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## Nutlin-3a efficacy in sarcoma predicted by transcriptomic and epigenetic profiling

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### **Running Title**

MDM2 amplification doesn't confer Nutlin-3a sensitivity

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### **Conflict of Interest**

Authors disclose no conflict of interest

## Abstract

Nutlin-3a is a small molecule antagonist of p53/MDM2 that is being explored as a treatment for sarcoma. In this study, we examined the molecular mechanisms underlying the sensitivity of sarcomas to Nutlin-3a. In an *ex vivo* tissue explant system, we found that *TP53* pathway alterations (*TP53* status, *MDM2/MDM4* genomic amplification/mRNA overexpression, *MDM2* SNP309, and *TP53* SNP72) didn't confer apoptotic or cytostatic responses in sarcoma tissue biopsies (n=24). Unexpectedly, *MDM2* status did not predict Nutlin-3a sensitivity. RNA sequencing revealed that the global transcriptomic profiles of these sarcomas provided a more robust prediction of apoptotic responses to Nutlin-3a. Expression profiling revealed a subset of *TP53* target genes which were transactivated specifically in sarcomas that were highly sensitive to Nutlin-3a. Of these target genes, the *GADD45A* promoter region was shown to be hypermethylated in 82% of wild-type *TP53* sarcomas that did not respond to Nutlin-3a, thereby providing mechanistic insight into the innate ability of sarcomas to resist apoptotic death following Nutlin-3a treatment. Collectively, our findings argued that the existing benchmark biomarker for MDM2 antagonist efficacy (MDM2 amplification) should not be used to predict outcome, but rather global gene expression profiles and epigenetic status of sarcomas dictate their sensitivity to p53/MDM2 antagonists.

## Introduction

Sarcomas constitute a heterogeneous group of bone and soft tissue malignancies that account for approximately 15% of all paediatric and 1% of all adult cancers (1). Many sarcoma subtypes are resistant to cytotoxic agents, and even for those subtypes that are chemo-sensitive, long term responders are rarely seen due to cumulative toxicity and a therapeutic ceiling that has now been reached. Overall five-year survival rates for patients diagnosed with soft tissue sarcomas remains at 60%, highlighting the pressing need for new targeted systemic therapies particularly for those patients with recurrent or advanced disease. In parallel with this, new approaches to better understand the biological predictors of patient response to these agents is critical, and remains the key enabler for us to be able to move away from empiricism to a more targeted approach. In essence, the implementation of genetic markers into treatment practices will allow us to match the correct drug with the right patient.

Detailed molecular analysis of the oncogenic pathways involved in sarcomagenesis has revealed that the integrity of the p53 tumor suppressor protein and its downstream pathways remain largely intact in the majority of sarcomas (2). A mechanism commonly employed by these malignancies to subvert the anti-tumor activity of p53 during sarcomagenesis and disease progression is through amplification or overexpression of *MDM2*, the principal ubiquitin ligase for p53 (3, 4) . Such characteristics suggest that sarcomas are ideal candidates for therapies that inhibit MDM2, thus reactivating the p53 pathway to subsequently drive apoptosis. To date, the most clinically advanced MDM2 antagonist is Nutlin-3a (RG7112, Hoffmann La Roche). Identified by Vassilev et al., this cis-

imidazoline molecule blocks the hydrophobic p53-binding pocket of MDM2 resulting in rapid stabilisation of biologically active p53 protein (5).

The importance of *MDM2* in sarcomagenesis has emanated from the observation that its gene amplification is present in over 90% of well/de-differentiated liposarcomas (6). Promising results from preclinical studies have demonstrated that sarcoma cell lines with *MDM2* amplification are more sensitive to Nutlin-3a than non-*MDM2*-amplified cell lines (7, 8). Thus, it has been much anticipated to evaluate the clinical efficacy of targeting MDM2 in *MDM2*-amplified sarcomas. The first clinical trial of Nutlin (RG7112) was recently conducted in 20 patients with well/de-differentiated liposarcoma (highly enriched for *MDM2* amplification). Surprisingly, poor clinical response was reported, with only one patient achieving a partial response (9). So, does this mean that MDM2 inhibition is not an attractive therapeutic approach, or alternatively have we over-estimated the role that *MDM2* amplification plays in the response to MDM2 antagonists? A clear answer to this question will be critical as new MDM2 inhibitors in addition to Nutlin (RG7112, RG7388), such as MI-773/SAR405838 (Sanofi) and JNJ-26854165 (Johnson & Johnson) enter phase I clinical testing (10-12).

Using primary human sarcoma tissue biopsies from broad range of sarcoma subtypes with varying MDM2 status; this study has re-evaluated the capacity for *MDM2* amplification as a biomarker to identify those sarcoma patients most likely to respond to Nutlin-3a. We conclusively demonstrate that *MDM2* genomic amplification is a remarkably poor predictor of Nutlin-3a sensitivity, and subsequently used transcriptomic approaches to identify more robust biomarkers for MDM2 antagonists.

## **Materials and Methods**

### ***Clinical Material***

Tumor specimens were collected from 24 sarcoma patients undergoing surgical resection or core biopsy at three clinical institutions; the Royal Adelaide Hospital, Calvary Wakefield Hospital and St Andrew's Hospital between November 2010 and September 2012. The patient cohort included fifteen males and nine females with a mean age at diagnosis of 56.5 years (range, 18-87 years). Written informed consent was obtained from each patient (RAH Protocol #100505). Tumour subtype was evaluated by experienced sarcoma pathologists, according to the Histologic Type World Health Organization (WHO) Classification and French Federation of Cancer Centres (FNCLCC) grading systems. No patients received neo-adjuvant chemotherapy or radiotherapy within one year prior to tissue collection.

### ***Ex vivo tissue explant system***

The tissue explant system was adapted from Singh et al., 2002 (13). Fresh tumor samples were collected immediately following surgical excision, dissected into 1mm<sup>3</sup> pieces and explanted onto gelatine sponges (Spongostan, Johnson and Johnson) immersed in RPM1 1640 media (PAA Laboratories, Pasching, Austria), supplemented with 5% Fetal Calf Serum, 2mM/L L-glutamine, 100 units Penicillin, 100µg Streptomycin, 0.25µg Amphotericin B, 19nM Insulin, and 27.5µM Hydrocortisone. Explants were incubated with Nutlin-3a (10µM) or vehicle control (DMSO) in a humidified atmosphere at 37°C containing 5% CO<sub>2</sub> and collected 48hrs post treatment. (-)- Nutlin-3a was sourced from Cayman Biochemicals (Ann Arbor, MI).



## **Immunohistochemistry**

FFPE sections were deparaffinised and quenched in 3% hydrogen peroxide (10mins) to eliminate endogenous peroxidase activity. After citrate buffer (0.001M, pH 6.0) antigen retrieval, sections were blocked with normal goat serum (20mins) and immunolabelled with the following antibodies: activated-caspase 3 (ab4051, 1:100, Abcam, Cambridge, UK), Ki-67 (ab16667, 1:100, Abcam) and anti-human p53 (DO-7, 1:300, Dako, Carpinteria, CA) overnight at 4°C. Sections were subsequently incubated with biotinylated secondary antibody, anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) or anti-mouse IgG (1:250, Vector Laboratories) for 30mins at room temperature, followed by incubation with avidin-biotin peroxidase complexes (Vector Laboratories) for an additional 30mins. Immuno-complexes were visualised using DAB (Invitrogen, Grand Island, NY) and counterstained with haematoxylin. Digital images were acquired using a Nanozoomer Digital Pathology Scanner (Hamamatsu, Japan), magnification of x40.

Total p53 protein expression was scored according to the intensity and percentage of positive cells from four fields of view. Staining intensity was scored: 1= not detectable, 2=weak, 3=moderate and 4=strong. Percentage of positive cells was scored: 1=0%, 2= 1-20%, 3=21-50% and 4= > 51%). The final p53 histoscore was calculated by multiplying the intensity scores with the extent of positivity scores (minimum score 1, maximum score 16).

## ***TP53* and MDM2 Genotype**

Exons 2-11 of *TP53* were sequenced using primers and parameters listed in Table S1. Sequencing reactions were processed on an ABI Hitachi 3730 DNA analyser. *TP53* status for patients 5 and 20 was determined from exome sequencing of sarcoma DNA (Roche, SeqCap

EZ Human Exome v3.0) (ACRF, Cancer Genomics Facility, Adelaide, Australia). MDM2 SNP309 tumor genotype was determined as previously described (14).

### ***Real-time PCR analysis***

Tissue samples were homogenized using a Micra RT homogenizer (ART-Labortechnik, Germany) with total RNA extracted using the RNeasy mini kit (Qiagen, Valencia, CA), using on-column RNase-free DNase digestion according to the manufacturer's instructions. cDNA was synthesized by reverse transcribing the total RNA using Random Primers (Promega, Madison, WI) and Moloney murine leukaemia virus reverse transcriptase (H-; Promega) as previously described (15). Genomic DNA was extracted using DNeasy mini kit (Qiagen), with copy number variation (CNV) determined using a previously described method (7) and normalized using the alpha albumin (AFM) housekeeping gene. Real-time PCR reactions were processed and normalised as previously described (16). Primers are listed Table S1.

### ***Expression microarray analysis***

Expression profiling was performed using Affymetrix Human Gene 1.0 ST array as per manufacturer's protocol from RNA extracted from six sarcoma patient samples (patients 1, 2, 9, 11, 14 and 22) treated with Nutlin-3a or vehicle control. Data was processed by applying the RMA algorithm using Partex Genomics Suite 6.5. Genes were ranked in order of; Criteria 1: their magnitude of fold activation following Nutlin-3a treatment (vehicle control versus Nutlin-3a treatment) averaged for all cases and Criteria 2: the correlation coefficients between increased gene activation by Nutlin-3a and percentage increase of apoptotic cells induced by Nutlin-3a. The cumulative total of these two independently

ranked criteria provided the basis for the final ranking of these genes. Microarray data has been deposited in the Gene Expression Omnibus database (GEO GSE48296).

### ***RNA-deep sequencing***

Illumina TruSeq LT (San Diego, CA) protocol was used to generate cDNA and libraries. Nine individual samples were multiplexed and sequenced on two HiSeq2000 sequencers (2 x 100bp). Tumor RNA from patients 1, 2 and 10 were sequenced at the Australian Genome Research Facility (AGRF, Melbourne, Australia) with the remaining patient samples (patients 3, 5, 12, 13, 17 and 23) sequenced at ACRF. This resulted in 41.5-80.1 million fragments per sample, using the Illumina 2x100 base paired-end protocol. Reads were mapped to the human genome (Version hg19) with Tophat2 (Version 2.0.4, using bowtie2, mate-innerdist -20, mate-std-dev 180, UCSC splice junction gtf) (17). Gene expression was quantified using HTSeq (Version 0.5.3p9) (18) and log<sub>2</sub> transformed after regularizing peaks containing no reads to one. Comparisons between samples using hierarchical clustering (single linkage) and heatmaps were carried out using Spearman's rank correlation coefficient as the basis for the distance measure in order to avoid any normalization artefacts. However, after normalization of the samples using the median of the top quartile, clustering based on Pearson's correlation coefficient, yielded similar results. Variation between technical repeats of the transcriptome of patient 10, sequenced at two different facilities, was minimal (Fig S1).

### ***GADD45A Methylation***

*GADD45A* CpG methylation approximately 700bp upstream of the *GADD45A* transcriptional start site was analysed as previously described (19). Represented here is the

methylation of the first CpG residue out of a string of four CpG residues described in Perugini (19). Consistent with Perugini (2012), methylation of this individual CpG residue was representative of methylation across this site. Genomic DNA extracted from untreated sarcoma specimens or sarcoma cell lines was submitted to AGRF for *GADD45A* promoter methylation analysis using Sequenom MassARRAY on bisulphite converted genomic DNA. Matching patient blood samples and normal human osteoblasts were used as reference controls.

### ***GADD45A Overexpression***

TC252 *GADD45A* overexpressing cells were generated using retroviral spin-infection as previously described (20). Overexpression of *GADD45A* mRNA was determined through real-time PCR.

### ***Cell Culture***

Ewing sarcoma cell lines were maintained as previously described (16) and supplied by G. Hamilton (University of Vienna, Austria) (TC252), F. van Valen (Westfälische-Wilhelms-University, Germany) (WE-68, VH-64), J. Sonnemann (University Children's Hospital Jena, Germany) (CADO-ES1), and P. Ambros (St. Anna Children's Hospital, Austria) (STA-ET1). Cell lines were not authenticated by our laboratory.

### ***Cell cycle and viability assays***

For Decitabine/Nutlin-3a synergy experiments cells were seeded in 96-well microtiter plates ( $2.5 \times 10^4$  cells/well) and left to adhere overnight. Cells were pre-treated with 0-1000nM of 5'Aza-2'Deoxyctidine (Decitabine) (Calbiochem, San Diego, CA) for 24hrs before the addition of Nutlin-3a (0-10 $\mu$ M) for an additional 24hrs. For apoptosis (7-amino-

actinomycin-D) and cell cycle analysis (propidium iodide), cells were harvested and processed (FACS Calibur flow cytometer, Becton Dickinson Immunocytometry Systems) as previously described (16).

### ***Statistical Analysis***

$p$  values were calculated using Student's  $t$ -test using Graph Pad Prism version 6 (La Jolla, CA). Difference was considered significant when  $p$  values were 0.05 or less.

## Results

### ***Nutlin-3a induces robust apoptosis in a subset of sarcomas***

To assess the biological response of sarcoma tissue to Nutlin-3a, tumour tissue was collected immediately following surgical resection and cultured *ex vivo* on gelatine sponges immersed in media containing Nutlin-3a (10 $\mu$ M) or vehicle control for 48hrs (Fig 1A/B). This concentration of Nutlin-3a falls well within the range of saturating tissue concentrations which can be achieved in mouse tissue (21). The native tissue architecture, proliferative capacity and cellular integrity of the tumor was maintained throughout the treatment period (Fig 1C). Clinical characteristics and histopathology of the 24 sarcoma patients are outlined in Table 1.

Immunohistochemical analysis of explanted tissues for activated-caspase 3, demonstrated robust apoptotic responses (>25% increase in apoptotic cells) in 3/24 (12.5%) of sarcomas following Nutlin-3a treatment (Fig 1D/E). The biological activity of Nutlin-3a was not restricted to specific sarcoma histotypes, as these major responses were observed in tissues from a myxoid liposarcoma (patient 1), Ewing sarcoma (patient 2), and a chondrosarcoma (patient 3). Tissues were additionally stained for Ki-67 to assess the degree of cytostatic responses induced by Nutlin-3a. A marked decrease in proliferating cells (>50% compared to vehicle control) was exhibited from patients 2, 9, 15 and 22 (Ewing sarcoma, de-differentiated liposarcoma, angiosarcoma and de-differentiated liposarcoma respectively) (Fig S2A/B). No correlation between apoptotic and cytostatic responses following Nutlin-3a treatment was observed (Fig S2C).

### ***MDM2 amplification doesn't confer Nutlin-3a sensitivity***

In an effort to understand why sarcomas from patients 1, 2 and 3 showed strong apoptotic responses, we performed a genetic study to assess the role of known *TP53* pathway alterations on Nutlin-3a sensitivity. Currently, *MDM2* amplification is the benchmark biomarker required for enhanced apoptotic response (7, 8, 22). Genomic amplification and mRNA overexpression of *MDM2* (defined as >3 fold) was detected in 6/24 (25%) sarcomas (Table 1). A positive correlation between *MDM2* genomic amplification and mRNA overexpression was observed ( $r^2=0.58$ ) (Fig S3A). Surprisingly, neither *MDM2* amplification nor mRNA expression levels showed significant correlation with cellular fate following Nutlin-3a treatment (Fig 2A and Fig S3B). Even in the presence of *MDM2* genomic amplification (sarcomas 4, 6, 9, 13, 21 and 22), weak or absent apoptotic responses following Nutlin-3a treatment were observed. Furthermore, *MDM2* amplification wasn't observed in any of the three sarcoma cases that elicited the most significant apoptotic responses (sarcomas 1, 2 and 3). These results highlight the limited role that *MDM2* amplification plays as a predictor of Nutlin-3a-induced apoptosis and counters the accepted view from preclinical studies that *MDM2* amplification sensitises cultured cell lines and xenografts to Nutlin-3a.

Several *in vitro* studies have demonstrated that the ability of Nutlin-3a to activate the *TP53* pathway is severely compromised in mutant *TP53* tumor cells (23, 24). Sequencing revealed 20/24 (83%) sarcomas were wild-type for *TP53*, with the remaining four cases harbouring DNA binding domain mutations (exons 5-8) (Table 1). Although those sarcomas that underwent robust apoptosis in response to Nutlin-3a all were wild-type for *TP53*, no significant association between *TP53* status and cellular response following Nutlin-3a

treatment was observed (Fig 2B, Fig S3C). This suggests that although wild-type *TP53* may be necessary for Nutlin-3a to induce an apoptotic response, it is not useful as a stand-alone biomarker.

Finally we assessed whether the sequence polymorphisms *TP53* SNP72, *MDM2* SNP309 and *MDM4* genomic amplification/mRNA overexpression mediate Nutlin-3a sensitivity to Nutlin-3a. *MDM2* SNP309 tumor genotypes were TT (58.3%), TG (25.0%) and GG (16.7%). *TP53* SNP72 tumor genotypes were as follows 17/24 (71%) arginine, 7/24 (29%) proline. No significant statistical association was observed between any of these *TP53* pathway alterations and cellular response following Nutlin-3a treatment (Table 1, Fig 2C/D Fig S3D/E).

### ***The global transcriptomic profile of sarcomas predicts response to Nutlin-3a***

Given that known genetic p53 pathway alterations were poor predictive markers of Nutlin-3a efficacy, RNA sequencing was used (in nine cases where sufficient RNA was available) to determine the basal gene expression profiles of sarcomas within our cohort. Hierarchical clustering analysis (single-linkage clustering agglomerative) of these transcriptomes demonstrated that those sarcomas that were highly sensitive to Nutlin-3a shared similar basal gene expression profiles (Fig 3). This was intriguing given that these three sarcomas are of different histopathological classifications, suggesting that molecular subtyping of sarcomas may be a useful approach to identify those sarcoma patients most likely to respond to Nutlin-3a.



### ***Activation of the canonical p53 pathway correlates with apoptotic responses***

In an effort to define the downstream events induced by Nutlin-3a, induction of p53 protein levels in response to Nutlin-3a treatment was investigated through immunohistochemical analysis. A significant increase in p53 protein induction following Nutlin-3a treatment was observed in sarcomas undergoing apoptotic responses ( $p=0.006$ ) (Fig 4 A/B). In contrast, p53 protein induction by Nutlin-3a was not an indicator of cytostatic responses (Fig 4C) suggesting that activation of the canonical p53 pathway by Nutlin-3a in sarcomas is specifically linked to apoptotic, but not cytostatic outcomes. Furthermore, cytostatic responses (>20% reduction in proliferating cells) were observed in two sarcomas harboring *TP53* mutations (patients 20 and 24), providing additional evidence that the cytostatic capabilities of Nutlin-3a may be mediated through a p53-independent pathway. Given that canonical p53 pathway activation was significantly associated with Nutlin-3a induced apoptosis, we subsequently investigated downstream p53 target genes that may mediate this response. Gene expression microarray analysis was employed to identify downstream *TP53* target genes that were specifically activated only in those sarcomas that underwent significant apoptosis following Nutlin-3a treatment. The top seven genes are listed in Table 2 and includes four established *TP53* target genes *GADD45A*, *BBC3*, *RPS27L*, and pre-mir34A (25).

### ***GADD45A methylation: A mechanism of Nutlin-3a resistance***

We next examined whether epigenetic silencing is a mechanism through which these *TP53* target genes were not activated by *TP53* in sarcomas that didn't respond to Nutlin-3a.

Aberrant proximal promoter methylation of the *GADD45A*, approximately 700bp upstream of the transcriptional start site, has been reported in solid tumors (26-28) and haematological malignancies (19). Using Sequenom MassARRAY on bisulphite converted genomic DNA, *GADD45A* CpG promoter methylation was highly prevalent in our cohort (0-89% methylation) (Fig 5A). Methylation of five matching germline control samples ranged from 1-12%. Hypermethylation was defined as methylation exceeding the highest level observed in germline samples (>12%). Hypermethylation of *GADD45A* was absent from the three sarcomas in which the strongest apoptotic responses to Nutlin-3a were observed. In contrast, *GADD45A* hypermethylation was evident in 14/17 (82%) of wild-type *TP53* sarcomas that didn't show robust apoptosis to Nutlin-3a. Indeed, hypermethylation of *GADD45A* was significantly correlated with poor apoptotic response following Nutlin-3a treatment ( $p=0.009$ ), thus providing a possible mechanism through which several sarcomas suppress the downstream apoptotic pathways of *TP53*.

Based on histological subtype, hypermethylation of *GADD45A* was observed for all wild-type *TP53* well/de-differentiated liposarcoma, undifferentiated pleomorphic sarcoma and myxofibrosarcomas (Fig S4A). Interestingly, none of the mutant *TP53* tumours showed evidence of *GADD45A* promoter methylation, with methylation levels significantly lower than wild-type *TP53* tumours ( $p=0.042$ ) (Fig S4B). We next assessed whether hypermethylation of *GADD45A* was a reflection of global tumor hypermethylation. No significant correlation between *GADD45A* methylation and total global methylation (5-methylcytosine levels) was observed suggesting that *GADD45A* methylation was a defined event (Fig S4C/D). Together, these results imply that epigenetic silencing of *TP53* target

genes such as *GADD45A* is one mechanism by which sarcomas possess intrinsic resistance to Nutlin-3a.

### ***Overexpression of GADD45A induces sarcoma apoptosis***

We subsequently investigated whether reactivation of *GADD45A* using the clinically approved DNA methyltransferase inhibitor Decitabine could enhance Nutlin-3a induced apoptosis. Out of a panel of five Ewing sarcoma cell lines, TC252 cells displayed the highest level of *GADD45A* CpG methylation (Fig S5A). TC252 cells were pre-treated with the Decitabine for 24hrs prior to addition of Nutlin-3a for a further 24hrs. Increased apoptosis was observed when these two agents were combined (Fig 5B) both in TC252 cells and an additional *GADD45A* hypermethylated cell line, STA-ET1. These findings providing a rationale for the use of epigenetic-based anti-tumor agents to enhance Nutlin-3a induced apoptosis.

Given that *GADD45A* transactivation by Nutlin-3a was associated with apoptotic outcomes (Table 2), we explored the biological effects of induced *GADD45A* expression in sarcomas. Retroviral-mediated ectopic expression of *GADD45A* in TC252 cells resulted in a significant increase in apoptosis (Fig 5C), which is consistent with previous studies showing a pro-apoptotic role for *GADD45A* (29-32). Colony formation assays comparing control and *GADD45A* overexpressing TC252 cells plated at low density also showed an 82% reduction in the number of discrete colonies formed, 10 days post seeding ( $p < 0.0001$ ) (Fig 5D).

## Discussion

Substantial improvements in overall survival rates for most sarcoma patients have not been achieved particularly for those with metastatic disease. Future progress will be dependent not on the discovery of new cytotoxic chemotherapeutics, but rather from the development of targeted therapies. The unique characteristic of sarcomas to retain functional wild-type *TP53* status suggests that sarcomas are ideal candidates for targeted therapies that reactivate the *TP53* pathway. The efficacy of such agents will be contingent on the identification of biomarkers that can prospectively identify patients most likely to respond to this novel therapeutic approach.

To date, the *in vitro* study of immortalised cancer cell lines has been the primary method used to examine the therapeutic potential of p53-MDM2 antagonists. Such culture systems have several limitations, and do not accurately recapitulate the complex genetic characteristics and cellular heterogeneity of the original tumor from which the cell line was derived. To circumvent these limitations, we employed an *ex vivo* methodology to directly assess the cellular response and clinical importance of genetic and epigenetic determinants underlying sarcoma apoptotic sensitivity to Nutlin-3a.

Our results demonstrate that restoration of the *TP53* pathway through Nutlin-3a effectively induces apoptotic and cytostatic cellular fates in a subset of sarcoma biopsies that is not dependent on histological subtype. This is supported by several preclinical *in vitro* and mouse xenograft studies which demonstrated that Nutlin-3a can effectively activate the *TP53* pathway resulting in cell cycle arrest or apoptosis in Ewing sarcoma (16, 33), liposarcoma (7), osteosarcoma (8), rhabdomyosarcoma (34), and synovial sarcoma (35).

The initial rationale for the development of MDM2 antagonists was for the targeted treatment of tumors with almost universal *MDM2* amplification, such as well/de-differentiated liposarcomas. Several groups have demonstrated through cell culture and mouse xenograft studies that *MDM2* amplification predisposes cells to undergo enhanced apoptotic responses following Nutlin-3a treatment (7, 8, 22). Based on these preclinical studies, it was presumed that *MDM2* amplification is the key benchmark biomarker required for Nutlin-3a sensitivity. Although, genomic amplification and/or mRNA overexpression of *MDM2* was observed in 25% of sarcomas in this study, strong apoptotic responses were not elicited in any of these sarcomas following Nutlin-3a treatment. Furthermore, neither *MDM2* amplification nor overexpression was observed in any of the three sarcomas in which the most robust apoptotic responses were elicited (patient 1, 2 and 3). These results are supported by a recent Nutlin (RG7112) early phase clinical study conducted in patients with well/de-differentiated liposarcomas. Only one partial response was observed in a patient harboring *MDM2* amplification (9). Indeed poor sensitivity to RG7112 was observed as changes in the pharmacodynamic markers *TP53*, *CDKN1A* and *MDM2* mRNA (baseline versus tumor samples collected during early treatment or resection) were not significantly correlated with drug exposure. Taken together our observations imply that *MDM2* amplification is not significantly associated with apoptotic outcomes induced by Nutlin-3a and questions the use of *MDM2* amplification as a biomarker to guide patient recruitment in clinical trials involving MDM2 antagonists.

In an effort to identify biomarkers that would better predict Nutlin-3a sensitivity, gene expression array analysis was undertaken to identify alterations in key downstream *TP53* target genes that drive apoptosis. We identified that one of these target genes

(*GADD45A*) was hypermethylated in 82% (14/17) of wild-type *TP53* sarcomas that didn't respond to Nutlin-3a. Indeed, absence of methylation was significantly correlated with apoptotic response following Nutlin-3a treatment ( $p=0.0090$ ), suggesting that this may be a plausible mechanism through which sarcomas acquire an innate resistance to Nutlin-3a. Interestingly, methylation of *GADD45A* was not evident in mutant *TP53* tumours supporting the hypothesis that inactivation of *GADD45A* may be a central mechanism of resistance to p53 induced apoptosis and is therefore not selected for in *TP53* mutant tumours.

In addition to co-ordinating DNA excision repair and cell cycle regulation, growing evidence has implicated that GADD45 proteins possess pro-apoptotic functions through activation of the JNK/p38 signalling pathway (36, 37). Recently, it has been reported that MDM2 functions as an E3 ubiquitin ligase for GADD45A. Enhanced association between MDM2 and RPS7 following arsenite treatment, attenuated MDM2-mediated GADD45A ubiquitination resulting in GADD45A protein stabilisation and apoptosis (38). Interestingly, our microarray analysis identified RPS27L, a direct *TP53* target that modulates cell fate in response to genotoxic stress (39), to be up-regulated only in those sarcomas in which robust apoptotic responses were observed. RPS27L has been shown to be translocated from the cytoplasm to the nucleoplasm where it co-localizes with MDM2 in response to p53 inducing signals (40). Like RPL7, RPS27L is a physiological substrate of MDM2, and binds MDM2 via its central acidic domain (40, 41). Therefore it may be plausible for sarcomas in which enhanced apoptotic response was observed that Nutlin-3a exposure induced the pro-apoptotic activity of GADD45A by blocking its constitutive ubiquitination and degradation by enhancing the interaction between ribosomal proteins such as RPS27L and MDM2.

However, it is clear that further investigations are required to elucidate the role of *GADD45A* in Nutlin-induced apoptosis.

Epigenetic alterations (hypermethylation of 5' regulatory CpG genomic regions) have been implicated in the repression and inactivation of cellular pathways that contribute to tumorigenesis such as those governing apoptosis. Promoter methylation of *GADD45A* has been associated with poor overall survival in both acute myeloid leukemia and gastric cardia adenocarcinoma (19, 28). This is the first study to link *GADD45A* promoter methylation with innate resistance to MDM2 antagonists. Indeed, treatment of a *GADD45A* hypermethylated Ewing sarcoma cell lines with Decitabine augmented the apoptotic potential of Nutlin-3a. Collectively our findings suggest that promoter methylation of *GADD45A* represents a novel marker that could potentially be used to stratify sarcoma patients in future clinical trials, and provides a rationale for combining MDM2 antagonists with demethylating agents in selected patients, an combinational approach that has not been investigated in any disease type prior to this study.

In summary, our findings provide a clear insight into the current lack of understanding of predictive markers that mediate Nutlin-3a sensitivity and offers a rationale to redefine the inclusion criteria for patient recruitment in clinical trials involving MDM2 antagonists.

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## Table Legend

Table 1: Clinical characteristics and histopathology of the sarcoma cohort

Table 2: Genes identified by microarray analysis involved in Nutlin-3a induced apoptosis

## Figure Legends

### Fig 1. Nutlin-3a induces apoptotic responses in sarcoma patient tissue *ex vivo*

(A) Sarcoma tissue was collected immediately following surgical resection, and explanted onto gelatine sponges immersed in culture media containing either Nutlin-3a (10 $\mu$ M) or vehicle control for 48hrs. (B) Representative image of sarcoma tumor pieces cultured on a gelatine sponge. (C) H&E and Ki67 immunostaining of sarcoma tissue biopsies collected following surgical resection (untreated) and 48hrs *ex vivo* incubation with vehicle control. Images depict sarcoma tissue acquired from patients 8, 18 and 20 (myxofibrosarcoma, leiomyosarcoma and undifferentiated pleomorphic sarcoma respectively). Scale bar= 300 $\mu$ M. Number of Ki67 positive cells was averaged from four fields of view (mean  $\pm$  SE shown). (D) Percentage increase in apoptosis compared to vehicle control following Nutlin-3a treatment. Data represents average percentage of apoptotic cells determined from immunohistochemical analysis of activated-caspase, counted from four fields of view. (D) Representative images of activated caspase 3 immunohistochemistry. Scale bar= 100 $\mu$ m.

### Fig 2. *MDM2* genomic amplification doesn't confer Nutlin-3a induced apoptosis

Correlation between (A) *MDM2* copy number/mRNA expression levels, (B) *TP53* status, (C) *MDM2* SNP309, (D) *TP53* SNP72 and percentage increase in apoptotic cells following Nutlin-

3a treatment (10 $\mu$ M). *MDM2* amplification or overexpression defined as > 3 fold. Horizontal bars indicate the mean of data. Open symbols denote sarcomas 1, 2 and 3 (strongest apoptotic response).

**Fig 3. Global transcriptomic profile of sarcomas predicts response to Nutlin-3a**

(A) Hierarchical clustering of transcriptomic response to Nutlin-3a. Strongest apoptotic responders highlighted in bold. (B) Heatmap of Spearman's rank correlation coefficients, range: 0.68 to 0.92, average within-group correlation being 0.87 vs. an average across-group correlation of 0.75.

**Fig 4. p53 protein levels following Nutlin-3a treatment predict apoptotic response**

(A) Representative images of p53 immunohistochemistry from sarcoma biopsies (patient 1, 2 and 16) treated with Nutlin-3a (10 $\mu$ M) and vehicle control. Scale bar= 300 $\mu$ m. Correlation between induction of p53 protein levels (compared to vehicle control) and (B) percentage increase in apoptotic cells, and (C) percentage decrease in proliferating cells following Nutlin-3a treatment (10 $\mu$ M). Horizontal bars indicate the mean of data. Asterisk denotes statistical significance (\*\* $p < 0.01$ ). Open symbols denote sarcomas 1, 2 and 3 (strongest apoptotic response).

**Fig 5. *GADD45A* is hypermethylated in sarcoma patient samples**

(A) Sarcoma *GADD45A* promoter CpG methylation levels. Asterisk denotes statistical significant difference in apoptosis induced by Nutlin-3a (\*\* $p < 0.01$ ). B) TC252 and STA-ET1

Ewing sarcoma cells were pre-treated with the Decitabine for 24hrs (0-1000nM) before the addition of Nutlin-3a (0-10 $\mu$ M) for a further 24hrs. Cell viability was determined through 7AAD staining and analyzed by flow cytometry. The percentage of cell death  $\pm$  STDEV from duplicate reactions for each treatment is shown. (C) TC252 cells were transduced with control or GADD45A virus. Percentage of apoptotic cells (SubG1) was determined through propidium iodide staining. Data represents mean percentage of cells in SubG1  $\pm$  STDEV from duplicate reactions. (D) TC252 control and GADD45A overexpressing cells were seeded at low density (4x10<sup>3</sup> cell per well), fixed and stained with Giemsa (10 days post seeding). Data represents mean number of colonies  $\pm$  STDEV from duplicate wells. Asterisk denotes statistical significance (\*\* $p$ <0.01, \*\*\* $p$ <0.001).



Table 1: Clinical characteristics and histopathology of the sarcoma cohort

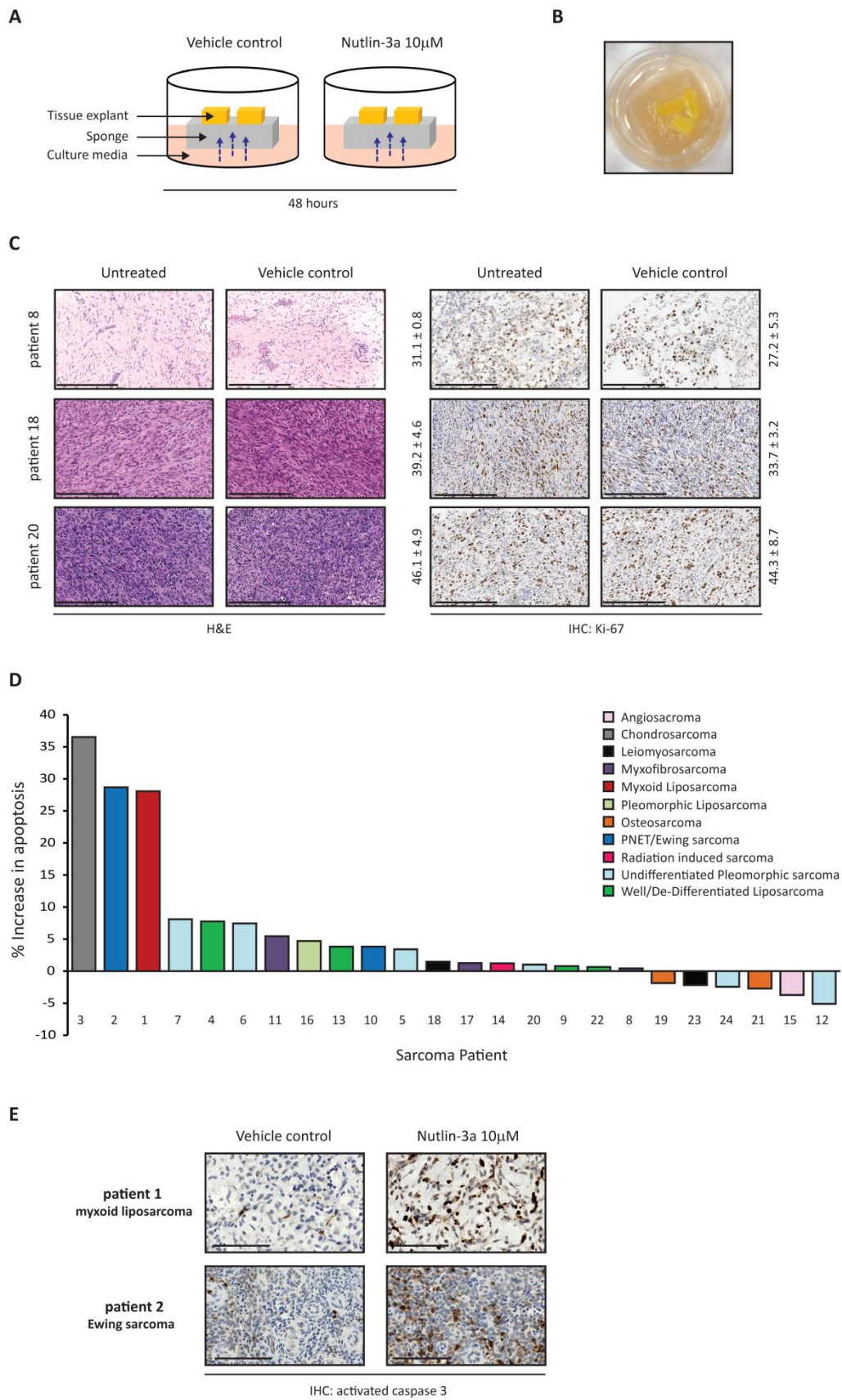
Sarcoma Patient	Gender	Age (Years)	Histological Subtype	Site	TP53 Status	p53 SNP72	MDM2 SNP309	MDM2 Copy Number	MDM2 mRNA level	MDM4 Copy Number	MDM4 mRNA level
1	Male	51	Myxoid liposarcoma	Leg	Wildtype	Pro	TG	0.81 ± 0.02	0.85 ± 0.00	0.81 ± 0.03	1.43 ± 0.11
2	Male	18	PNET/Ewing sarcoma	Heel	Wildtype	Arg	TG	0.80 ± 0.02	0.99 ± 0.01	0.79 ± 0.00	0.98 ± 0.05
3	Male	52	Chondrosarcoma	Thigh	Wildtype	Arg	TT	0.57 ± 0.05	1.11 ± 0.09	0.54 ± 0.08	0.11 ± 0.07
4	Male	76	Dedifferentiated Liposarcoma	Retroperitoneum	Wildtype	Arg	TT	12.84 ± 0.43	24.54 ± 3.06	0.91 ± 0.03	0.12 ± 0.12
5	Female	77	Undifferentiated Pleomorphic sarcoma	Scapula	V272M	Arg	TT	0.96 ± 0.03	1.90 ± 0.07	0.76 ± 0.06	0.39 ± 0.12
6	Male	84	Undifferentiated Pleomorphic sarcoma	Humerus	Wildtype	Arg	TT	8.26 ± 0.23	36.34 ± 1.12	1.21 ± 0.02	0.30 ± 0.04
7	Female	82	Undifferentiated Pleomorphic sarcoma	Thigh	Wildtype	Pro	TG	1.22 ± 0.04	1.12 ± 0.06	0.95 ± 0.02	0.16 ± 0.05
8	Female	72	Myxofibrosarcoma	Groin	Wildtype	Pro	TT	1.04 ± 0.03	1.99 ± 0.14	0.89 ± 0.05	1.04 ± 0.07
9	Male	33	De-differentiated Liposarcoma	Retroperitoneum	Wildtype	Arg	TT	80.46 ± 3.28	54.54 ± 1.00	1.04 ± 0.02	0.68 ± 0.03
10	Female	19	Ewing sarcoma	Sacrum	Wildtype	Pro	TT	0.78 ± 0.06	1.33 ± 0.16	0.62 ± 0.07	1.68 ± 0.16
11	Female	59	Myxofibrosarcoma	Chest Wall	Wildtype	Arg	TG	0.65 ± 0.03	1.28 ± 0.28	0.54 ± 0.02	0.49 ± 0.16
12	Male	50	Undifferentiated Pleomorphic sarcoma	Shoulder	Wildtype	Arg	TT	0.66 ± 0.07	0.86 ± 0.33	0.61 ± 0.04	0.21 ± 0.30
13	Male	77	Well-differentiated Liposarcoma	Groin	Wildtype	Arg	TT	36.73 ± 2.58	23.15 ± 0.73	0.74 ± 0.01	0.71 ± 0.17
14	Female	60	Radiation Induced sarcoma	Chest Wall	Wildtype	Arg	TT	0.43 ± 0.04	0.50 ± 0.01	0.64 ± 0.05	0.13 ± 0.02
15	Male	57	Angiosarcoma	Thigh	Wildtype	Pro	GG	0.72 ± 0.05	1.16 ± 0.11	0.62 ± 0.04	0.24 ± 0.16
16	Male	70	Pleomorphic Liposarcoma	Chest Wall	Wildtype	Pro	TT	0.56 ± 0.10	0.84 ± 0.06	1.17 ± 0.11	0.32 ± 0.07
17	Female	70	Myxofibrosarcoma	Thigh	Wildtype	Arg	TT	0.39 ± 0.04	1.76 ± 0.09	0.20 ± 0.01	0.12 ± 0.02
18	Male	76	Leiomyosarcoma	Stomach	Wildtype	Arg	TT	0.37 ± 0.03	0.62 ± 0.26	0.47 ± 0.05	0.23 ± 0.12
19	Male	18	Osteosarcoma	Femur	Wildtype	Arg	TG	0.69 ± 0.03	3.16 ± 0.27	0.53 ± 0.06	0.08 ± 0.06
20	Male	57	Undifferentiated Pleomorphic Sarcoma	Trapezius	V203#	Arg	TT	0.60 ± 0.02	1.00 ± 0.32	0.51 ± 0.02	0.25 ± 0.25
21	Female	29	Osteosarcoma	Fibula	Wildtype	Arg	GG	9.39 ± 0.40	10.05 ± 0.42	0.63 ± 0.04	0.20 ± 0.08
22	Male	41	De-differentiated Liposarcoma	Retroperitoneum	Wildtype	Arg	GG	12.10 ± 0.90	49.84 ± 3.55	0.67 ± 0.02	0.39 ± 0.02
23	Male	40	Leiomyosarcoma	Abdomen	H168R	Pro	GG	1.58 ± 0.24	2.22 ± 0.03	1.17 ± 0.22	0.48 ± 0.13
24	Female	87	Undifferentiated Pleomorphic Sarcoma	Groin	R196*	Arg	TG	0.94 ± 0.03	1.21 ± 0.02	0.64 ± 0.04	0.40 ± 0.02

# 16bp deletion resulting in a frameshift (Exon 6); \* Stop codon

Table 2: Genes identified by microarray analysis involved in Nutlin-3a induced apoptosis

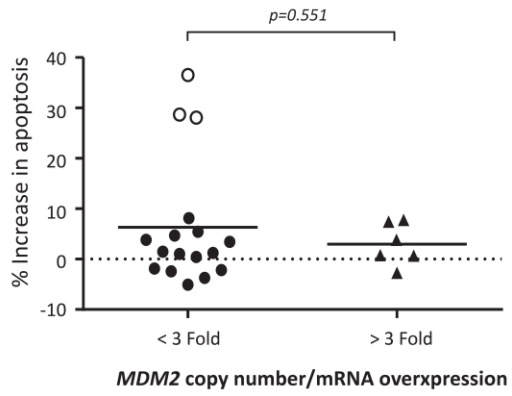
<b>Accession Number</b>	<b>Gene Symbol</b>	<b>Gene Name</b>	<b>Cellular Function</b>
NM_001071	TYMS	Thymidylate synthetase	Nucleotide biosynthesis
NR_002578	GAS5	Growth arrest-specific 5	Noncoding RNA
NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha	DNA damage repair and growth arrest
EF592573	Pre-mir34A	MicroRNA 34a	Noncoding RNA
NM_014578	RHOD	Ras homolog family member D	Muscle differentiation
NM_015920	RPS27L	RPS27L ribosomal protein S27-like	Ribonucleoprotein
NM_001127240	BBC3	BCL2 binding component 3	Apoptosis

**Figure 1**

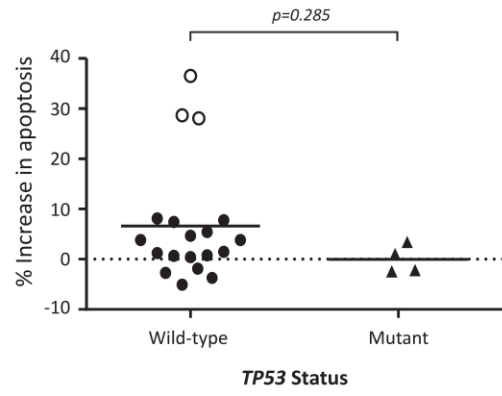


**Figure 2**

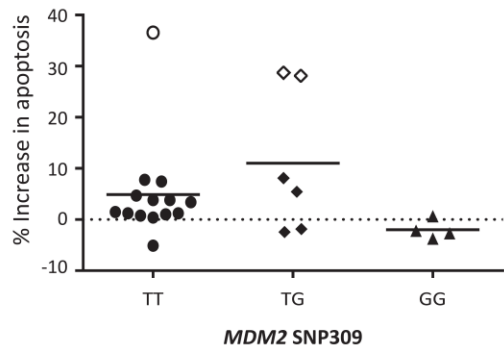
**A**



**B**



**C**



**D**

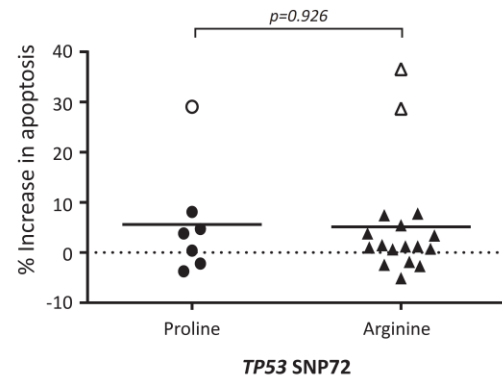
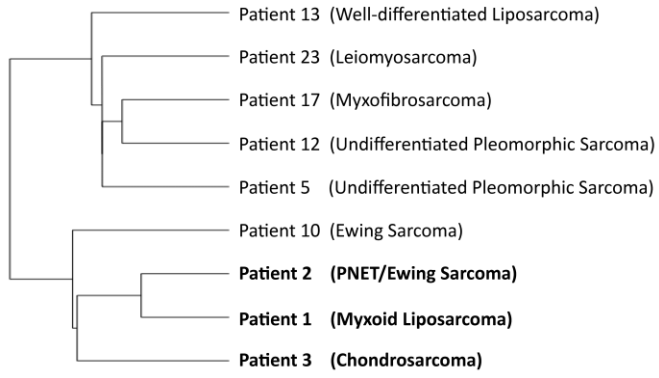
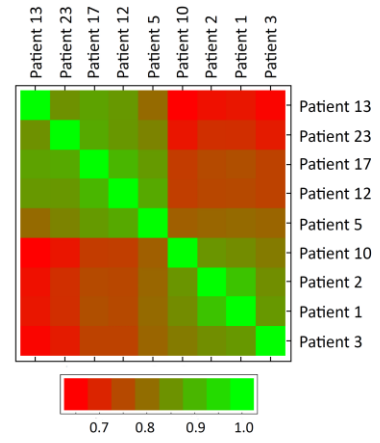


Figure 3

A

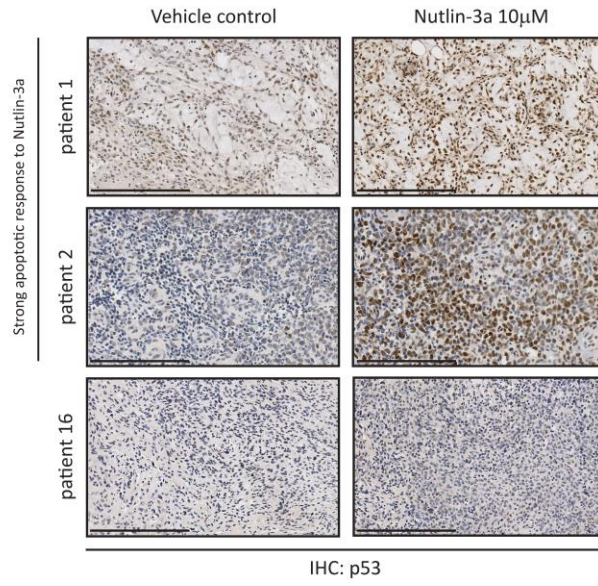


B

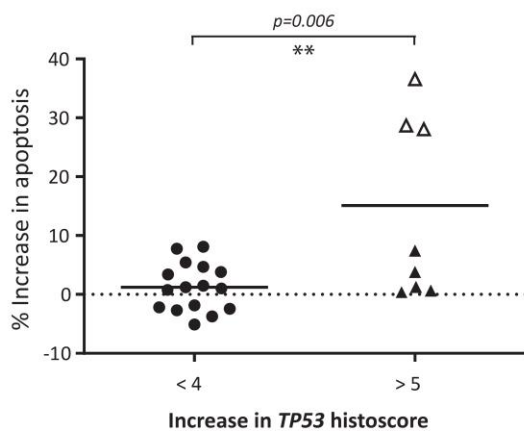


**Figure 4**

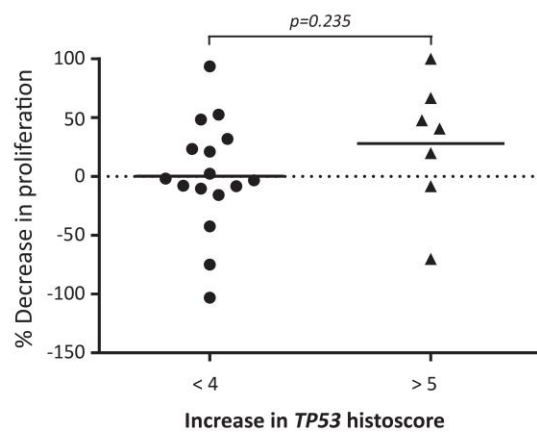
**A**



**B**



**C**



**Figure 5**

