Regulation of Cortisol Secretion in Humans: Relation to Vasopressin Action at the Adrenals in Macronodular and Micronodular Adrenocortical Tumours; and Well-Being in Addison's Disease

Lucia Gagliardi

MBBS, FRACP

for the Degree of Doctor of Philosophy

School of Medicine, University of Adelaide

March 2011

Table of Contents

Abstract vii
Declaration ix
Acknowledgements x
Publications xiii
List of Figures xiv
List of Tables xx
Abbreviations xxii

Chapter 1: Introduction

1.1 Hypothalamic-pituitary-adrenal axis function
1.1.1 Historical developments 1
1.1.2 The physiology of the hypothalamic-pituitary-adrenal axis 2
1.1.3 Hypothalamic regulation of adrenocorticotropic hormone production 4
1.1.4 Regulation of cortisol synthesis 10
1.1.5 Adrenal steroidogenesis 10
1.1.6 Circadian variation in daily cortisol production 15
1.1.7 Genomic and non-genomic effects of cortisol 19

1.2 Clinical states of glucocorticoid excess
1.2.1 Cushing's syndrome 20
1.2.2 Subclinical Cushing's syndrome 21
1.2.3 The Pseudo-Cushing's state 21

1.3 Primary adrenal Cushing's syndrome
Genetic syndromes associated with Cushing's syndrome and/or adrenocortical tumours
1.3.1 Multiple endocrine neoplasia type 1 syndrome 22
1.3.2 McCune-Albright syndrome 23
1.3.3 Primary pigmented nodular adrenocortical disease (PPNAD) 23
1.3.3.1 The Protein kinase A pathway in normal adrenal and in PPNAD 25
1.3.3.2 The canonical Wnt signalling pathway in normal adrenal and in PPNAD 26
1.3.4 ACTH-independent macronodular adrenal hyperplasia (AIMAH)
1.3.4.1 Introduction 28
1.3.4.2 Clinical presentation 29
1.3.4.3 Diagnosis 30
1.3.4.4 Histopathology 31
1.3.4.5 Inefficient steroidogenesis 31
1.3.4.6 Pathogenesis of AIMAH 32
1.3.4.7 Aberrant G-protein coupled receptors in AIMAH 33
1.3.4.8 Vasopressin-sensitive AIMAH and other adrenocortical tumours 37
1.3.4.9 Familial AIMAH 38
1.3.4.10 Genetics of AIMAH tumours 41
1.3.4.11 Treatment 43

1.4 Approaches to discovery of the genetic basis of Mendelian disease
1.4.1 Introduction 44
1.4.2 Meiosis 45
1.4.3 Monogenic *vs* polygenic inheritance 48
1.4.4 Methods of Mendelian disease gene discovery 50
1.4.4.1 Linkage analysis 50
1.4.4.2 Loss of heterozygosity studies 54
1.4.4.3 Somatic copy number variation analysis 56
1.4.4.4 Next-generation sequencing 56
1.4.5 Gene expression studies
1.4.5.1 Introduction 62
1.4.5.2 Experimental design 65
Principles of microarray data analysis
1.4.5.3 Normalisation 66
1.4.5.4 Array quality 66
1.4.5.5 Statistical analysis 66
1.4.5.6 Biological interpretation of data 67
1.4.5.7 Validating microarray data 69
1.4.5.8 Comparing microarray studies 69

1.5 Screening for subclinical Cushing's syndrome in type 2 diabetes mellitus
1.5.1 Definition 70
1.5.2 The morbidity of subclinical Cushing's syndrome and treatment outcomes 71
1.5.3 Screening tests for hypercortisolism 73
1.5.3.1 The 1mg dexamethasone suppression test (DST) 73
1.5.3.2 Twenty-four hour urinary free cortisol (UFC) 75
1.5.3.3 Midnight serum cortisol 75
1.5.3.4 Nocturnal salivary cortisol 76
1.5.4 Subclinical Cushing's syndrome in type 2 diabetes mellitus 77

1.6 Continuous subcutaneous hydrocortisone infusion therapy in Addison's disease
1.6.1 Exogenous glucocorticoid replacement 79
1.6.2 Mortality in Addison's disease 80
1.6.3 Subjective health status (health-related quality of life) in Addison's disease 81
1.6.3.1 Impaired subjective health status and DHEA 82
1.6.3.2 Impaired subjective health status and glucocorticoid replacement 83
1.6.4 Circadian glucocorticoid replacement 83

1.7 Summary 85

1.8 Aims and Hypotheses 86

Chapter 2: Familial ACTH-independent Macronodular Adrenal Hyperplasia (AIMAH): Phenotyping data of three kindreds

2.1 Introduction 89
2.2 Research Methods 93
2.3 Results and Discussion 98
2.4 Conclusion 121

Chapter 3: The Biochemical Profile of Inefficient Steroidogenesis in AIMAH-01

3.1	**Introduction**	123
3.2	**Research Methods**	126
3.3	**Results and Discussion**	129
3.4	**Conclusion**	132

Chapter 4: Genome-wide Gene Expression Profiling of AIMAH-01 tumours: Comparison with normal adrenal cortex and other adrenocortical tumours

4.1	**Introduction**	133
4.2	**Research Methods**	141
4.3	**Results and Discussion**	153
4.4	**Conclusion and Future studies**	203

Chapter 5: Genome-wide Linkage Analysis Studies, Somatic Copy Number Variation and Loss of Heterozygosity Studies of Familial ACTH-Independent Macronodular Adrenal Hyperplasia

5.1	**Introduction**	204
5.2	**Research Methods**	208
5.3	**Results and Discussion**	214
5.4	**Conclusion and Future studies**	263

Chapter 6: Next-Generation Sequencing Studies of Familial ACTH-Independent Macronodular Adrenal Hyperplasia, in kindred AIMAH-01

6.1	**Introduction**	264
6.2	**Research Methods**	266
6.3	**Results and Discussion**	273
6.4	**Conclusion and Future studies**	299

Chapter 7: A Study of the Adrenocortical Response to Low-dose Vasopressin in Functioning and Non-Functioning Adrenal Adenomas

7.1	**Introduction**	303
7.2	**Research Methods**	307
7.3	**Results and Discussion**	309
7.4	**Conclusion and Future studies**	317

Chapter 8: Screening for Subclinical Cushing's syndrome in patients with Type 2 Diabetes Mellitus and the Metabolic Syndrome

8.1	**Introduction**	318
8.2	**Research Methods**	322
8.3	**Results and Discussion**	326
8.4	**Conclusion and Future studies**	336

Chapter 9: Continuous Subcutaneous Hydrocortisone Infusion therapy in Addison's disease: a pilot study of the effect on subjective health status

9.1 Introduction 338
9.2 Research Methods 342
9.3 Results and Discussion 350
9.4 Conclusion and Future studies 359

Chapter 10: Final Discussion 362

Appendices

Appendix 1: Laboratory Methods 366

Appendix 2: Familial AIMAH-01 Affymetrix Human Gene 1.0 ST array expression data 377

Appendix 3: Ingenuity Pathway Analysis of differentially expressed genes in familial AIMAH-01 Affymetrix Human Gene 1.0 ST array expression data 390

Appendix 4: Gene set enrichment analysis of familial AIMAH-01 Affymetrix Human Gene 1.0 ST array expression data 399

Appendix 5: Ancillary linkage data 404

Appendix 6: Common regions of copy number variation between tumours from III-2 and III-3 (AIMAH-01) 411

Appendix 7: The 712 genes captured in the targeted exon capture. 412

Appendix 8: Health-related quality of life assessments. 415

Bibliography 426

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis exhibits tight physiological regulation on a circadian and ultradian basis in humans. Key central regulators include the peptides corticotrophin-releasing hormone (CRH) and arginine vasopressin (VP), acting at the pituitary, and at peripheral structures relevant to the HPA axis and other components of the stress system. Altered regulation has many causes, frequently related to tumorigenesis, and can lead to disease due to an excess of the HPA axis end-organ hormone cortisol, as in Cushing's syndrome (CS), or cortisol deficiency, as in Addison's disease. More subtle alterations of HPA axis function have been associated with many diseases. It may be that a lack of normal circadian and ultradian regulation leads to altered well-being.

Studies of three families with the rare cause of cortisol excess, ACTH-independent macronodular adrenal hyperplasia (AIMAH) revealed that adrenal function could be directly stimulated by an aberrant response to exogenous vasopressin (VP; VPs-AIMAH). In addition, it appeared possible to define subtle forms of adrenal dysregulation or early tumour formation, short of clinical CS, thereby expanding the range of phenotypic expression of this disorder for the first time, and further highlighting the familial nature of VPs-AIMAH. Studies of germline DNA, as well as expression of genes potentially relevant to the VP response in adrenal tumours, did not reveal any abnormality to explain heritable VPs-AIMAH. A SNP-based linkage study in the largest (seven affected) family revealed a single potential locus (LOD score 1.83) leading to sequencing of a number of positional candidates. Further studies have included gene expression studies of the familial AIMAH tumours, the most extensive of these studies internationally, *in vivo* stimulation studies of adrenal steroid intermediates, and finally whole exome capture and next-generation sequencing, all of which has led to increased knowledge in the AIMAH field, but without final gene/mutation identification to date.

Parallel studies examined VP responses in a convenience sample of patients presenting with adrenocortical hormone hypersecretion states or incidentally discovered adrenal tumours, and an attempt to simultaneously examine the negative predictive value of nocturnal salivary cortisol (NSC) sampling to detect the prevalence of mild CS in patients with type 2 diabetes mellitus led to the conclusion that aberrant VP responses are less frequent in adrenocortical tumours, the NSC has a low false positive rate compared with other screening tests, and that mild CS is not prevalent in local diabetes cohorts, consistent with more recent international data.

Finally, a study aimed at determining the importance of circadian and ultradian HPA axis responses was embarked upon in patients with Addison's disease, a patient group with an unmet need relating to poor well-being. Dose-response dynamic biochemical studies established the feasibility of continuous subcutaneous hydrocortisone infusion (CSHI) to produce physiological ultradian responses to daily life stress. The feasibility of longer term CSHI was studied in a randomised, double-blind, placebo-controlled clinical trial. Recruitment rates have led to this study being adopted at a multicentre level. Ultimately, this study will address the question of the importance of cortisol rhythmicity and responsiveness to well-being in humans.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Lucia Gagliardi and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

……

Lucia Gagliardi

March 2011

Acknowledgements

I would like to thank my supervisors, David Torpy, Ian Chapman and Hamish Scott for their support and guidance over the past three years. David, as principal supervisor, you have been a great source of inspiration for me to undertake a research career. Thank you for providing me with projects that have challenged me in so many ways, and which continue to captivate me – I have been enriched, both personally and professionally, for having undertaken this work. Ian, thank you for your support – you have been a source of much-needed advice for me on many occasions. Hamish, for the positive influence you have had on the course of this PhD, I am truly grateful.

I would also like to thank:

The many people in the Molecular Pathology lab who have spent many hours teaching me laboratory techniques or helped with the statistical analyses for the AIMAH project – Chris, Michael, Joe, Chung, Peter, Milena, Glenice, Zoe – I am very grateful for your efforts.

Wilton Braund, Cheri Hotu and Constantine Stratakis, for referring the other AIMAH families we have studied.

Patrick Phillips, for supporting Queen Elizabeth Hospital participation in the subclinical Cushing's syndrome screening study.

All study participants – especially the AIMAH families and the Addison's study recruits.

Medtronic for loaning us the infusion pumps to use in the Addison's study.

I am very appreciative of the funding support I have had during this PhD:

Scholarship funding: Endocrine Society of Australia Higher Research Degree Scholarship (January 2008 – August 2008) and NHMRC Medical Postgraduate Scholarship (August 2008 – present).

Research funding: RACP Novartis Oncology Endocrinology Fellowship Award (2008); Novo Nordisk Regional Diabetes Support Scheme (2008); RAH/IMVS Clinical Project Grant (2009).

I gratefully acknowledge the contribution of others to the work presented in this thesis.

Chapter 2:

Dr Cheri Hotu, Auckland General Hospital, phenotyped AIMAH-03. Mr King-Hwa (Michael) Ling, Molecular Pathology Research, Centre for Cancer Biology, SA Pathology, assisted with the vasopressin receptor RT-qPCR.

Chapter 4:

Ms Rosalie Kenyon and Mr Mark Van der Hoek, Adelaide Microarray Centre, SA Pathology, performed the array labelling and hybridisation for the microarray studies.

Mr Chung Kok, Department of Haematology, Centre for Cancer Biology, performed the statistical analysis of the raw microarray data and generated the heat map; Associate Professor Richard D'Andrea contributed to discussions regarding data analysis. Mr Joe Carolan, Molecular Pathology Research, assisted with Partek analyses. Dr Mikhail Pachkov, Swiss Institute of Bioinformatics, Biozentrum, University of Basel, Switzerland, performed the Motif activity response analysis (MARA) of the microarray data.

Mr Michael Ling and Mr Peter Brautigan, Molecular Pathology Research, assisted with primer design and microarray validation by RT-qPCR, respectively.

Chapter 5:

The SNP-typing was performed by the Australian Genome Research Facility (AGRF), Parkville, Victoria.

Ms Catherine Bromhead and Dr Melanie Bahlo, Bioinformatics Division, Walter and Eliza Hall Institute of Medical Research, performed the linkage analyses, generated the LOD score plots and haplotype pictures. Mr Joe Carolan assisted with the Partek analyses. The

sequencing reactions in Chapters 5 and 6 were performed by the Sequencing Department, SA Pathology.

Chapter 6:

Targeted exon capture was performed by NimbleGen (Madison, Wisconsin) and sequencing performed by AGRF. Mr Joe Carolan performed downstream sequencing analyses. The whole exome capture and next-generation sequencing was performed in collaboration with Beijing Genomics Institute. Mr Joe Carolan assisted with downstream sequencing analyses.

Chapter 9:

Ms Jenny von der Borch, Diabetes Educator, Royal Adelaide Hospital, instructed the participants in the use of the infusion pump. Dr Tilenka Thynne, Endocrine Registrar, Royal Adelaide Hospital, supervised the last of the participants recruited to date.

Unless otherwise specified, assays were performed by SA Pathology. Research funding subsidised the assays performed purely for research purposes.

And last, but not least, I would like to thank my family and friends for love, support and being a source of light (and stress) relief whenever it was needed. I would especially like to thank my sister, Rita, for her support, and my parents, Maria and Orazio, for their unwavering belief and confidence in me. I dedicate this thesis to you.

Publications

Peer-reviewed publications arising during candidature:

Original work

Gagliardi, L., Hotu, C., Casey, G., Braund, W.J., Ling, K.H., Dodd, T., Manavis, J., Devitt, P.G., Cutfield, R., Rudzki, Z., Scott, H.S., Torpy, D.J., 2009. Familial Vasopressin-sensitive ACTH-Independent Macronodular Adrenal Hyperplasia (VPs-AIMAH): Clinical Studies of Three Kindreds. Clin Endocrinol (Oxf) 70, 883-91.

Gagliardi, L., Chapman, I.M., O'Loughlin, P., Torpy, D.J., 2010. Screening for subclinical Cushing's syndrome in type 2 diabetes mellitus: low false-positive rates with nocturnal salivary cortisol. Horm Metab Res 42, 280-4.

Review articles/Book chapters

Gagliardi, L., Ho, J.T., Torpy, D.J., 2010. Corticosteroid-binding globulin: significance of altered levels and heritable mutations. Mol Cell Endocrinol 316, 24-34.

Gagliardi, L., Thynne, T., ACTH-Independent Macronodular Adrenal Hyperplasia. Advances in Medicine and Biology, Volume 23. (In press)

Letters

Gagliardi, L., Torpy, D.J., 2010. Subclinical Cushing's syndrome in adrenal incidentaloma: a common problem or an artefact of current diagnostic testing? Clin Endocrinol (Oxf) 72, 277-8.

Submitted

Gagliardi, L., Ling, K.H., Kok, C.H., Carolan, J., Brautigan, P., Kenyon, R., D'Andrea, R.J., Van der Hoek, M., Hahn, C.N., Torpy, D.J., Scott, H.S. Genome-wide gene expression studies in familial vasopressin-sensitive ACTH-independent macronodular adrenal hyperplasia.

List of Figures

Figure 1.1	Regulation of the hypothalamic-pituitary-adrenal axis.	3
Figure 1.2	Synthesis of adrenocorticotropic hormone (ACTH).	3
Figure 1.3	The central regulation of adrenocorticotropic hormone (ACTH) secretion.	5
Figure 1.4	The regulation of steroidogenesis by ACTH and aberrantly expressed G-protein coupled receptors.	11
Figure 1.5	The adrenal steroid synthetic pathway.	12
Figure 1.6	Plasma cortisol concentrations over a 24-hour period in normal humans.	16
Figure 1.7	The cortisol-awakening response.	17
Figure 1.8	Serum cortisol profiles in normal men during the fasting and postprandial states.	17
Figure 1.9	The cortisol response to stress.	18
Figure 1.10	Molecular mechanisms leading to cAMP pathway activation and steroidogenesis in hyperfunctioning adrenocortical tumours and hyperplasias.	24
Figure 1.11	Protein Kinase A.	25
Figure 1.12	The canonical Wnt signalling pathway.	27
Figure 1.13	Aberrant receptor screening protocol.	35
Figure 1.14	Meiosis.	46
Figure 1.15	Recombinant and nonrecombinant gametes.	47
Figure 1.16	Patterns of Mendelian inheritance.	49
Figure 1.17	Knudson’s two hit hypothesis of tumorigenesis.	55
Figure 1.18	The Sanger sequencing method.	58
Figure 1.19	Next-generation sequencing.	59
Figure 1.20	An Affymetrix GeneChip®.	64
Figure 1.21	Serum cortisol in normal subjects and after thrice-daily hydrocortisone.	80
Figure 1.22	Serum cortisol-time profiles after delayed-release hydrocortisone.	84

Figure 1.23	Serum and salivary cortisol profiles during continuous subcutaneous hydrocortisone infusion therapy.	85
Figure 2.1	Pedigree of kindred AIMAH-01.	99
Figure 2.2	The aberrant cortisol response to vasopressin in AIMAH-01.	99
Figure 2.3	Resected macronodular adrenal glands from III-1 (AIMAH-01).	101
Figure 2.4	Histopathology of AIMAH tumour.	101
Figure 2.5	Adrenal scintigraphy in III-2 (AIMAH-01).	103
Figure 2.6	Resected macronodular right adrenal gland from III-2 (AIMAH-01).	104
Figure 2.7	Adrenal scintigraphy in III-3 (AIMAH-01).	105
Figure 2.8	Resected macronodular right adrenal gland from III-3 (AIMAH-01).	105
Figure 2.9	Expression of vasopressin receptors by AIMAH-01 adrenal tumours.	107
Figure 2.10	Pedigree of kindred AIMAH-02.	112
Figure 2.11	The aberrant cortisol response to vasopressin in AIMAH-02.	112
Figure 2.12	Pedigree of kindred AIMAH-03.	117
Figure 2.13	The aberrant cortisol response to vasopressin in AIMAH-03.	118
Figure 3.1	The steroidogenic pathway showing the enzyme defects in AIMAH-01, and elevated steroid intermediates (in bold) as suggested by measurement of basal and ACTH-stimulated adrenocortical hormones and steroid intermediates**.**	124
Figure 4.1	Dendrogram showing hierarchical clustering of gene expression in sporadic AIMAH.	138
Figure 4.2	Motif activity response analysis activity profiles.	147
Figure 4.3	Gene set enrichment analysis.	148
Figure 4.4	Principal components analysis plot of AIMAH-01 tumours and normal adrenal cortex expression data.	154
Figure 4.5	Heat map of 100 most differentially expressed (according to fold-change) genes in the AIMAH-01 tumours.	155

Figure 4.6	MA plot for all AIMAH *vs* normal.	156
Figure 4.7	Selected significantly differentially expressed genes in AIMAH-01 tumours.	159
Figure 4.8	Familial AIMAH-01 enrichment plot for upregulated (Panel A) and downregulated (Panel B) genes in sporadic AIMAH.	169
Figure 4.9	Gene Set Enrichment Analysis Plots.	170
Figure 4.10	The adrenal steroid synthetic pathway in AIMAH-01.	182
Figure 4.11	Expression of enzymes and transcription factors involved in the steroidogenic pathway in AIMAH-01 tumours.	184
Figure 4.12	Expression of *TGFB2* in AIMAH-01 tumours.	189
Figure 4.13	Cyclic AMP-independent protein kinase A (PKA) activation involving TGFβ.	192
Figure 4.14	Putative somatic copy number variation involving *GNB2* in III-2.	193
Figure 4.15	Putative somatic copy number variation involving *PRKAR2A* in III-2.	195
Figure 4.16	The Wnt/β-catenin signalling pathway in AIMAH-01.	199
Figure 4.17	Putative somatic copy number variation involving *AXIN* in III-2.	202
Figure 4.18	Putative somatic copy number variation involving *UBB* in III-2.	202
Figure 5.1	Pedigree of kindred AIMAH-04.	209
Figure 5.2	Autosomal nonparametric linkage analysis scores for kindreds AIMAH-01, -02 and -03.	215
Figure 5.3	Autosomal nonparametric linkage analysis scores for kindred AIMAH-04.	219
Figure 5.4	Autosomal parametric linkage analysis scores for kindreds AIMAH-01, -02 and -03.	224
Figure 5.5	Autosomal parametric linkage analysis scores for kindred AIMAH-04.	228
Figure 5.6	Possible locus in AIMAH-01.	232

Figure 5.7	Haplotype analysis of AIMAH-02 (AIMAH-01 locus).	237
Figure 5.8	Haplotype analysis of AIMAH-03 (AIMAH-01 locus).	238
Figure 5.9	Haplotype analysis of AIMAH-04 (AIMAH-01 locus).	239
Figure 5.10	Possible locus in AIMAH-04.	241
Figure 5.11	Haplotype analysis of AIMAH-01 (AIMAH-04 locus).	243
Figure 5.12	Haplotype analysis of AIMAH-02 (AIMAH-04 locus).	244
Figure 5.13	Haplotype analysis of AIMAH-03 (AIMAH-04 locus).	246
Figure 5.14	Putative somatic copy number loss in the AIMAH-01 locus.	252
Figure 5.15	Putative somatic copy number loss involving 2p16.	253
Figure 5.16	Putative somatic copy number loss involving 3p21.	254
Figure 5.17	Putative somatic copy number loss involving 11q13.	255
Figure 5.18	Putative somatic copy number loss involving 17q24.	256
Figure 5.19	Putative somatic copy number loss in region containing *ZCCHC8*.	258
Figure 5.20	Loss of heterozygosity on 16p13 in tumour from III-3 (AIMAH-01)	258
Figure 5.21	RT-PCR of ataxin 3 mRNA from III-2 and III-3 (AIMAH-01).	262
Figure 6.1	The SureSelect Target Enrichment System Capture Process.	269
Figure 6.2	Pipeline of bioinformatics analyses.	271
Figure 6.3	Exon capture alignment data for *NRP1* and *PKHD1*.	274
Figure 6.4	The putative SNV (“T to C”) in neuropilin 1 (*NRP1*) in one patient.	276
Figure 6.5	The putative SNV (“C to T”) in Polycystic kidney and hepatic disease 1 (autosomal recessive) (*PKHD1*) in one patient.	277
Figure 6.6A	Collagen, type VII, alpha 1 (*COL7A1*) - Whole exome capture SNV validation.	286
Figure 6.6B	Distribution of the Collagen, type VII, alpha 1 (*COL7A1*) SNV in AIMAH-01.	286
Figure 6.7A	A kinase (PRKA) anchor protein 13 (*AKAP13*) - Whole exome capture SNV validation.	288

Figure 6.7B	Distribution of the A kinase (PRKA) anchor protein 13 (*AKAP13*) SNV in AIMAH-01.	288
Figure 6.8	RAS protein activator like 1 (GAP1 like) (*RASAL1*) - Whole exome capture SNV validation.	289
Figure 6.9A	Coiled-coil domain containing 88A (*CCDC88A*) - Whole exome capture SNV validation.	290
Figure 6.9B	Distribution of the Coiled-coil domain containing 88A (*CCDC88A*) SNV in AIMAH-01.	290
Figure 6.10A	IQ motif containing GTPase activating protein 1 (*IQGAP1*) – Whole exome capture SNV validation.	291
Figure 6.10B	Distribution of the IQ motif containing GTPase activating protein 1 (*IQGAP1*) SNV in AIMAH-01.	291
Figure 6.11A	Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) - Whole exome capture SNV validation.	293
Figure 6.11B	Distribution of the Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV in AIMAH-01.	293
Figure 6.12	High resolution melt analysis of Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV.	294
Figure 6.13	Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV in a normal individual.	294
Figure 6.14A	Phosphodiesterase 11A (*PDE11A*) INDEL in AIMAH-01.	298
Figure 6.14B	Distribution of the Phosphodiesterase 11A (*PDE11A*) INDEL in AIMAH-01.	298
Figure 7.1	Classification of ACTH-cortisol responses to physiologic-dose vasopressin.	308
Figure 8.1	Subclinical Cushing’s syndrome screening protocol.	324

Figure 8.2 Midnight salivary cortisol concentrations using the Roche Elecsys Cortisol Electrochemiluminescence Immunoassay in healthy controls, Cushing's syndrome (CS) and suspected CS. 325
Figure 9.1 Continuous subcutaneous hydrocortisone infusion pump study protocol. 344
Figure 9.2 The serum cortisol profiles after bolus subcutaneous hydrocortisone in a single patient with Addison's disease. 351
Figure 9.3 The serum cortisol profiles after basal-bolus subcutaneous hydrocortisone infusion in a single patient with Addison's disease. 352
Figure 9.4 Participant recruitment for the Continuous Subcutaneous Hydrocortisone Infusion study. 355

List of Tables

Table 2.1	Summary of familial ACTH-Independent Macronodular Adrenal Hyperplasia.	90
Table 2.2	Phenotyping data of kindred AIMAH-01.	109
Table 2.3	Phenotyping data of kindred AIMAH-02.	115
Table 2.4	Phenotyping data of kindred AIMAH-03.	119
Table 3.1	Basal and ACTH-stimulated (Synacthen®) steroids and steroid intermediate concentrations measured before in III-2 and III-3 (AIMAH-01).	130
Table 4.1	Summary of published gene expression data in AIMAH.	135
Table 4.2	Gene sets used for Gene Set Enrichment Analysis.	149
Table 4.3	Ten highly differentially expressed genes in AIMAH-01.	158
Table 4.4	AIMAH-01 expression data for selected genes, comparison with normal adrenal cortex.	164
Table 4.5	Expression of enzymes and transcription factors involved in the steroidogenic pathway, in AIMAH-01.	183
Table 5.1	Mutations associated with sporadic ACTH-Independent Macronodular Adrenal Hyperplasia.	205
Table 5.2	Genes in locus 14q32.11-14q32.12.	233
Table 5.3	Selected LOD score data for AIMAH-01.	247
Table 5.4	Selected LOD score data for AIMAH-04.	248
Table 5.5	Coding variations of sequenced candidate genes in possible AIMAH-01 locus.	261
Table 6.1	Summary of single nucleotide variants in a DNA sample using targeted exon capture.	275
Table 6.2	Summary of the quality of sequencing data generated from whole exome capture and next-generation sequencing.	279

Table 6.3	Coding exons within the AIMAH-01 possible region of linkage which were not resequenced to an average four-fold depth by whole exome capture.	280
Table 6.4	Summary of SNVs and INDELs for whole exome capture samples.	281
Table 6.5	The basis for selection for validation of six SNVs detected by whole exome capture.	284
Table 7.1	Characteristics of participants and their ACTH and cortisol responses to physiologic-dose vasopressin.	310
Table 8.1	Prevalence studies of Cushing's syndrome or subclinical Cushing's syndrome in patients with diabetes mellitus.	319
Table 8.2	Clinical and metabolic parameters of the study participants (*n*=100).	327
Table 8.3	Clinical characteristics and results of study participants requiring further evaluation for possible hypercortisolism.	329
Table 10.1	Hypothalamic-pituitary-adrenal axis regulation, relation to vasopressin and relevance to well-being in humans.	363

Abbreviations

ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACT	adrenocortical tumour
ACTH	adrenocorticotropic hormone
AIMAH	ACTH-independent macronodular adrenal hyperplasia
ATP	adenosine triphosphate
AVPR1A	vasopressin receptor type 1A
AVPR1B	vasopressin receptor type 1B
AVPR2	vasopressin receptor type 2
CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
CNC	Carney complex
CNV	copy number variation
CRF	corticotrophin-releasing factor(s)
CRH	corticotrophin-releasing hormone
CS	Cushing’s syndrome
CSHI	continuous subcutaneous hydrocortisone infusion
CV	coefficient of variation
CYP11A1	cholesterol side-chain cleavage enzyme
CYP11B2	aldosterone synthase
CYP17A1	17α-hydroxylase
CYP21A2	21-hydroxylase
dbSNP	SNP database; National Centre for Biotechnology Information
DEG	differentially expressed genes

DHEA/S	dehydroepiandrosterone/sulphate
DNA	deoxyribose nucleic acid
DST	dexamethasone suppression test
FC	fold-change
FDR	false-discovery rate
GIP	gastric-inhibitory polypeptide
GPCR	G-protein coupled receptor
GSEA	gene set enrichment analysis
HbA_{1c}	glycosylated haemoglobin
HPA	hypothalamic-pituitary-adrenal
IM	intramuscular/ly
INDEL	insertion/deletion
IPA	Ingenuity Pathway Analysis
I.U.	international units
IV	intravenous/ly
LOD	logarithm of the odds
LOH	loss of heterozygosity
MARA	motif activity response analysis
MAS	McCune-Albright syndrome
MEN1	multiple endocrine neoplasia type 1
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NFAI	non-functioning adrenal incidentaloma
NGS	next-generation sequencing
NPL	nonparametric linkage
NSC	nocturnal salivary cortisol
OMIM	Online Mendelian Inheritance in Man

PCA	principal components analysis
PCR	polymerase chain reaction
PD-VP	physiologic dose vasopressin
PKA	protein kinase A
POMC	pro-opiomelanocortin
PPNAD	primary pigmented nodular adrenocortical disease
PRKAR1A	protein kinase A regulatory subunit 1A
RNA	ribonucleic acid
RR	reference range
RT-qPCR	reverse transcription-quantitative PCR
SCS	subclinical Cushing's syndrome
SF-1	steroidogenic factor-1
SF-36	short form-36 (health survey)
SNP	single nucleotide polymorphism
SNV	single nucleotide variant/variation
Sp1	transcription factor Sp1
StAR	steroidogenic acute regulatory protein
3βHSD	3β-hydroxysteroid dehydrogenase
T2DM	type 2 diabetes mellitus
TGFβ	transforming-growth factor β
UCSC	University of California, Santa Cruz
UFC	urinary free cortisol
VP/-s	vasopressin/-sensitive
WEC	whole exome capture