGTAGTCCAGGAGGTTC	R	GGTTCCCGTAGGTCATG	5464.62
W2	CGT	E355G	c.1064A>G/C	ACGTTGGATGTACATGGCCACTCAGATCTC	ACGTTGGATGGTTTCGGGCAGCAAGATCTC	F	CAGCCATGGAGTACCTGG	5795.8
W2	CGT	T315A	c.943A>G	ACGTTGGATGAGCTCCTTGGGGTCTGCAC	ACGTTGGATGTCAGGTAGTCCAGGAGGTTC	F	GCCCCCGTTCTATATCATC	5945.9
W2	CGT	Control_R_G_W2	c.1263C	ACGTTGGATGGCCATAGGTAGCAATTTCCC	ACGTTGGATGCTGGCCTACAACAAGTTCTC	R	AATACTCCAAATGCCCAGAC	6310.2
W2	CGT	M244V	c.730A>G	ACGTTGGATGACAAGTGGGAGATGGAACGC	ACGTTGGATGTATTTCTTCCACACGCCCTC	F	ATGGAACGCACGGACATCACC	6680.4
W2	CGT	F359C	c.1076T>G	ACGTTGGATGGTTTCGGGCAGCAAGATCTC	ACGTTGGATGTACATGGCCACTCAGATCTC	R	GCAGCAAGATCTCTGTGGATG	6733.4
W3	TC	Q252H	c.756G>C/T	ACGTTGGATGATGGAACGCACGGACATCAC	ACGTTGGATGGGCTGTATTTCTTCCACACG	F	GCTGGGCGGGGGCCA	4922.27
W3	TC	F317L_L	c.951C>A/G	ACGTTGGATGAGCCCCCGTTCTATATCATC	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	R	AGGTTCCCGTAGGTCAT	5448.6
W3	TC	V299L_L	c.895G>C/T	ACGTTGGATGTGGAGGTGGAAGAGTTCTTG	ACGTTGGATGCGGGTGCAGACCCCAAGGA	F	AGATCAAACACCCTAACCTG	6286.1
W3	TC	Control_F_T_W3	c.1263C	ACGTTGGATGCTGGCCTACAACAAGTTCTC	ACGTTGGATGGCCATAGGTAGCAATTTCCC	F	ACGTCTGGGCATTTGGAGTA	6435.2
W4	TG	G250E V11-3	c.749G>A	ACGTTGGATGATGGAACGCACGGACATCAC	ACGTTGGATGGGCTGTATTTCTTCCACACG	R	CCCCGTACTGGCCC	4407.9
W4	TG	L248V	c.742C>G	ACGTTGGATGATGGAACGCACGGACATCAC	ACGTTGGATGGGCTGTATTTCTTCCACACG	F	TCACCATGAAGCACAAG	5419.6
W4	TG	T315I	c.944C>T	ACGTTGGATGCGGGAGCCCCCGTTCTATA	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	F	CCCCCGTTCTATATCATCA	5905.9
W4	TG	E459K	c.1375G>A	ACGTTGGATGCAACATGCTCGCATGAGTTC	ACGTTGGATGAGCTGCTAGAGAAGGACTAC	R	GGGCAGCCTTCTGGGCGCT	6083.9
W4	TG	Control_R_G_W4	c.1263C	ACGTTGGATGGCCATAGGTAGCAATTTCCC	ACGTTGGATGCTGGCCTACAACAAGTTCTC	R	AATACTCCAAATGCCCAGAC	6310.2
W4	TG	E279K	c.835G>A	ACGTTGGATGAGGTTAGGGTGTTTGATCTC	ACGTTGGATGTGAAGACCTTGAAGGAGGAC	R	TGTTTCAAGAACTCTTCCACCT	6867.44
W4	TG	E255K	c.763G>A	ACGTTGGATGATGGAACGCACGGACATCAC	ACGTTGGATGGGCTGTATTTCTTCCACACG	R	CTTCCACACGCCCTCGTACACCT	7087.6
W4	TG	M351T v11.2	c.1052T>C	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGTACATGGCCACTCAGATCTC	R	TTTTTTTTCTTCTCCAGGTACTCC	7472.87
W5	CGT	T315L_M	T315L 943A>C 944C>T 945T>A; T315M 944 C>T	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGGAGGACACCATGGAGGTGGA	R	CCCGTAGGTCATGAACTC	5706.7

			945 T>G					
W5	CGT	E450G	c.1349A>G	ACGTTGGATGTTGGGAAATTGCTACCTATG	ACGTTGGATGCCTGCAGCAAGGTACTCACA	F	GACCTGTCCCAGGTGTATG	6091
W5	CGT	I502L	c.1504A>C	ACGTTGGATGTTGGGAAATTGCTACCTATG	ACGTTGGATGCCTGCAGCAAGGTACTCACA	F	GTTCCAGGAATCCAGT	5128.4
W5	CGT	Control L323 >G	c.969C	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGGAGGACACCATGGAGGTGGA	R	CACTCCCTCAGGTAGTCCAG	6325.1
W6	TG	F311I	c.931T>A	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGGAGGACACCATGGAGGTGGA	R	TGAACTCAGTGATGATATAGA	6764.5
W6	TG	Control L323 >G	c.969C	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGGAGGACACCATGGAGGTGGA	R	TCCCTCAGGTAGTCCAG	5433.6
W7	TGA	A337V	c.1010C>T	ACGTTGGATGGAGGACACCATGGAGGTGGA	ACGTTGGATGCAGCAAGATCTCTGTGGATG	F	GGCAGGAGGTGAACG	4954.3
W7	TGA	C464W	c.1392C>G	ACGTTGGATGTTGGGAAATTGCTACCTATG	ACGTTGGATGCCTGCAGCAAGGTACTCACA	F	GCGCCCAGAAGGCTG	4850.2
W7	TGA	V468F	c.1402G>T	ACGTTGGATGCCTGCAGCAAGGTACTCACA	ACGTTGGATGTTGGGAAATTGCTACCTATG	R	GCTCGCATGAGTTCATAGA	6075
W7	TGA	F497L	c.1491C>G	ACGTTGGATGTTGGGAAATTGCTACCTATG	ACGTTGGATGCCTGCAGCAAGGTACTCACA	F	GCCTTTGAGACAATGTT	5447.6
W7	TGA	Control G321 >G	C961G	ACGTTGGATGGAGGACACCATGGAGGTGGA	ACGTTGGATGCAGCAAGATCTCTGTGGATG	F	CACTGAGTTCATGACCTAC	5994.9
W8	AC	A337V	c.1010C>T	ACGTTGGATGCAGCAAGATCTCTGTGGATG	ACGTTGGATGGAGGACACCATGGAGGTGGA	R	TGTACAGCAGCACCACG	5435.6
W8	AC	G463S	c.1387G>A	ACGTTGGATGTTGGGAAATTGCTACCTATG	ACGTTGGATGCCTGCAGCAAGGTACTCACA	F	GCATGGAGCGCCCAGAA	5500.6
W8	AC	P465S	c.1393C>T	ACGTTGGATGCCTGCAGCAAGGTACTCACA	ACGTTGGATGTTGGGAAATTGCTACCTATG	R	AGTTCATAGACCTTCTCTG	6025
W8	AC	F497L	c.1491C>G	ACGTTGGATGCCTGCAGCAAGGTACTCACA	ACGTTGGATGTTGGGAAATTGCTACCTATG	R	TCTGAGATACTGGATTCCTG	6370.2
W8	AC	Control L323 >C	c.969C	ACGTTGGATGGAGGACACCATGGAGGTGGA	ACGTTGGATGCAGCAAGATCTCTGTGGATG	F	AGTTCATGACCTACGGGAACCT	6966.6

†Amplification primers contain a 10-mer tag (ACGTTGGATG) at the 5’ end, according the iPLEX protocol (Agena).

Appendix 4: PCR primer set up

PCR primers received at 100uM each
PCR primer pools contain ~500nM of each mutation PCR primer
PCR primers stored in PCR room (1-218-H) freezer (asset number 49186)

Wells 1-4

Primer name	First PCR primer sequence	Primer volume (ul)	Check	Primer name	Second PCR primer sequence	Primer volume (ul)	Check	Water to be added (ul)
W1 PCRP mix								
E255V_10.09_PCRP1	ACGTTGGATGGGCTGTATTTCTTCCACACG	8		E255V_10.09_PCPR2	ACGTTGGATGACATCACCATGAAGCACAAG	8		936
H396R_P_10.09_PCRP1	ACGTTGGATGTTTGGCCTGAGCAGGTTGAT	8		H396R_P_10.09_PCPR2	ACGTTGGATGGGTGCAGTCCATTTGATGGG	8		
F486S_10.09_PCRP1	ACGTTGGATGAGCATGTTGGCAGTGGAATC	5		F486S_10.09_PCPR2	ACGTTGGATGTTGTTTCAAAGGCTTGGTGG	5		
Control_10.09_PCRP1	ACGTTGGATGGCCATAGGTAGCAATTTCCC	1		Control_10.09_PCPR2	ACGTTGGATGCTGGCCTACAACAAGTTCTC	1		
F317I_L_V_10.09_PCRP1	ACGTTGGATGAGCCCCCGTTCTATATCATC	5		F317I_L_V_10.09_PCPR2	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	5		
D276G_10.09_PCRP1	ACGTTGGATGGTGGCCGTGAAGACCTTGA	5		D276G_10.09_PCRP2	ACGTTGGATGTCTTTCATGACTGCAGCTTC	5		
V11 W2 PCRP mix								
T315A_10.09_PCRP1	ACGTTGGATGAGCTCCTTGGGGTCTGCAC	8		T315A_10.09_PCRP2	ACGTTGGATGTCAGGTAGTCCAGGAGGTTC	8		942
Control_10.09_PCRP1	ACGTTGGATGGCCATAGGTAGCAATTTCCC	1		Control_10.09_PCPR2	ACGTTGGATGCTGGCCTACAACAAGTTCTC	1		
M244V_10.09_PCRP1	ACGTTGGATGACAAGTGGGAGATGGAACGC	12		M244V_10.09_PCRP2	ACGTTGGATGTATTTCTTCCACACGCCCTC	8		
M351T_10.09_PCPR2	ACGTTGGATGGTTTCGGGCAGCAAGATCTC	8		M351T_10.09_PCRP1	ACGTTGGATGTACATGGCCACTCAGATCTC	8		
V10 W3 PCRP mix								
V299L_L_10.09_PCRP1	ACGTTGGATGTGGAGGTGGAAGAGTTCTTG	15		V299L_L_10.09_PCRP2	ACGTTGGATGCGGGTGCAGACCCCAAGGA	15		928
F317I_L_V_10.09_PCRP1	ACGTTGGATGAGCCCCCGTTCTATATCATC	12		F317I_L_V_10.09_PCPR2	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	12		
G250E_10.09_PCRP1	ACGTTGGATGGGACATCACCATGAAGCACA	8		E255V_10.09_PCRP1	ACGTTGGATGGGCTGTATTTCTTCCACACG	8		
Control_10.09_PCPR2	ACGTTGGATGCTGGCCTACAACAAGTTCTC	1		Control_10.09_PCRP1	ACGTTGGATGGCCATAGGTAGCAATTTCCC	1		
V11 W4 PCRP mix								
E279K_10.09_PCRP1	ACGTTGGATGAGGTTAGGGTGTTTGATCTC	5		E279K_10.09_PCRP2	ACGTTGGATGTGAAGACCTTGAAGGAGGAC	5		948

Q252H_10.09_PCRP1	ACGTTGGATGATGGAACGCACGGACATCAC	5		E255V_10.09_PCRP1	ACGTTGGATGGGCTGTATTTCTTCCACACG	5	
T315I_10.09_PCRP1	ACGTTGGATGCGGGAGCCCCCGTTCTATA	5		F317I_L_V_10.09_PCPR2	ACGTTGGATGTTGCACTCCCTCAGGTAGTC	5	
E459K_10.09_PCRP1	ACGTTGGATGCAACATGCTCGCATGAGTTC	5		E459K_10.09_PCRP2	ACGTTGGATGAGCTGCTAGAGAAGGACTAC	5	
Control_10.09_PCRP1	ACGTTGGATGGCCATAGGTAGCAATTTCCC	1		Control_10.09_PCPR2	ACGTTGGATGCTGGCCTACAACAAGTTCTC	1	
M351T_10.09_PCRP1	ACGTTGGATGTACATGGCCACTCAGATCTC	5		M351T v11 FPCRP	ACGTTGGATGCAGCAAGATCTCTGTGGATG	5	

Wells 5-8

Primer name	First PCR primer sequence	Primer volume (ul)	Check	Primer name	Second PCR primer sequence	Primer volume (ul)	Check	Water to be added (ul)
V1 W5 PCRP mix								
ABL1_MA_GLOBAL_1R	ACGTTGGATGCAGCAAGATCTCTGTGGATG	5		ABL1_MA_GLOBAL_1F	ACGTTGGATGGAGGACACCATGGAGGTGGA	5		980
ABL1_MA_GLOBAL_2F	ACGTTGGATGTTGGGAAATTGCTACCTATG	5		ABL1_MA_GLOBAL_2R	ACGTTGGATGCCTGCAGCAAGGTACTCACA	5		
V1 W6 PCRP mix								
ABL1_MA_GLOBAL_1R	ACGTTGGATGCAGCAAGATCTCTGTGGATG	5		ABL1_MA_GLOBAL_1F	ACGTTGGATGGAGGACACCATGGAGGTGGA	5		990
V1 W7 PCRP mix								
ABL1_MA_GLOBAL_1F	ACGTTGGATGGAGGACACCATGGAGGTGGA	5		ABL1_MA_GLOBAL_1R	ACGTTGGATGCAGCAAGATCTCTGTGGATG	5		980
ABL1_MA_GLOBAL_2F	ACGTTGGATGTTGGGAAATTGCTACCTATG	5		ABL1_MA_GLOBAL_2R	ACGTTGGATGCCTGCAGCAAGGTACTCACA	5		
V1 W8 PCRP mix								
ABL1_MA_GLOBAL_1R	ACGTTGGATGCAGCAAGATCTCTGTGGATG	5		ABL1_MA_GLOBAL_1F	ACGTTGGATGGAGGACACCATGGAGGTGGA	5		980
ABL1_MA_GLOBAL_2F	ACGTTGGATGTTGGGAAATTGCTACCTATG	5		ABL1_MA_GLOBAL_2R	ACGTTGGATGCCTGCAGCAAGGTACTCACA	5		

Appendix 5: UEP primer set up

1. Enter volume of final UEP mix (orange square) (Usually 1000ul)
2. Pool displayed volumes of UEP stock (green squares) and add molecular grade water to make up final UEP volume
3. Take aliquot and fire on MALDI-TOF (2ul UEP mix + 25ul water)
4. Adjust UEP mix according to primer adjustment report and spectrum results
5. Pool the UEP mixes together (2uL of each UEP mix + 19ul water) and fire on MALDI-TOF and adjust to ~ intensity 30

** If necessary reconstitute primers to 500nM with molecular grade water

FINAL UEP MIX VOLUME:
1000

Well 1	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A01	E255V	500		5060.3	0.455	4.75	9.51
A02	H396R_P	500		5524.6	1.154	12.06	24.11
A03	F486S	500		5636.7	1.176	12.28	24.56
A04	D276G V11.2	500		6003.9	1.452	15.17	30.34
A05	Control_R_G_W1	500		6039	0.812	8.48	16.97
A06	F317I_L_V	500		6645.3	1.494	15.60	31.20
A07	F359I_V	500		6850.5	1.262	13.18	26.36

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME	Water volume
334.02	665.98

Well 2	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A01	Y253F	500		4385.91	0.742	7.75	15.50
A02	Y253H	500		5042.3	0.957	10.00	20.00
A03	F317C_S	500		5217.42	0.922	9.63	19.27
A04	E355G	500		5524.6	1.154	12.06	24.11
A05	T315A	500		5674.7	1.284	13.41	26.82
A06	Control_R_G_W2	500		6039	0.812	8.48	16.97
A07	M244V	500		6409.2	1.023	10.68	21.36
A08	F359C	500		6486.2	1.290	13.48	26.95

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME	Water volume
245.51	754.49

Well 3	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A01	Q252H	500		4675.08	0.733	7.66	15.32
A02	F317L_L	500		5201.4	0.912	9.52	19.04
A03	V299L_L	500		6038.9	1.088	11.37	22.73
A04	Control_F_T_W3	500		6188	0.835	8.72	17.43

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME	Water volume
262.44	737.56

Well 4	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A01	G250E V11 -3	500		4160.7	0.471	4.92	9.83
A02	L248V	500		5172.4	1.012	10.57	21.14
A03	T315I	500		5658.7	1.174	12.26	24.52
A04	E459K	500		5836.8	1.281	13.38	26.75
A05	Control_R_G_W4	500		6039	0.812	8.48	16.97
A06	E279K	500		6620.24	1.479	15.45	30.90
A07	E255K	500		6840.4	1.306	13.64	27.29
A08	M351T v11.2	500		7201.7	1.460	15.25	30.51

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME Water volume
187.90 812.10

Well 5	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A01	ABL1_T315L-M_EXT_R	500		5459.6	0.850	8.88	17.76
A02	ABL1_E450G_EXT_F	500		5819.8	1.079	11.27	22.55
A03	ABL1_I502L_EXT_F	500		4881.2	0.800	8.36	16.71
A04	Control_W5	500		6038	0.983	10.27	20.54

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME Water volume
77.56 922.44

Well 6	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A05	ABL1_F311I_EXT_R	500		6493.3	0.822	8.59	17.17
A06	Control_W6	500		5146.4	0.637	6.65	13.30

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME Water volume
30.48 969.52

Well 7	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A07	ABL1_A337V-2_EXT_F	500		4707.1	0.909	9.49	18.98
A08	ABL1_C464W_EXT_F	500		4603	0.636	6.65	13.29
A09	ABL1_V468F_EXT_R	500		5827.8	0.927	9.68	19.36
A10	ABL1_F497L_EXT_F	500		5200.4	1.021	10.67	21.33
A11	Control_W7	500		5747.8	0.902	9.42	18.84

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME Water volume
91.81 908.19

Well 8	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)
A12	ABL1_A337V_EXT_R	500		5164.4	0.709	7.40	14.80
B01	ABL1_G463S_EXT_F	500		5229.4	0.967	10.10	20.20
B02	ABL1_P465S_EXT_R	500		5753.8	0.936	9.77	19.55
B03	ABL1_F497L_EXT_R	500		6123	0.936	9.78	19.56
B04	Control_W8	500		6719.4	0.884	9.23	18.46

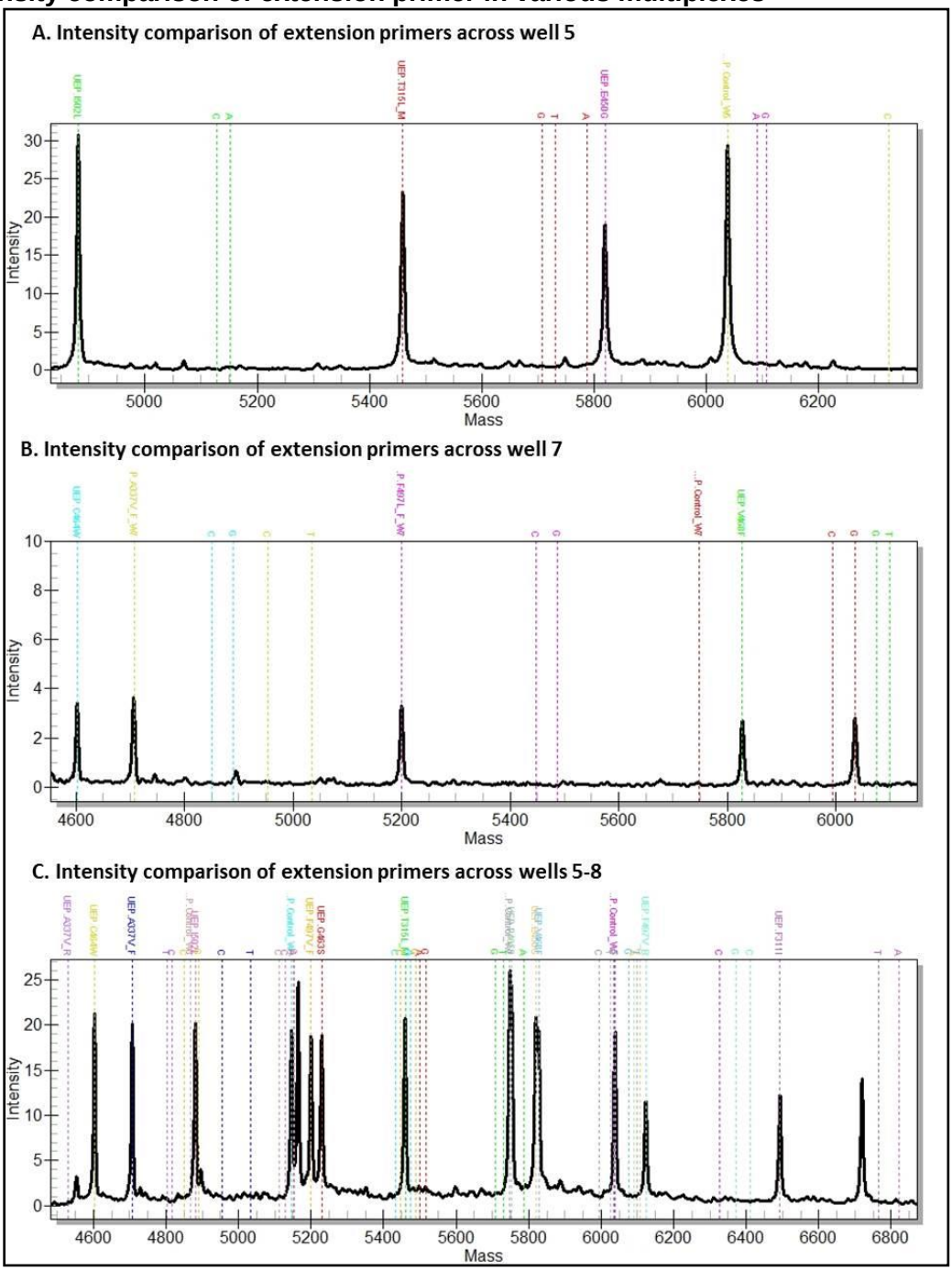
Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

POOLED STOCK VOLUME	Water volume
92.57	907.43

Appendix 6: Extension primer optimization

The extension primers in each of the multiplex reaction were optimized on the MassARRAY® Analyser 4 MALDI-TOF Mass Spectrometer to confirm primer location and intensity, aiming for an intensity of approximately 30 (Figure 1). Utilizing the Primer Adjustment Report generated to evaluate the signal to noise ratio and to calculate the additional volume required to be added into the primer pool.

Figure 4: Intensity comparison of extension primer in various multiplexes



(A) Intensity comparison of primers in well 5 demonstrating that most primers are approaching the target intensities; (B) In comparison to figure 1A, the intensity comparison in well 7 demonstrates that a substantial adjustment is required to reach the target intensity; (C) Intensity spread across all primers in wells 5-8 confirming minimal overlap between designed primers.

Appendix 7: Oligo Spike set up

Instructions:

1. Enter volume of final UEP mix + 10% (orange square)
2. Add displayed volume of oligo QC stock to 3 separate tubes (pink square)
3. Add displayed volume of molecular grade water to each tube (yellow square)
4. Pool 0.5ul of each oligo spike in one tube (0.5ul of the 3 oligo spikes + 25.5ul water) and run on mass spectrometry to ensure the intensities match and adjust as required
5. Make up enough volume for 10 wells to get average reading
6. Adjust oligo spikes according to the primer adjustment report and spectrum results

FINAL Oligo spike MIX VOLUME:	Oligo Spike QCs - 3 separate tubes
	Reactions
1000	2000

TUBE	Assay	Original stock concentration	Misc.	UEP_MASS	Target Reaction Concentration (uM)	Target Working Concentration (uM)	Volume from Stock (ul)	Percent extra to add	Amount extra to add (ul)	Total Amount of oligo to add	Water
PCR Oligo QC	PCR	500		4145.7	0.510	4.88	18.35	158	29.0	47.4	952.65
SAP Oligo QC	SAP	500		4185.7	0.519	4.97	18.70	0	0	18.7	981.30
EXT Oligo QC	EXT	500		4225.8	0.529	5.06	19.04	0	0	19.0	980.96

Relation between MASS and Peak INTENSITY: Intensity = -301Ln(Mass) + 2731

**** To use**: Add 0.5ul/reaction of the QC Oligo spike to the respective mastermix (i.e. PCR QC Oligo Spike to the PCR mastermix; SAP QC Oligo Spike to the SAP mastermix; EXT QC Oligo Spike to the iPLEX Extension mastermix)

Appendix 8: Final design of mass spectrometry assay including asciminib-resistant mutations

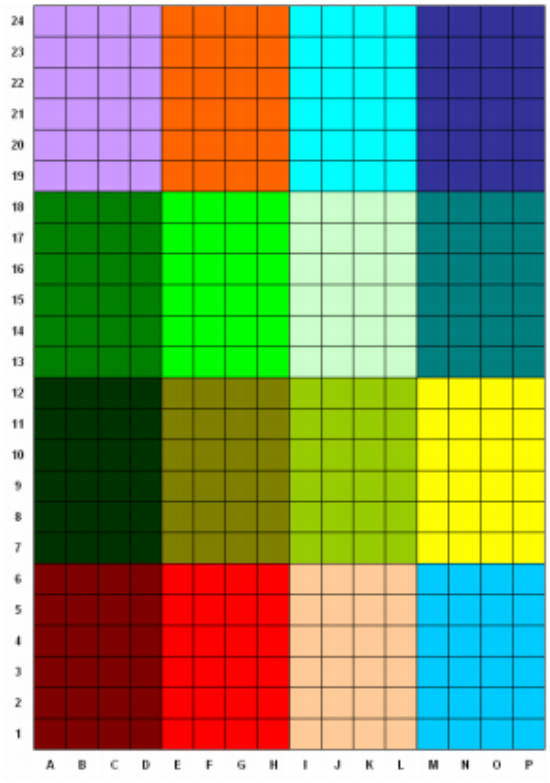
Multiplex Well	Mutation mix	Standards	Positive control cell line	Negative cluster standards	Negative control
1	D276G, D421*, E255V, F317I, F317L, F317V, F359I, F359V, F486S, H396P, H396R	2% plasmid, 0.5% plasmid, 0.2% plasmid	BV173	2x *BCR-ABL1* negative donors, 2x Hela	100% wild type *BCR-ABL1* plasmid
2	D421*, E355G, F317C, F317S, F359C, M244V, T315A, Y253F, Y253H	2% plasmid, 0.5% plasmid, 0.2% plasmid		2x *BCR-ABL1* negative donors, 2x Hela	
3	D428*, F317L, F317L, Q252H, Q252H, V299L, V299L	2% plasmid, 0.5% plasmid, 0.2% plasmid, 0.05% plasmid		2x *BCR-ABL1* negative donors, 1x Hela	
4	D421*, E255K, E279K, E459K, G250E, L248V, M351T, T315I	2% plasmid, 0.5% plasmid, 0.2% plasmid, 0.05% plasmid		2x *BCR-ABL1* negative donors, 1x Hela	
5	E450G, I502L, L323*, T315L†, T315M◊	2% gBlock, 1.5% gBlock, 1% gBlock, 0.5% gBlock	KU812	2x *BCR-ABL1* negative donors, 1x Hela	100% wild type gBlock
6	F311I, L323*	2% gBlock, 1.5% gBlock, 1% gBlock, 0.5% gBlock		2x *BCR-ABL1* negative donors, 1x Hela	
7	A337V, C464W, G321*, V468F	2% gBlock, 1.5% gBlock, 1% gBlock, 0.5% gBlock		2x *BCR-ABL1* negative donors, 1x Hela	
8	A337V, F497L G463S, L323*, P465S,	2% gBlock, 1.5% gBlock, 1% gBlock, 0.5% gBlock		2x *BCR-ABL1* negative donors, 1x Hela	

*Sample and biochemistry positive control
†Detection of this mutation is dependent on the T315A assay in well 2 detecting the first nucleotide (C nucleotide) of the three nucleotide change
◊Detection of this mutation is dependent on the T315I assay in well 4 detecting the first nucleotide (T nucleotide) of the two nucleotide change

Appendix 9: Plate-to-chip mapping (adapted from the MassARRAY® Nanodispenser RS1000 user guide v1.2)

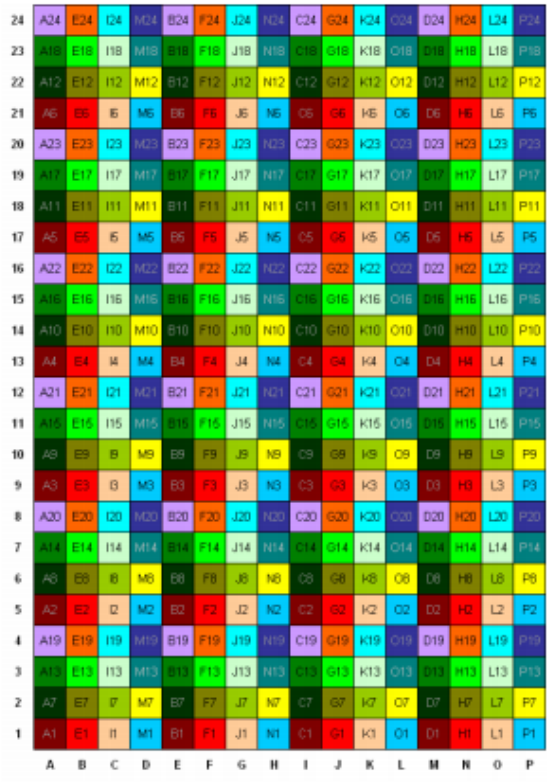
The diagrams represent a single 384-plate (left) and a 384-chip (right). The mapping scheme below is recommended which will mean that when using the 4x6 pin system will deposit samples on the chip as shown in the right hand figure.

384-well microtiter plate:



Each cell in this diagram represents a plate well. The 24-pin array is designed to fit into a 4 X 6 group of contiguous wells. In this diagram, 4 X 6 groups are color-coded to indicate that analytes in wells of the same color are loaded in the same load cycle.

384 SpectroCHIP Array:



Each cell in this diagram represents a chip pad. Each pad is labelled with the number of the plate well from which it receives analyte. Note that the pins are spaced so they land on every fourth pad; a 4 X 6 group of plate wells is spread out across every fourth chip pad.

Appendix 10. Official nomenclature for *BCR-ABL1* mutations

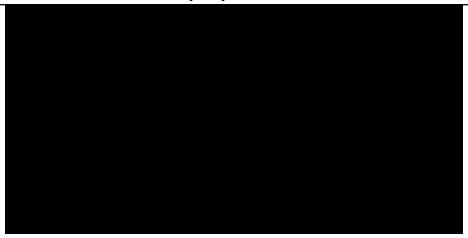
ABL1 reference sequence NM_005157. Confirm the current version of the reference sequence at the time of reporting.

Kinase domain mutation	Nucleotide position change	HGVS nomenclature
M244V	730A>G	*ABL1* NM_005157.6:c.730A>G**:**p.(Met244Val):chr21(GRCh37):g.133738330
L248V	742C>G	*ABL1* NM_005157.6:c.742C>G**:**p.(Leu248Val):chr21(GRCh37):g.133738342
G250E	749G>A	*ABL1* NM_005157.6:749G>A:p.(Gly250Glu):chr21(GRCh37):g.133738349
Q252H	756G>C	*ABL1* NM_005157.6:756G>C:p.(Gln252His):chr21(GRCh37):g.133738349
Q252H	756G>T	*ABL1* NM_005157.6:756G>T:p.(Gln252His):chr21(GRCh37):g.133738357
Y253H	757T>C	*ABL1* NM_005157.6:757T>C:p.(Tyr253His):chr21(GRCh37):g.133738357
Y253F	758A>T	*ABL1* NM_005157.6:758A>T:p.(Yyr253Phe):chr21(GRCh37):g.133738358
E255K	763G>A	*ABL1* NM_005157.6:763G>A:p.(Glu255Lys):chr21(GRCh37):g.133738363
E255V	764A>T	*ABL1* NM_005157.6:764A>T:p.(Glu255Val):chr21(GRCh37):g.133738364
D276G	827A>G	*ABL1* NM_005157.6:827A>G:p.(Asp276Gly):chr21(GRCh37):g.133747520
E279K	835G>A	*ABL1* NM_005157.6:835G>A:p.(Glu279Lys):chr21(GRCh37):g.133747528
V299L	895G>C	*ABL1* NM_005157.6:895G>C:p.(Val299Leu):chr21(GRCh37):g.133747587
V299L	895G>T	*ABL1* NM_005157.6:895G>T:p.(Val299Leu):chr21(GRCh37):g.133747587
F311I	931T>A	*ABL1* NM_005157.6:931T>A:p.(Phe311Iso):chr21(GRCh37):g.133748270
T315A	943A>G	*ABL1* NM_005157.6:943A>G:p.(The315Ala):chr21(GRCh37):g.133748282
T315I	944C>T	*ABL1* NM_005157.6:944C>T:p.(The315Iso):chr21(GRCh37):g.133748283
T315L	943A>C 944C>T 945T>A	*ABL1* NM_005157.6:c.943_945ACT>CTA: p.(The315Leu):chr21(GRCh37):g.133748282
T315M	944 C>T 945 T>G	*ABL1* NM_005157.6:c.944_945CT>TG**:**p.(The315Met):chr21(GRCh37):g.133748283
F317I	949T>A	*ABL1* NM_005157.6:949T>A**:** p.(Phe317Iso):chr21(GRCh37):g.133748288
F317L	949T>C	*ABL1* NM_005157.6:949T>C**:** p.(Phe317Leu):chr21(GRCh37):g.133748288
F317V	949T>G	*ABL1* NM_005157.6:c.949T>G:p.(Phe317Val):chr21(GRCh37):g.133748288
F317C	950T>G	*ABL1* NM_005157.6:950T>G:p.(Phe317Cys):chr21(GRCh37):g.133748289
F317S	950T>C	*ABL1* NM_005157.6:950T>C:p.(Phe317Ser):chr21(GRCh37):g.133748289
F317L	951C>G	*ABL1* NM_005157.6:951C>G:p.(Phe317Leu):chr21(GRCh37):g.133748290
F317L	951C>A	*ABL1* NM_005157.6:951C>A:p.(Phe317Leu):chr21(GRCh37):g.133748290
A337V	1010C>T	*ABL1* NM_005157.6:1010C>T:p.(Ala337Val):chr21(GRCh37):g.133748349
M351T	1052T>C	*ABL1* NM_005157.6:1052T>C:p.(Met351Thr):chr21(GRCh37):g.133748391
E355G	1064A>G	*ABL1* NM_005157.6:1064A>G:p.(Glu355Gly):chr21(GRCh37):g.133748403
F359I	1075T>A	*ABL1* NM_005157.6:1075T>A:p.(Phe359Iso):chr21(GRCh37):g.133748414
F359V	1075T>G	*ABL1* NM_005157.6:1075T>G:p.(Phe359Val):chr21(GRCh37):g.133748414
F359C	1076T>G	*ABL1* NM_005157.6:1076T>G:p.(Phe359Cya):chr21(GRCh37):g.133748415
H396P	1187A>C	*ABL1* NM_005157.6:1187A>C:p.(His396Pro):chr21(GRCh37):g.133750356
H396R	1187A>G	*ABL1* NM_005157.6:1187A>G:p.(His396Pro):chr21(GRCh37):g.133750356
E450G	1349A>G	*ABL1* NM_005157.6:1349A>G:p.(Glu450Gly):chr21(GRCh37):g.133753889
E459K	1375G>A	*ABL1* NM_005157.6:1375G>A:p.(Glu459K):chr21(GRCh37):g.133753906
G463S	1387G>A	*ABL1* NM_005157.6:1387G>A:p.(Gly463Ser):chr21(GRCh37):g.133753918
C464W	1392C>G	*ABL1* NM_005157.6:1392C>G:p.(Cys464Trp):chr21(GRCh37):g.133753923
P465S	1393C>T	*ABL1* NM_005157.6:1393C>T:p.(Pro465Se):chr21(GRCh37):g.133753924
V468F	1402G>T	*ABL1* NM_005157.6:1402G>T:p.(Val468Phe):chr21(GRCh37):g.133753933
F486S	1457T>C	*ABL1* NM_005157.6:1457T>C:p.(Phe486Ser):chr21(GRCh37):g.133755489
F497L	1491C>G	*ABL1* NM_005157.6:1491C>G:p.(Phe497Leu):chr21(GRCh37):g.133755522
I502L	1504A>C	*ABL1* NM_005157.6:c.1504A>C:p.(Ile502Leu):chr9(GRCh37):g.133755535

Appendix J Statement of authorship

Published manuscripts included in Chapter 1: The literature review

Title of paper	**Early Management of CML**
Publication Status	Published
Publication Details	Current hematologic malignancy reports
Reference (if published)	Shanmuganathan N, Hughes TP: Early Management of CML. *Current hematologic malignancy reports*. 2019, 14(6):480-491.
Digital Object identifier (if available)	https://doi.org/10.1007/s11899-019-00550-8

Name of principal author	Naranie Shanmuganathan (candidate)
Contribution to the paper	Literature review Manuscript draft and revision
Overall percentage (%)	90%
Certification	This paper involves detailed expansion on topics introduced in sections of my literature review that was published during the candidature of my Higher Degree by Research and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
Signature	
Date	23 September 2022

Co-authorship contributions:
By signing the statement of authorship, each author certified that:

- The candidate's stated contribution to the publication is accurate (as detailed above);
- Permission is granted for the candidate to include the publication in the thesis; and
- The sum of all co-authorship contributions is equal to 100% less the candidate's stated contribution

Name of Co-author	Timothy Hughes
Contribution to the paper	Outline of paper Manuscript supervision and review
Signature	[redacted]



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Publication: Current Hematologic Malignancy Reports
Publisher: Springer Nature
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Publication Details	Blood
Reference (if published)	Shanmuganathan N, Hughes TP. Molecular monitoring in CML: How deep? How often? How should it influence therapy? *Blood*. 2018;132(20):2125-2133.
Digital Object identifier (if available)	https://doi.org/10.1182/blood-2018-05-848630

Name of principal author	Naranie Shanmuganathan (candidate)
Contribution to the paper	Literature review Manuscript draft and revision
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Molecular monitoring in CML: how deep? How often? How should it influence therapy?

Author: Naranie Shanmuganathan,Timothy P. Hughes
Publication: Blood
Publisher: Elsevier
Date: 15 November 2018

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Title of paper	**Multiplex technologies for the assessment of minimal residual disease and low-level mutation detection in leukaemia: mass spectrometry versus next-generation sequencing**
Publication Status	Published
Publication Details	British Journal of Haematology
Reference (if published)	Shanmuganathan N, Branford S. Multiplex technologies for the assessment of minimal residual disease and low-level mutation detection in leukaemia: mass spectrometry versus next-generation sequencing. *British Journal of Haematology*. 2022;196(1):19-30.
Digital Object identifier (if available)	https://doi.org/10.1111/bjh.17623

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Title	Precision Medicine in CML
Institution name	University of South Australia
Expected presentation date	Jan 2023
Order reference number	15102022R2
Requestor Location	Naranie Shanmuganathan Royal Adelaide Hospital North Terrace Adelaide, SA 5000 Australia Attn:
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Title of paper	**What's NEXT for CML-NGS mutation screening**
Publication Status	Published
Publication Details	Blood
Reference (if published)	Shanmuganathan N, Hughes TP. What's NEXT for CML-NGS mutation screening. *Blood*. 2020;135(8):515-516.
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Publisher: Elsevier
Date: 20 February 2020

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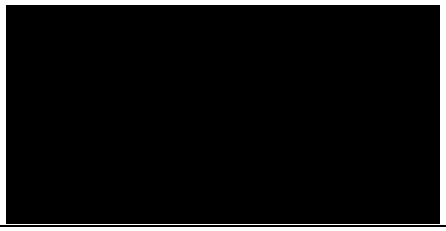
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Title of paper	**The Hidden Pathogenesis of CML: Is BCR-ABL1 the First Event?**
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Publication Details	Current Hematologic Malignancy Reports
Reference (if published)	Shanmuganathan N, Branford S. The Hidden Pathogenesis of CML: Is BCR-ABL1 the First Event? Current Hematologic Malignancy Reports. 2019;14(6):501-506.
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Title	Precision Medicine in CML
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Title of paper	**Asciminib for chronic myeloid leukaemia: Next questions**
Publication Status	Published
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Asciminib for chronic myeloid leukaemia: Next questions

Author: Timothy P. Hughes, Naranie Shanmuganathan
Publication: British Journal of Haematology
Publisher: John Wiley and Sons
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Title of paper	**Management of TKI resistant chronic phase CML**
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Reference (if published)	Hughes TP, Shanmuganathan N: Management of TKI-resistant chronic phase CML. *Hematology* 2022, 2022(1):129-137.
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Published manuscripts included as part of chapter 2

Title of paper	**Early *BCR-ABL1* kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia**
Publication Status	Published
Publication Details	Blood
Reference (if published)	Shanmuganathan N, Pagani IS, Ross DM, et al. Early *BCR-ABL1* kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia. *Blood*. 2021;137(9):1196-1207.
Digital Object identifier (if available)	https://doi.org/10.1182/blood.2020005514

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Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia

Author:
Naranie Shanmuganathan,Ilaria S. Pagani,David M. Ross,Sahee Park,Agnes S.M. Yong,Jodi A. Braley,Haley K. Altamura,Devendra K. Hiwase,David T. Yeung,Dong-Wook Kim,Susan Branford,Timothy P. Hughes

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Published manuscript included as part of Chapter 3

Title of paper	**Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission**
Publication Status	Published
Publication Details	Blood
Reference (if published)	Shanmuganathan N, Braley JA, Yong ASM, et al. Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission. *Blood*. 2019;134(1):85-89.
Digital Object identifier (if available)	https://doi.org/10.1182/blood.2019000120

Name of principal author	Naranie Shanmuganathan (candidate)
Contribution to the paper	Design of project Data collection and analysis including statistics Drafting and revision of manuscript
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Contribution to the paper	Patient provision Manuscript review
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Name of Co-author	Devendra Hiwase
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Modeling the safe minimum frequency of molecular monitoring for CML patients attempting treatment-free remission

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Published manuscript included as part of chapter 5

Title of paper	**RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia**
Publication Status	Published
Publication Details	Journal of Molecular Diagnostics
Reference (if published)	Shanmuganathan N, Wadham C, Thomson D, et al. RNA-based targeted gene sequencing improves the diagnostic yield of mutant detection in chronic myeloid leukemia. J Mol Diagn. 2022;24(7):802–822.
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Name of principal author	Naranie Shanmuganathan (candidate)
Contribution to the paper	Design of project Sample collection and processing Data collection and analysis including statistics Drafting and revision of manuscript
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RNA-Based Targeted Gene Sequencing Improves the Diagnostic Yield of Mutant Detection in Chronic Myeloid Leukemia

Author:
Naranie Shanmuganathan,Carol Wadham,Daniel Thomson,Nur Hezrin Shahrin,Chloe Vignaud,Vanessa Obourn,Shalini Chaturvedi,Feng Yang,Jinghua Feng,Verity Saunders,Chung H. Kok,David Yeung,Rob M. King,Rosalie R. Kenyon,Ming Lin,Paul Wang,Hamish Scott et al.

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Manuscript submitted for publication included as part of chapter 6

Title of paper	**Genetic abnormalities at diagnosis of chronic myeloid leukemia adversely affect molecular response and clinical outcome**
Publication Status	Under review with Haematologica
Publication Details	Not available
Reference (if published)	Not available
Digital Object identifier (if available)	Not available

Name of principal author	Naranie Shanmuganathan (candidate)
Contribution to the paper	Design of project Sample collection and processing Data collection and analysis including statistics Drafting and revision of manuscript
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Appendix K: Response to examiners' comments and/or corrections

Examiner 1

1.1 In Chapter 2, on page 33, the patients' TFR rate was compared based on the quartile of halving time (Q1 to Q4). The results showed a significant difference in TFR rates of 80% vs. 4% between the Q1 and Q4 groups. However, in practice, clinicians would like to know the best binary cut-off value. Have you tried other statistical methods, such as ROC or binary recursive partitioning methods? Additionally, presenting sensitivity, specificity, positive and negative predictive values, and accuracy (e.g., F1 score or MCC score) together would be helpful for clinicians in terms of clinical relevance.

While the published manuscript evaluated the impact of halving time quartiles on treatment free remission outcomes, I have investigated the optimal cut off for halving time using receiver operating curves. However, a binary cutoff value did not provide an adequate indicator for outcomes. Similar findings were obtained when binary recursive partitioning was applied.

Calculations for patients with a halving time ≤9.35 days versus >9.35 days are shown below.

	TFR	No TFR
Halving time ≤9.35 days	20 (TP)	5 (FP)
Halving time >9.35 days	30 (FN)	47 (TN)

Sensitivity: 40%
Specificity: 91%
Positive predictive value: 80%
Negative predictive value: 61%
F1 score: 0.53

1.2 In Chapter 5, on page 128, Figure 1C shows some discrepancies in VAFs between DNA-based and RNA-based variant calling. The R2 value of 0.6107 is acceptable, but still much lower than the correlation between DNA capture panel & WES (R2 9605) or between RNA capture panel vs RNA seq (R2 0.9441). Please provide detailed information on the reason for this discrepancy, considering the mutated gene list and biological pathway.

Thank you for your comments and this discrepancy was noted and discussed in the manuscript. The discrepancy was attributed to nonsense mediated decay and allele specific expression.

1.3 Examiner 1 has requested inclusion of a paragraph detailing the future use of NGS based tools in the clinic.

With the increasing availability of NGS, there is expanded utilization in haematological disease to prognosticate as well as monitor patients based on minimal residual disease. For diseases such as AML and ALL, NGS is conventionally performed at diagnosis to evaluate for the presence of prognosis-defining variants such as *ASXL1* or

RUNX1. In CML however, NGS investigation has lagged behind other haematological malignancies but has been able to uncover some of the genomic landscape underpinning prognostication and progression. Development of a clinical grade NGS panel that can be deployed in the clinic will ideally be utilised in the diagnostic setting to identify the presence of variants. This will ideally inform on the potential for achieving the various molecular milestones which in turn have importance for eventual TFR eligibility and TKI discontinuation. TKI selection can also be guided by the NGS findings based on the identified variants as preliminary data would suggest that the more potent 2G-TKIs can ameliorate some of the negative impact of the AGAs. Furthermore, if variants that are considered very high risk are identified, this may highlight a subset of patients that should have close monitoring for risk of progression and early identification for a donor for a potential allograft. Low level variants of concern could be monitored for clonal expansion associated with pending resistance.

Examiner 2

2.1 Given the knowledge of the halving time of BCR::ABL1 and predictable kinetics of response, could the actual % of BCR::ABL1 at treatment response be predicted? This may give an idea of whether a threshold value exists at which point TFR is likely to be successful. This may be an idea for a future study.

Previous work from Branford et al. has been performed to predict subsequent BCR::ABL1 values and clinical outcomes based on specific values and relevant timepoints (e.g. the 3-month BCR::ABL1 value and future MR4.5 prediction). I believe my work demonstrated in chapter 2 indicates that the kinetics of response is a far better predictor of response compared to specific BCR::ABL1 values at relevant timepoints.

2.2 Could halving time be relevant to other diseases where MRD is monitored (e.g. NPM1 in AML or BCR::ABL1/IGHV in B-ALL)? Why or why not? It would be interesting to discuss the generalisability of this further.

This is theoretically possible assuming that the kinetics of the disease is demonstrated to be linear. This may be possible for CLL or multiple myeloma where initial therapy is generally consistent with similar drugs/dosages being administered through the different cycles. A similar model may be challenging for transplant eligible AML patients due to the intensity and variability of treatment (e.g. induction followed by 2-3 cycles of consolidation) but may be possible if patients are receiving regular cycles of azacitidine and/or venetoclax.

2.3 The sensitivity of NGS is stated to be 3% without reference. There is no intrinsic sensitivity limitation of NGS, and new techniques with UMI correction/duplex sequencing can achieve 10^-6 sensitivity.

I have referenced the papers which assigned the sensitivity of kinase domain mutation testing to 3% which includes Soverini et al. and Kizilors et al. Higher sensitivity is certainly achievable with the duplex sequencing and UMI correction.