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

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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.

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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

Chapter 1: Introduction

1.1 Hypothalamic-pituitary-adrenal axis function

1.1.1 Historical developments

The anatomy of the adrenal glands was first described by Bartolomeo Eustachius in 1563 (Eustachius, 1774). However, he offered no hypothesis regarding their function. The zonation of the adrenal cortex and its distinction from the medulla were reported shortly thereafter. Little progress was made towards understanding the function of the adrenal glands until the pioneering work of Thomas Addison.

It was whilst Addison was studying "…a very remarkable form of general anaemia, occurring without any discoverable cause whatever…" but which "with scarcely a single exception, was followed, after a variable period, by the same fatal result" that he observed "…the existence of diseased supra-renal capsules" (Addison, 1855). He noted "general languor and debility, irritability of the stomach, and a peculiar change of colour in the skin" and "…failure of appetite…body (wasting)…in connexion (sic) with (this) diseased condition of the 'supra-renal capsules'" (Addison, 1855). This was the first clinical description of primary adrenocortical failure, even though, "…at the present moment, the functions of the supra-renal capsules, and the influence they exercise in the general economy, are almost or altogether unknown" (Addison, 1855).

From Addison's first descriptions of adrenocortical insufficiency in 1855 until 1930, attempts at treatment were unsuccessful, and death occurred within a year or two after diagnosis (Dunlop, 1963). Many advances in adrenocortical hormone isolation, synthesis and clinical application occurred during the 1930s and 1940s. In the early 1950s cortisone acetate became widely available, with a marked improvement in the survival of patients with Addison's

disease (Dunlop, 1963). Soon after, oral fludrocortisone became available for mineralocorticoid replacement.

The control of adrenocortical function by a pituitary factor was demonstrated in the 1920s; a concept supported by the work of Harvey Cushing, who associated his clinical observations, a "polyglandular syndrome" caused by pituitary basophilism, with adrenal hyperactivity (Cushing, 1932). Adrenocorticotropic hormone (ACTH) was isolated in the sheep in 1943 (Li *et al.,* 1943).

1.1.2 The physiology of the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is the humoral component of an integrated and intricate neural and endocrine system that responds to internal and external challenges to homeostasis (stressors) (Figure 1.1). The system comprises the neuronal pathways linked to catecholamine release from the adrenal medulla (fight-or-flight response) and the hypothalamic control of ACTH release which regulates cortisol production from the zona fasciculata of the adrenal cortex. The HPA axis is tightly regulated by negative feedback – ACTH and cortisol attenuate hypothalamic corticotrophin-releasing hormone (CRH) release, and cortisol also attenuates pituitary ACTH production and secretion (Figure 1.1).

Cortisol regulates CRH and ACTH secretion *via* a "slow" negative feedback loop involving inhibition of pro-opiomelanocortin (POMC; the precursor of ACTH) (Figure 1.2) gene transcription in the anterior pituitary and CRH (and vasopressin, discussed later) gene transcription and peptide secretion in the hypothalamus (Lundblad and Roberts, 1988; Davis *et al.,* 1986; Eberwine *et al.,* 1987; Keller-Wood and Dallman, 1984). "Fast" feedback also occurs, and may be due to cortisol inhibition of an event in stimulus-secretion coupling, e.g., cyclic adenosine monophosphate (cAMP) production (Keller-Wood and Dallman, 1984).

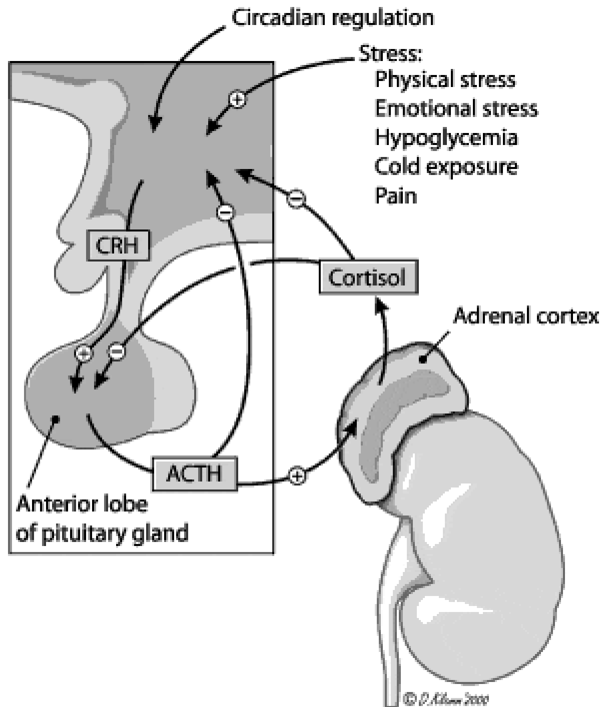

Figure 1.1. Regulation of the hypothalamic-pituitary-adrenal axis.
Vasopressin is not shown. (Kirk, Jr, *et al.,* 2000).

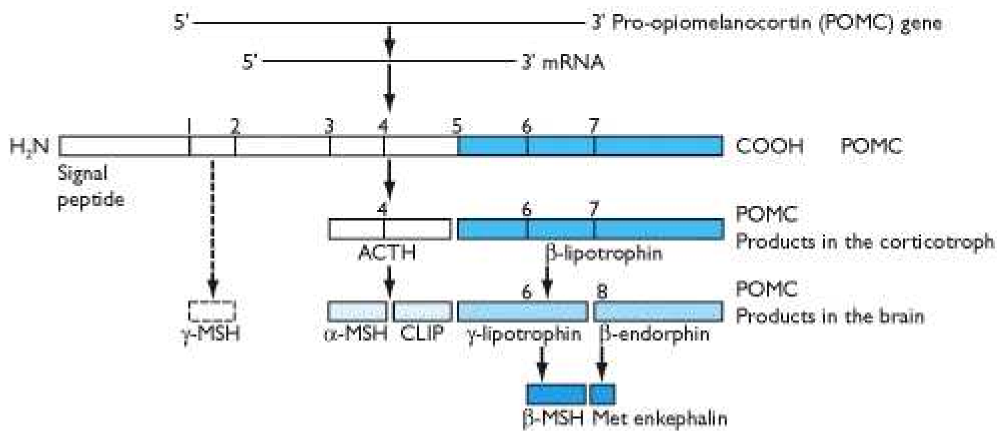

Figure 1.2. Synthesis of adrenocorticotropic hormone (ACTH).
The pro-opiomelanocortin (POMC) gene codes for a large pro-hormone plus signal sequence that is subsequently cleaved into smaller active molecules, as indicated, under the action of peptidases. The processing of POMC is tissue-specific, as indicated in the figure.

1.1.3 Hypothalamic regulation of adrenocorticotropic hormone production

In the mid-1950s it was demonstrated that hypothalamic factors could increase ACTH secretion from the pituitary gland incubated *in vitro* or maintained in organ culture (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). Vasopressin (VP) and noradrenaline were amongst these corticotrophin-releasing factors (CRFs).

In 1981, Vale *et al.,* isolated (from ovine hypothalami), sequenced and synthesized a 41-residue peptide CRF that was highly active in stimulating the secretion of corticotrophin-like immunoactivity *in vitro* (primary cultures of rat pituitary cells) and *in vivo* (Vale *et al.,* 1981). This was a more potent secretagogue than other known CRFs; later becoming known as corticotrophin-releasing hormone (CRH), consistent with its role as the primary corticotrophin secretagogue (Vale and Rivier, 1977; Vale *et al.,* 1983). CRH binds to the G-protein coupled CRH type 1 receptor expressed on pituitary corticotrophs, stimulating adenylate cyclase activation, cAMP generation, and hence, ACTH secretion (Chen *et al.,* 1993; Chang *et al.,* 1993; Giguere and Labrie, 1982).

Several lines of evidence led to the identification of VP, a hypothalamic nonapeptide usually associated with the posterior pituitary and with antidiuretic properties, as also having corticotropic activity. It was observed that certain hypothalamic injuries in animals, particularly involving the median eminence, were associated with an absence of circulating ACTH during acute stress (McCann and Sydnor, 1954). Subsequently, it was shown that reduced ACTH production and release induced by hypothalamic lesions could be restored by administering large doses of VP (McCann and Brobeck, 1954). Intravenous administration of VP to humans also stimulated an increase in 17-hydroxycorticosteroid and cortisol; later studies verified the ACTH dependency of the response (McDonald and Weise, 1956; McDonald *et al.,* 1956; Bähr *et al.,* 1991).

In the hypothalamus, VP derived from the magnocellular supraoptic hypothalamic nuclei has vasoconstrictor and antidiuretic properties, operating *via* VP type 1a (AVPR1A) and type 2 (AVPR2) receptors, respectively (Figure 1.3). However, VP secreted by the parvicellular neurons of the paraventricular nuclei into the hypothalamic-portal circulation has intrinsic and synergistic (with CRH) activity as an ACTH secretagogue (Figure 1.3) (Buckingham, 1985). The intrinsic secretagogue activity of VP is mediated by a specific VP receptor (type 1b or 3, AVPR1B) expressed on anterior pituitary corticotrophs (Baertschi and Friedli, 1985; Koch and Lutz-Bucher, 1985).

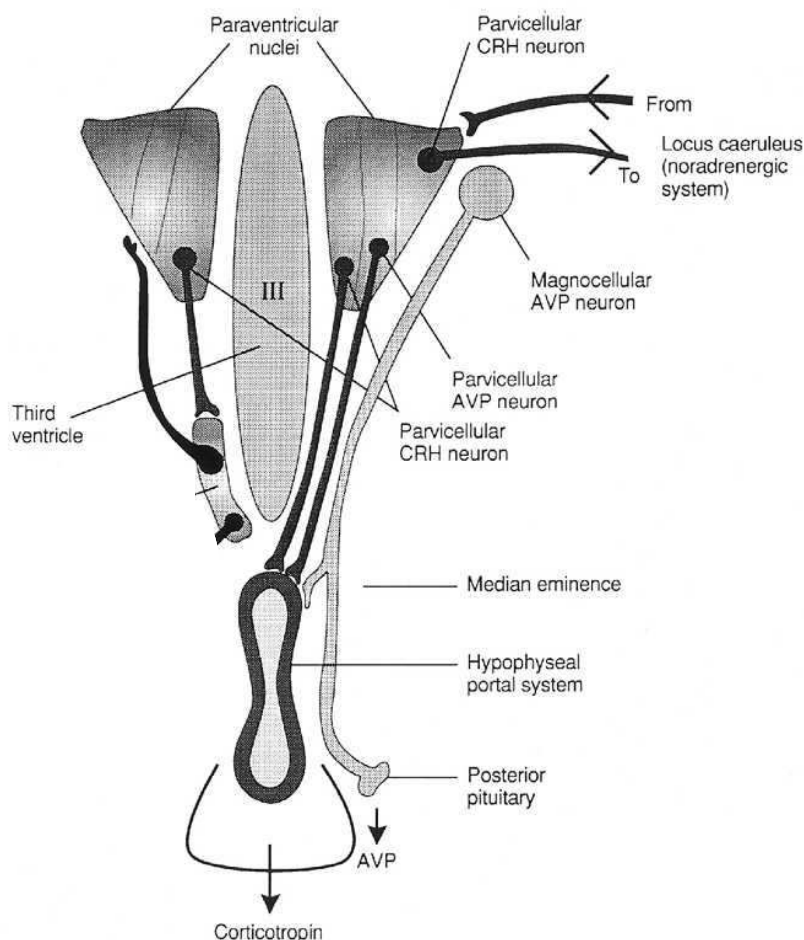

Figure 1.3. The central regulation of adrenocorticotropic hormone (ACTH) secretion. Parvicellular vasopressin (AVP/VP) neurons secrete VP into the hypophyseal portal system, whereafter corticotropin (adrenocorticotropic hormone) secretion is stimulated. (Torpy, 2010).

Serendipitous observations during experiments on CRFs led to the discovery of the synergistic action of VP with CRH to stimulate ACTH release in rats and in humans (Gillies *et al.,* 1982; Rivier and Vale, 1983; DeBold *et al.,* 1984; Liu *et al.,* 1983; Lamberts *et al.,* 1984). It had been observed that the HPA axis response to tested materials was greater in dehydrated (for 24 hours) rats and in newly arrived animals to the laboratory, than in those kept in the laboratory for more than one day, suggesting that endogenous VP release might increase the sensitivity of the anterior pituitary to CRFs (Yates *et al.,* 1971). The interaction involves VP-mediated potentiation of CRF-induced cAMP accumulation in corticotrophs, augmenting ACTH release (Giguere and Labrie, 1982).

Within the paraventricular nucleus of the hypothalamus there are two approximately equal subpopulations of CRH neurosecretory cells; those secreting only CRH (CRH+/VP-), distributed throughout the medial parvicellular subdivision, and those also secreting VP (CRH+/VP+), found within the dorsal third of this subdivision (Whitnall *et al.,* 1987b; Whitnall, 1988; Whitnall and Gainer, 1988). In CRH+/VP+ cells, the secretagogues are co-localised within neurosecretory vesicles (Whitnall *et al.,* 1985).

The differential distribution of CRH+/VP- and CRH+/VP+ secretory cells in the hypothalamus suggested the cells were differentially regulated by stressors. Measurements of hypophysial portal blood collected before and after specific stressors confirmed stressor-specific evoked changes in CRH and/or VP (Plotsky *et al.,* 1985; Caraty *et al.,* 1990; Engler *et al.,* 1989; Canny *et al.,* 1989). In animal studies, the percentage of CRH+/VP+ neurons increased from 50 to 95% after adrenalectomy, suggesting these neurons were independently regulated by glucocorticoids (Whitnall *et al.,* 1987a). Acute stress selectively activates CRH+/VP+ neurons, suggesting that VP is important in the ACTH response to short-term stress (Whitnall, 1989). There are three distinct classes of secretagogue-responsive corticotrophs – those responsive to: (1) CRH only; (2) CRH and VP, but not to either alone;

and (3) CRH or VP (Jia *et al.,* 1991). The greater number of CRH-responsive corticotrophs could explain why CRH is the more potent ACTH secretagogue.

Thus, the central regulation of ACTH secretion occurs *via* independent mechanisms operating at the hypothalamus and pituitary. The specificity of the ACTH response to a stressor may protect the pituitary from ACTH secretory fatigue by reserving a corticotroph population which could produce a swift ACTH response to a subsequent stressor.

In vitro administration of CRH and VP to (rat) hypothalamic extracts potentiated the ACTH response to CRH by four-fold (Gillies *et al.,* 1982). Human studies showed a similar augmentation of the ACTH response to CRF (1µg/kg, intravenously (IV)) with VP (10 I.U. intramuscularly (IM)) (DeBold *et al.,* 1984; Liu *et al.,* 1983; Lamberts *et al.,* 1984). However, the large doses of VP administered would have produced supraphysiological concentrations of VP, potentially exaggerating its role in the regulation of ACTH secretion. In later studies, a lower dose (1 I.U./70kg) of IV VP (physiologic dose VP; PD-VP) achieved circulating VP levels of 46-60pmol/L and was sufficient to stimulate ACTH secretion (Hensen *et al.,* 1988; Torpy *et al.,* 1994). Basal and CRH-stimulated ACTH secretion is also augmented by physiological increases in endogenous VP (e.g., water deprivation or hypertonic saline infusion) (Watabe *et al.,* 1988; Rittmaster *et al.,* 1987; Bähr *et al.,* 1988; Milsom *et al.,* 1985). To the extent that changes in plasma VP reflect the relative concentration of VP in the pituitary-portal circulation which is accessible to corticotrophs, then these studies establish a physiological role of VP in regulating ACTH release.

In the non-stressed state, circulating VP levels are low, relate to hydration status and are insufficient to stimulate ACTH secretion (Hensen *et al.,* 1988). During a significant stress, such as hypotension or haemorrhage, peripheral VP levels increase and may exceed the threshold for ACTH stimulation (Robertson, 1977). In these situations, increases in

circulating VP may be sufficient to stimulate the HPA axis; otherwise circulating levels are generally too low to do so. However, concentrations of VP within the portal system are much higher (≈ 300-fold) than circulating levels; moreover within the hypophyseal-portal system, VP can directly stimulate ACTH secretion by corticotrophs (Zimmerman *et al.,* 1973). In an *AVPR1B* knockout mouse, resting circulating concentrations of ACTH and corticosterone (the predominant circulating glucocorticoid in rodents) were lower than in the wildtype mouse, impaired VP-induced ACTH release occurred *in vivo* and *in vitro*, and the ACTH increment after a forced swim stress was impaired (Tanoue *et al.,* 2004). These data provide further evidence for a role of VP in the physiological regulation of the HPA axis.

During pregnancy in human and non-human primates, cortisol levels increase throughout gestation, driven by placental production of CRH (homologous to hypothalamic CRH), increasing plasma ACTH, and reaching values found in Cushing's syndrome (Sasaki *et al.,* 1988; Magiakou *et al.,* 1997). The ACTH and cortisol responses to CRH are blunted (Goland *et al.,* 1990; Schulte *et al.,* 1990; Suda *et al.,* 1989). In contrast, the ACTH and cortisol responses to VP are enhanced in pregnant, non-human primates, augmenting further as gestation progresses (Goland *et al.,* 1991).

Although studies of VP responsiveness have not been performed in human pregnancy, the similarities of HPA axis changes during pregnancy suggest the non-human data could be extrapolated. Since CRH responsiveness is diminished in pregnancy, VP may be important in the dynamic modulation of the HPA axis. This may include a direct effect on stimulating adrenocortical production of cortisol, a phenomenon that occurs in some adrenocortical tumours, discussed later, and a focus of two of the studies presented in this thesis (Chapters 2 and 7). Alternatively, HPA axis regulation by VP may have evolved to stimulate vasoconstriction and protect against uterine haemorrhage. Administration of VP antiserum to cats with an intact HPA axis had no effect on the ACTH or cortisol response to haemorrhage.

However, under dexamethasone suppression, VP antiserum attenuated the ACTH and cortisol responses, suggesting that when there is CRH-ACTH suppression, VP is important in the HPA axis response to haemorrhage (Carlson and Gann, 1984).

Through its ability to mediate a stress response, and thus protect from real or perceived threats to homeostasis, the HPA axis is critical for survival of the organism. We postulate that the dual regulation of the axis has evolved as a protective mechanism, should a secretagogue become depleted or have diminished effectiveness on ACTH secretion. Furthermore, the presence of corticotrophs differentially responsive to CRH/VP is another protective strategy, preventing ACTH secretory fatigue and thereby ensuring a residual capacity to respond to a subsequent stressor.

Thus, the principal effect of VP on the regulation of cortisol secretion is via an ACTH-dependent mechanism. As will be discussed later, some adrenocortical tumours demonstrate VP-sensitivity; i.e., endogenous or exogenous modulation of VP concentration directly, and independently of ACTH, stimulates cortisol secretion. This may be due to adrenal tumour cells overexpressing VP receptors, which function analogously to the ACTH receptor, to stimulate steroidogenesis, described below. We studied VP-sensitivity in patients with a familial predisposition to develop VP-sensitive adrenal tumours (ACTH-independent macronodular adrenal hyperplasia – AIMAH) and Cushing's syndrome (Chapter 2) because in these families, VP sensitivity may be an early manifestation of the disease phenotype. We also studied VP sensitivity in patients with sporadic functioning and non-functioning solitary adrenal adenomas (Chapter 7) using PD-VP, because our preliminary studies in AIMAH indicated that this dose was sufficient to detect an aberrant response, but there were no data on the frequency of VP sensitivity in adrenocortical tumours using this dose.

1.1.4 Regulation of cortisol synthesis

Intra-adrenal cortisol storage is minimal; thus the capacity of the adrenal to rapidly increase steroidogenesis in response to a provocative stimulus is paramount to the physiological HPA axis response to a stressor. In many endocrine tissues, ligand binding to a specific G-protein coupled receptor (GPCR) is a key regulator of glandular function; ACTH is one such example. Cortisol is synthesized within thirty minutes of ACTH binding to the specific seven transmembrane-domain ACTH GPCR on zona fasciculata cells of the adrenal cortex (Catalano *et al.,* 1986). After ACTH binding, the Gsα subunit of the heterotrimeric (three subunit) coupled G protein becomes activated, subsequent adenylate cyclase activation generates the second messenger, cAMP, from the hydrolysis of adenosine triphosphate (ATP) (Figure 1.4). The main mediator of cAMP signalling is activated protein kinase A (PKA) (Scott, 1991). Cyclic AMP directly, and indirectly, via activation of the PKA pathway, stimulates steroidogenesis (Ferguson, Jr, 1963). The action of cAMP is terminated by its degradation by phosphodiesterases.

1.1.5 Adrenal steroidogenesis

The adrenal steroid synthetic pathway is depicted in Figure 1.5. The obligate substrate for steroidogenesis is cholesterol, which is derived from receptor-mediated endocytosis of plasma low density lipoproteins (LDL), but may also be synthesised *de novo* (Miller, 2008). LDL receptor activity and uptake of LDL cholesterol are stimulated by ACTH.

Cholesterol transport to the inner mitochondrial membrane, the site of the cholesterol side-chain cleavage enzyme (CYP11A1), is facilitated by steroidogenic acute regulatory (StAR) protein (Miller, 2007). CYP11A1 catalyses the first and rate-limiting step of steroidogenesis: conversion of cholesterol to pregnenolone, by three sequential reactions - 20α-hydroxylation, 22-hydroxylation and scission of the cholesterol side chain.

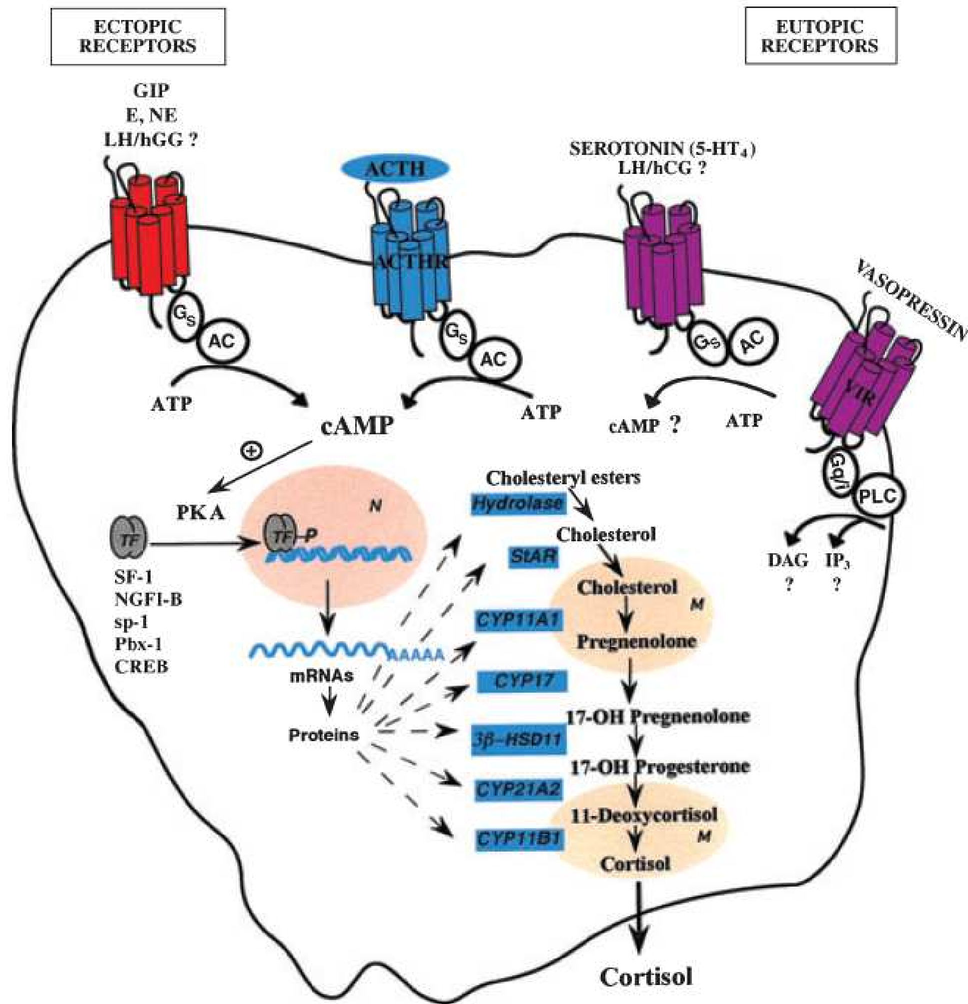

Figure 1.4. The regulation of steroidogenesis by ACTH and aberrantly expressed G-protein coupled receptors.

ACTH binds to its seven transmembrane G-protein coupled receptor, stimulating the Gs subunit of the heterotrimeric (three subunit) G protein. Adenylate cyclase is activated, adenosine triphosphate (ATP) is hydrolysed to cyclic AMP (cAMP). This activates protein kinase A (PKA) which stimulates steroidogenesis. Some adrenal tumours express aberrant receptors. These receptors may be ectopic (not found on normal adrenal cells) or eutopic overexpressed (found on normal adrenal cells, but not coupled to steroidogenesis) receptors that are coupled to steroidogenesis. In this situation, cortisol production is regulated by an aberrant, non-ACTH-dependent mechanism, which escapes the negative glucocorticoid feedback system. (Lacroix *et al.,* 2004).

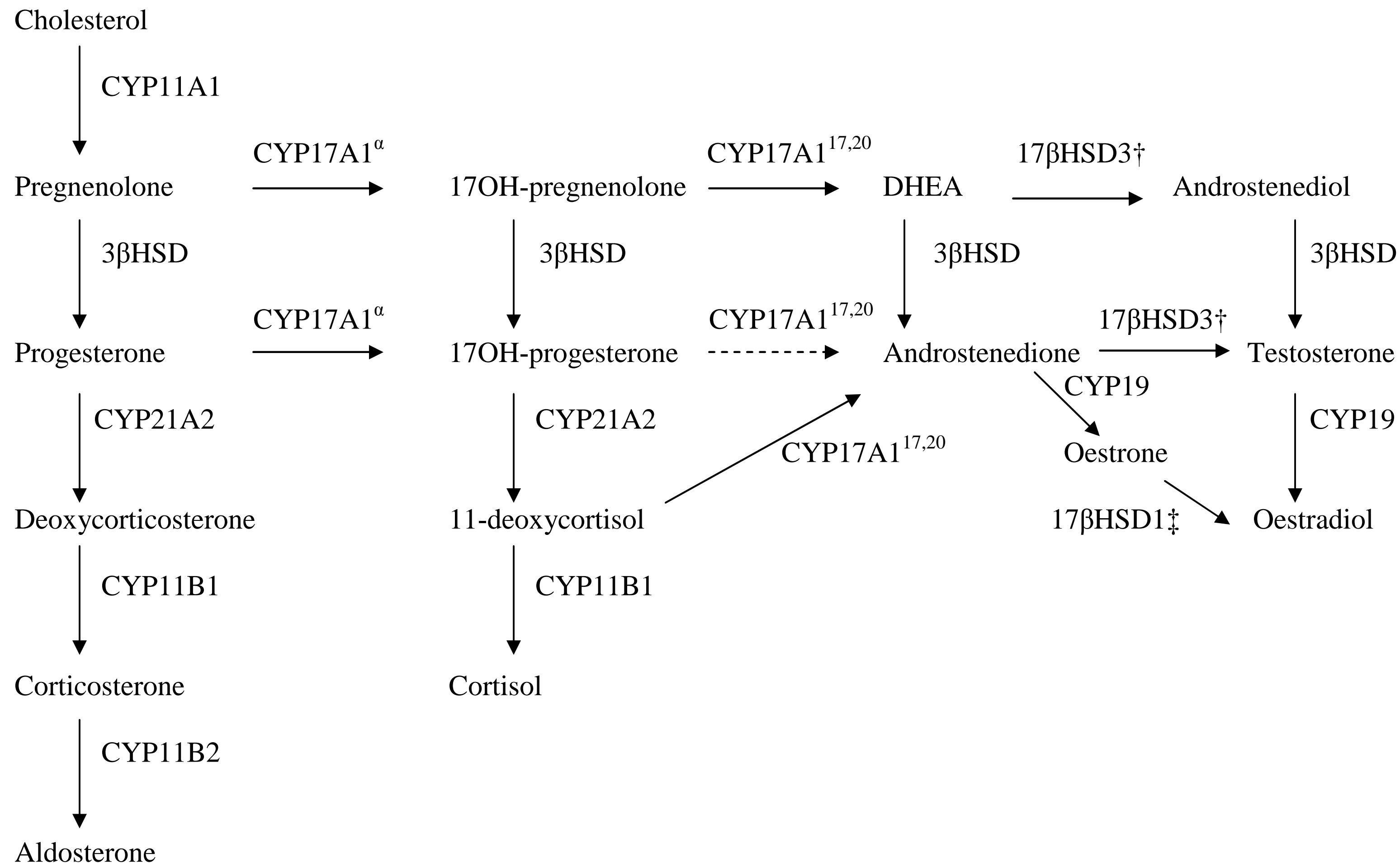

Figure 1.5. The adrenal steroid synthetic pathway.
CYP11A1 – cholesterol side chain cleavage enzyme; CYP11B1 - 11β-hydroxylase; CYP11B2 – aldosterone synthase; CYP17A1$^{\alpha}$ - 17α-hydroxylase of CYP17; CYP17A1^{17,20} – 17,20 lyase activity of CYP17; CYP19 – aromatase; CYP21A2 – 21-hydroxylase; DHEA - dehydroepiandrosterone; 3βHSD - 3β-hydroxysteroid dehydrogenase; 17βHSD1,3 - 17β-hydroxysteroid dehydrogenase 1,3; *insignificant amounts of 17OH-progesterone are converted to androstenedione in humans; †testis; ‡placenta, ovary

The subsequent oxidation-reduction reactions, by which, in the adrenal, pregnenolone is transformed into glucocorticoids, mineralocorticoids or androgens (Figure 1.5) is determined by specific steroidogenic enzyme expression in the zones of the adrenal cortex (fasciculata – cortisol; glomerulosa – aldosterone; reticularis – androgens). Since cholesterol delivery to CYP11A1 is crucial for the initiation of steroidogenesis; this StAR-dependent process is also considered a rate-limiting step of steroidogenesis (Clark *et al.,* 1994).

The acute regulation of steroidogenesis, where steroids are produced and secreted within minutes of a stimulus is *via* cAMP/PKA signalling which: (1) increases the availability of cholesterol substrate; (2) increases *StAR* transcription; and (3) *via* phosphorylation of specific serine residues, increases the biological activity of StAR (Arakane *et al.,* 1997; Clark et al, 1995; Sugawara *et al.,* 1995). The chronic regulation of adrenal steroidogenesis occurs over hours to days and involves increased cAMP-dependent transcription of genes encoding steroidogenic enzymes (Miller, 2008).

Steroidogenic factor 1 (SF-1; NR5A1) and transcription factor Sp1 (Sp1) are important transcriptional regulators of genes encoding proteins involved in steroidogenesis. Whilst SF-1 is specifically expressed in adrenal glands, gonads and the neural and pituitary elements of the HPA axis, Sp1 is ubiquitous (Ikeda *et al.,* 1995; Luo *et al.,* 1994). SF-1 confers basal and cAMP-dependent responsiveness to many of the genes encoding steroidogenic enzymes (Parker and Schimmer, 1997).

Adrenocortical expression of StAR is crucial to steroidogenesis. SF-1 is essential to both basal and cAMP-stimulated regulation of the *StAR* gene (Sugawara *et al.,* 1996). The human *StAR* gene promoter has three SF-1 binding sites; the distal SF-1 binding site is important for basal activity; whilst the proximal two binding sites are important for basal and cAMP-stimulated promoter activity (Sugawara *et al.,* 1997). The human *StAR* promoter also has two

consensus Sp1 sequences near the more distal SF-1 binding sites; *in vitro* studies suggest that the Sp1 binding sites enable the interaction of Sp1 with SF-1 (Sugawara *et al.,* 2000). Transforming growth factor β1 (TGFβ1), is an inhibitor of bovine adrenocortical steroidogenesis; this involves repression of *StAR* expression, which in part may be due to inhibition of *SF-1* transcription (Brand *et al.,* 1998; Lehmann *et al.,* 2005).

Since CYP11A1 mediates the first and rate-limiting step of steroidogenesis, its abundance is the critical determinant of the chronic steroidogenic capacity of each adrenocortical cell (Miller, 2008). In addition to their role in regulating transcription of StAR, SF-1 and Sp1 are also key transcriptional regulators of CYP11A1. The *CYP11A1* promoter region contains binding sites for SF-1 and Sp1 (Guo and Chung, 1999; Chung *et al.,* 1997; Watanabe *et al.,* 1994). Two adrenal-selective enhancers harbour Sp1 binding sites (Chou *et al.,* 1996). Other elements in the *CYP11A1* promoter bind proteins (e.g., transcriptional regulating protein of 132kDa) which physically bind SF-1, synergistically activating the promoter (Gizard *et al.,* 2001; Gizard *et al.,* 2002).

We studied the steroidogenic pathway in patients with a familial predisposition to develop adrenal tumours (ACTH-independent macronodular adrenal hyperplasia). This particular type of adrenal tumour is characterised by massive bilateral adrenal enlargement but relatively mild cortisol excess, due to steroidogenic enzyme defects. We sought to identify the specific defects in siblings with these familial tumours by measuring basal and ACTH-stimulated steroid hormones and intermediates (Chapter 3) and by performing gene expression studies of resected adrenal tumours (Chapter 4).

Although ACTH is the primary regulator of adrenocortical cortisol production, VP may also directly stimulate the normal adrenal gland to secrete cortisol, operating via the VP receptor, AVPR1A, expressed on cells of the zona fasciculata of the adrenal cortex (Guillon *et al.,*

1995; Perraudin *et al.,* 1993). Intra-adrenal VP has been detected in cells of the medulla, and more scarcely in the cortex; physiologically this may serve to coordinate the stress responses of the adrenal medulla and cortex (Perraudin *et al.,* 1993; Guillon *et al.,* 1995). The data suggest that VP may act as a paracrine and/or autocrine regulator of adrenal steroidogenesis. However in humans, pituitary corticotrophs are relatively more VP-sensitive; thus the cortisol response to VP is principally ACTH-dependent (Gwinup *et al.,* 1967; Bähr *et al.,* 1991).

1.1.6 Circadian variation in daily cortisol production

The secretion of cortisol occurs in a light-entrained circadian ("about a day") rhythm. The circadian pacemaker is located in the suprachiasmatic nuclei of the hypothalamus, and has an intrinsic and autonomous rhythm. Photic input to the suprachiasmatic nuclei from the photosensory system synchronises the circadian rhythm to a precise 24-hour period, coordinated with the solar light-dark cycle. In blind subjects, the circadian rhythm is "free-running" (not entrained to an external time cue and longer than 24 hours), suggesting that light, rather than other time cues, entrains the rhythm (Sack *et al.,* 1992).

The circadian rhythm produces a rise in cortisol in the early hours of the morning; cortisol levels decline throughout the day after waking, reaching a nadir at midnight (Figure 1.6). The circadian rhythm is comprised not of continuous cortisol secretion, but rather intermittent cortisol pulses, which follow ACTH pulses (Gallagher *et al.,* 1973; Hellman *et al.,* 1970). The circadian ACTH rhythm is mediated by increased ACTH pulse amplitude between 0500h and 0900h, and a reduction in pulse frequency between 1800h and 2400h (Veldhuis *et al.,* 1990; Horrocks *et al.,* 1990). Thus, the early morning rise in cortisol is due to increased frequency and duration of cortisol secretory episodes (Weitzman *et al.,* 1971). About half of the day's cortisol is produced in the early hours of the morning, during sleep (Hellman *et al.,* 1970).

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of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.6. Plasma cortisol concentration over a 24 hour period in normal humans. The mean values are shown as the continuous line; the magnitude of the standard deviation is shown by the box. Hourly mean value includes all plasma values obtained for each hour (20-minutely sampling) from measurements in six normal individuals. (Weitzman *et al.,* 1971).

Soon after waking, there is a cortisol surge which produces a 50-75% increase in free cortisol within 30 minutes of waking (Figure 1.7) (Pruessner *et al.,* 1997). This cortisol-awakening response is augmented by exposure to light and is thought to physiologically prepare the individual for the active period (Scheer and Buijs, 1999). HPA axis activation in response to a midday, protein-containing meal results in a mean increase in serum cortisol of 37ng/ml (Figure 1.8) (Ishizuka *et al.,* 1983; Quigley and Yen, 1979). Pituitary ACTH stimulates cortisol secretion, and gastrointestinal-pituitary signalling appears important, although the precise mechanisms are not known (Al-Damluji *et al.,* 1987; Benedict *et al.,* 2005). The physiological significance of this meal-related cortisol increase is not known; the lunch-time meal may be important as a short-term synchronizer of subsequent cortisol secretion or may regulate the disposition of metabolic fuels. Pulsatile cortisol release also occurs throughout the day (ultradian rhythm) and in response to stressors (Figure 1.9) (Kirschbaum *et al.,* 1996).

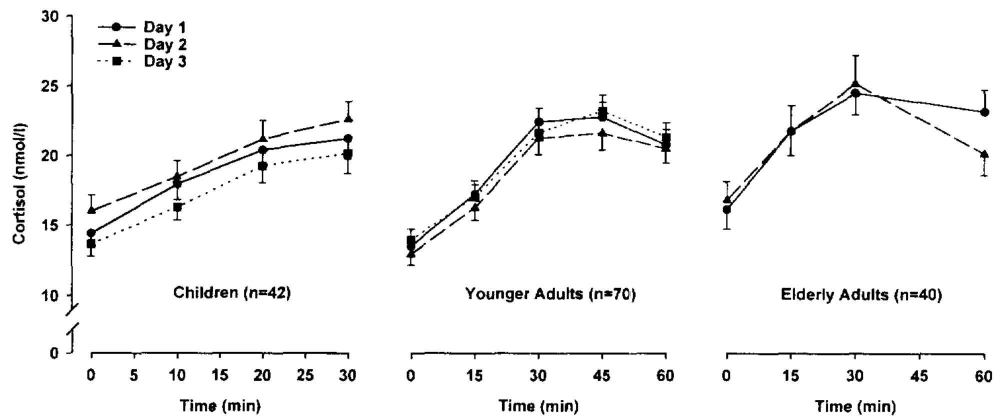

Figure 1.7. The cortisol-awakening response. Mean salivary cortisol levels after morning awakening. The error bars represent the standard error of the mean. Subjects studied: children aged 7-14, adolescents aged 19-37, elderly adults aged 59-82. (Pruessner *et al.,* 1997).

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Figure 1.8. Serum cortisol profiles in normal men during the fasting and postprandial states. The mean cortisol levels during fasting or after the ingestion of 80% protein, pure carbohydrate, or a pure fat meal, at 1200h, are shown. The error bars represent the standard error of the mean. (Ishizuka *et al.,* 1983).

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This figure is included on page 18 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.9. The cortisol response to stress. Mean salivary cortisol levels before and after stress. The error bars represent the standard error of the mean. The *shaded area* indicates the period of stress (Trier Social Stress Test) exposure. (Kirschbaum *et al.,* 1996).

Circadian variation in CRH, and perhaps VP, is likely to produce the diurnal variation in ACTH and cortisol. This is evidenced by restoration of normal basal ACTH and cortisol secretory patterns by pulsatile CRH in patients with secondary adrenal insufficiency but preserved corticotrophs (Avgerinos *et al.,* 1986). Preserved circadian ACTH release during constant CRH infusion suggests other factors, including perhaps VP, are important in the circadian variation of ACTH and cortisol secretion (Schulte *et al.,* 1985). A higher ACTH response to VP in the morning suggests that VP acts synergistically with higher endogenous CRH levels (Salata *et al.,* 1988).

Oral glucocorticoid replacement produces serum cortisol profiles that do not mimic the normal circadian variation (Mah *et al.,* 2004). As will be discussed later in this chapter, such nonphysiological glucocorticoid replacement has been postulated to account for the physical and mental fatigue frequently reported by patients with primary adrenocortical failure (Addison's disease) (Løvås *et al.,* 2002). One of the studies in this thesis aims to determine

the effect of attempted reproduction of circadian and ultradian variation in cortisol levels on physical and psychological well-being in Addison's disease (Chapter 9).

1.1.7 Genomic and non-genomic effects of cortisol

In 1942, it was noted that some steroids induced effects minutes after their application, even in the absence of their traditional biological actions, whilst the latter were only apparent hours or days later (Selye, 1942). These traditional actions are mediated by genomic effects which, for cortisol, are a result of its binding to the cytoplasmic glucocorticoid receptor, with subsequent activation of the cortisol-receptor complex through a process involving the dissociation of heat shock proteins (Pratt, 1993). After translocation to the nucleus, dimerized receptor-ligand complexes bind glucocorticoid response elements in the promoter region of target genes, regulating gene expression either by activating transcription (transactivation) or inhibiting the transactivating function of transcription factors (Beato and Sanchez-Pacheco, 1996). The diverse actions of cortisol are due to hundreds of glucocorticoid-responsive genes and ubiquitous expression of the glucocorticoid receptor (Stewart, 2003).

There are two glucocorticoid receptors in the brain: type 1 – the mineralocorticoid receptor – with high-affinity for cortisol; and type 2 – the low-affinity glucocorticoid receptor. The high-affinity type 1 receptor maintains glucocorticoid occupancy between cortisol pulses, whereas cortisol binding to the type 2 receptor follows peaks in circulating glucocorticoid levels produced by the ultradian rhythm and the stress response. Glucocorticoid receptors are distributed throughout the central nervous system; however they are particularly abundant in limbic structures crucial to learning and memory – the hippocampus, amygdala and parts of the prefrontal cortex. In mineralocorticoid target tissues, the mineralocorticoid receptor is shielded from cortisol by 11β-hydroxysteroid dehydrogenase 2 which inactivates cortisol to cortisone; this enzyme is absent in the hippocampus (de Kloet *et al.,* 1998). The distribution of the glucocorticoid receptor in the central nervous system explains why cortisol has effects

on cognitive arousal, sleep, behaviour, cognition, memory and affect; that these effects vary with hormone concentrations, and timing and duration of exposure, reflect the importance of diurnal and ultradian cortisol variations on psychological functioning (de Kloet *et al.,* 1990). These data are the basis for the hypothesis tested in Chapter 9: that impaired well-being in Addison's disease is due to nonphysiological glucocorticoid replacement.

The mechanisms by which glucocorticoids exert non-genomic effects are incompletely understood but may include specific interaction with the cytosolic glucocorticoid receptor, but not triggering gene transcription; nonspecific interaction with cellular membranes; or specific interaction with membrane-bound glucocorticoid receptor (Sapolsky *et al.,* 2000).

1.2 Clinical states of glucocorticoid excess

1.2.1 Cushing's syndrome

Cushing's syndrome (CS) is the clinical state associated with the effects of longstanding, excessive tissue glucocorticoid exposure. Frank CS due to endogenous hypersecretion of cortisol is rare, with an incidence of 0.7-2.4 per million per year (Lindholm *et al.,* 2001). The clinical features include: (1) central adiposity, facial plethora, amenorrhoea (> 80%); (2) hypertension, depression/emotional lability (70-75%); (3) proximal myopathy, glucose intolerance, easy bruising (60-65%); and (4) fragility fractures (50%) (Newell-Price *et al.,* 2006). There is a four- to five-fold excess mortality of untreated CS, mostly due to vascular disease (Etxabe and Vazquez, 1994; Lindholm *et al.,* 2001).

The most frequent cause of endogenous CS is ACTH-dependent and due to an ACTH-secreting pituitary tumour (Cushing's disease; 70% of cases) (Newell-Price *et al.,* 2006). Ectopic ACTH syndrome is rare (10%), and in the remainder of ACTH-dependent cases, the source of ACTH is unknown (Newell-Price *et al.,* 2006). Approximately 15-20% of CS is ACTH-independent; in this group unilateral adrenocortical tumours, mostly adenomas, are

predominant (> 90%) (Newell-Price *et al.,* 2006). Less frequent are bilateral adrenal adenomas, adrenocortical carcinoma, primary pigmented nodular adrenocortical disease, McCune-Albright syndrome and ACTH-independent macronodular adrenal hyperplasia (Newell-Price *et al.,* 2006).

1.2.2 Subclinical Cushing's syndrome

Subclinical Cushing's syndrome (SCS) was initially reported thirty years ago in patients studied for incidentally discovered adrenal masses ("adrenal incidentaloma") who were found by adrenal scintigraphy to have increased ^{131}I-19-iodocholesterol uptake by the adenoma and suppressed uptake by the contralateral adrenal, together with subtle biochemical abnormalities of the HPA axis (Beierwaltes *et al.,* 1974). SCS is conceptually defined as hypercortisolism in the absence of clinically apparent CS (Mantero *et al.,* 1997). Operationally, it has been defined as the presence of at least two abnormal basal or dynamic tests of the HPA axis (Mantero *et al.,* 1997). However, there is no international consensus on definition or criteria for diagnosis.

In autopsy series, the prevalence of adrenal incidentaloma is at least 3% in those aged over 50 and is two- to five-fold higher in those with type 2 diabetes mellitus (T2DM) or obesity (NIH Consensus statement, 2002; Kloos *et al.,* 1995). Estimates of the prevalence in adrenal incidentaloma (12-24%), place SCS at a higher overall prevalence than overt CS (Reincke *et al.,* 1992; Rossi *et al.,* 2000).

1.2.3 The Pseudo-Cushing's state

The pseudo-Cushing's state is defined as the presence of some or all of the features resembling CS, accompanied by biochemical evidence of hypercortisolism (Newell-Price *et al.,* 1998). It occurs mostly in the context of an underlying primary condition (depression, obesity, excessive alcohol consumption), although it may sometimes be idiopathic (Newell-

Price *et al.,* 1998; Lamberts *et al.,* 1979). The hypercortisolism of pseudo-Cushing's states may be due to increased CRH neuronal activity, which stimulates ACTH production and release (Gold *et al.,* 1986). The biochemical and clinical features of hypercortisolism associated with pseudo-Cushing's states disappear over time with the resolution of the primary condition.

1.3 Primary adrenal Cushing's syndrome

Genetic syndromes associated with Cushing's syndrome and/or adrenocortical tumours

These syndromes may be inherited or may occur sporadically. The studies in this thesis (Chapters 2 through 6) pertain to CS due to familial ACTH-independent macronodular adrenal hyperplasia (AIMAH). However, the other syndromes will be discussed here, since all may be associated with adrenocortical tumours. Furthermore, a spectrum of adrenal disease, from adenoma to AIMAH, may occur within some of these syndromes; and thus the genes and molecular pathways are prime candidates for involvement also in AIMAH, a disorder of presently unknown pathogenesis.

1.3.1 Multiple endocrine neoplasia type 1 syndrome

Multiple endocrine neoplasia type 1 (MEN1) syndrome is an autosomal dominant disease caused by mutations of the tumour suppressor gene, menin (*MEN1*). A germline inactivating (loss-of-function) mutation in one copy of the *MEN1* gene is inherited; tumours develop when a somatic mutation occurs in the second (normal) copy of the gene (Thakker *et al.,* 1989; Friedman *et al.,* 1989). Whilst primary hyperparathyroidism is the most common (90% by age 40) endocrine tumour, adrenal tumours are not infrequent (20-40%), although they are mostly nonfunctional (Skogseid *et al.,* 1992; Burgess *et al.,* 1996). The spectrum of adrenal disease in MEN1 spans from solitary, unilateral adrenal adenomas to bilateral macronodular adrenal tumours (AIMAH). *MEN1* mutations in sporadic adrenocortical tumours are rare (Görtz *et al.,* 1999; Heppner *et al.,* 1999).

1.3.2 McCune-Albright syndrome

McCune-Albright syndrome (MAS) is a sporadic disease characterised by polyostotic fibrous dysplasia, café-au-lait spots, and autonomous endocrine gland hyperfunction (McCune, 1936; Albright *et al.,* 1937). It is caused by a postzygotic, somatic, activating mutation of the *GNAS* complex locus (*GNAS*; guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1) gene (Weinstein *et al.,* 1991; Happle, 1986). *GNAS* encodes the Gsα subunit of the heterotrimeric G protein, which is involved in the signal transduction coupling ligand binding of a G protein coupled receptor to adenylate cyclase activation and cAMP generation (Figure 1.10). Activating mutations of Gsα lead to constitutive cell signalling *via* the adenylate cyclase-cAMP pathway; in adrenal nodules harbouring the mutation this results in constitutive steroidogenesis, cellular proliferation and tumorigenesis (Rosenberg *et al.,* 2002). ACTH-independent CS in MAS is rare and usually presents in infancy; in these patients the adrenal glands may contain nodular hyperplasia (AIMAH) or solitary adenomas (Kirk *et al.,* 1999).

1.3.3 Primary pigmented nodular adrenocortical disease

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare form of bilateral micronodular adrenocortical hyperplasia, characterized by pigmented nodular adrenal glands and frequently presenting with CS in childhood (Meador *et al.,* 1967). It may occur sporadically or as part of the Carney complex (CNC), an autosomal dominantly inherited multiple neoplasia syndrome comprising cardiac myxomas, lentiginoses and endocrine tumours (PPNAD, growth hormone secreting pituitary adenoma, thyroid adenoma or carcinoma, ovarian cyst, testicular tumour) (Carney *et al.,* 1985). The PKA and Wnt signalling pathways are involved in tumorigenesis in PPNAD.

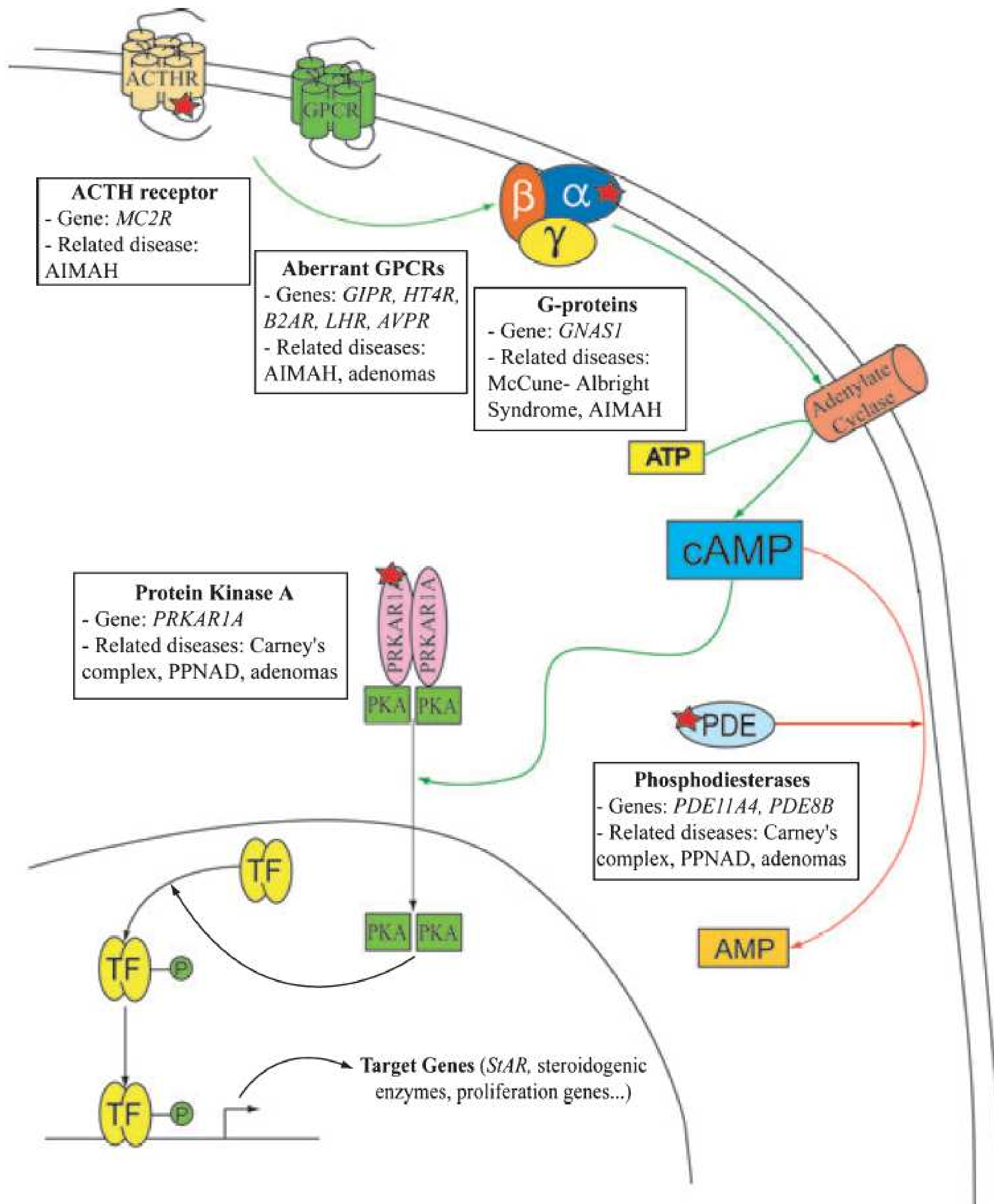


Figure 1.10. Molecular mechanisms leading to cAMP pathway activation and steroidogenesis in hyperfunctioning adrenocortical tumours and hyperplasias. Some affected genes have been identified at the indicated ★ levels of the signalling cascade (Lacroix *et al.*, 2010).

1.3.3.1 The Protein kinase A pathway in normal adrenal and in PPNAD

Protein kinase A (PKA) consists of two homodimers of regulatory (R) subunits and two catalytic (C) subunits (Figure 1.11) (Taskén *et al.,* 1997). Cyclic AMP binds to the R subunits resulting in their dissociation from, and release of, catalytic C subunits and PKA activation. The C subunits are kinases, and phosphorylate serine-threonine residues on cellular proteins. Translocation of the C subunit into the nucleus and phosphorylation of transcription factors (e.g., cAMP response element-binding protein – CREB; cAMP response element modulator – CREM) forms the basis of transcriptional regulation/activation by PKA (Daniel *et al.,* 1998).

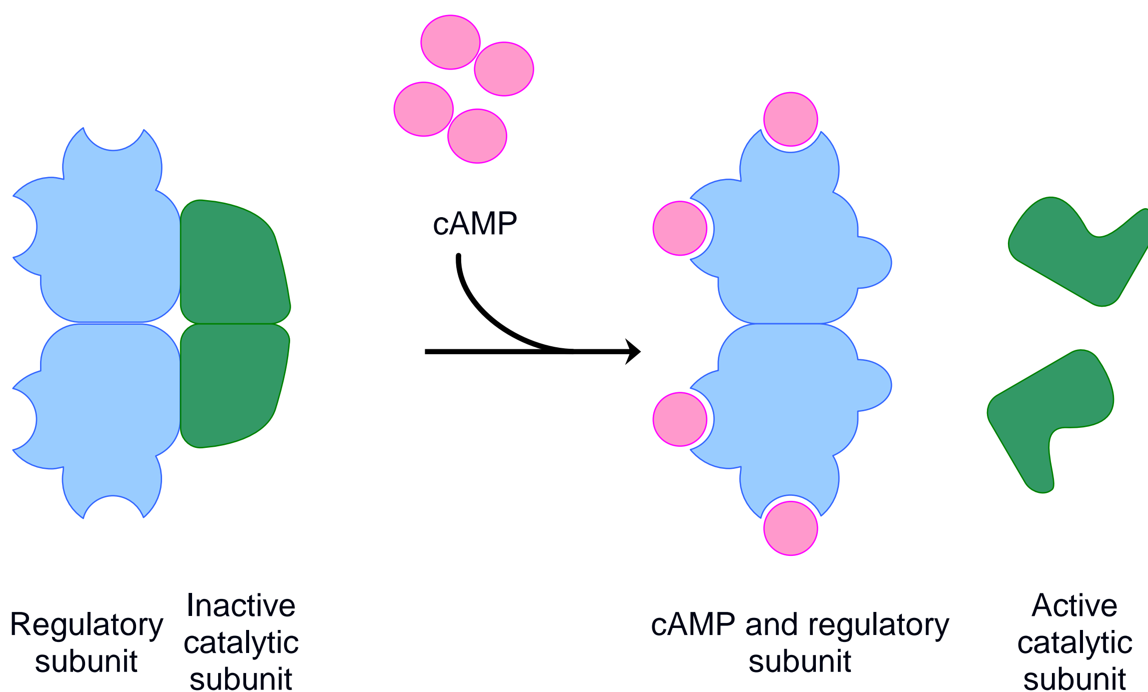


Figure 1.11. Protein Kinase A.
Protein Kinase A (PKA) is comprised of four regulatory subunits (R1A, R1B, R2A, R2B) and two catalytic subunits. In the non-cAMP bound state, the catalytic subunits are bound to the regulatory subunits and maintained in an inactive state. However, when cAMP binds to the regulatory subunits, they dissociate from the catalytic subunits, which are then able to mediate PKA signalling.

By interacting with other pathways (mitogen-activated protein kinase, protein kinases B and C), PKA may mediate cAMP effects indirectly (Robinson-White and Stratakis, 2002). The cAMP/PKA pathway regulates DNA replication, cellular metabolism, differentiation and has tissue-specific effects on proliferation (Roger *et al.,* 1995). The involvement of this pathway in adrenal steroidogenesis has been discussed.

Mutations of the regulatory 1A subunit of PKA (*PRKAR1A*; CNC1) gene (chromosome 17q23-24) are the most frequent germline mutations found in PPNAD, whether associated with other features of CNC or not (Figure 1.10) (Groussin *et al.,* 2002a; Groussin *et al.,* 2002b; Kirschner *et al.,* 2000a). The CNC alleles are functionally null mutations; loss of function of the regulatory subunit of PKA results in dysregulated cAMP-stimulated PKA catalytic activity (Kirschner *et al.,* 2000a). Germline inactivating mutations in phosphodiesterase 11A (*PDE11A*) are present in some patients with micronodular adrenal hyperplasia who do not have a *PRKAR1A* mutation (Figure 1.10) (Horvath *et al.,* 2006a). PDE11A catalyses the hydrolysis of cAMP; inactivating mutations could result in increased intracellular cAMP and tumorigenesis *via* PKA pathway activation (D'Andrea *et al.,* 2005). All *PDE11A* mutations were also found, albeit less frequently, in the normal population, suggesting that the variants are predisposing, but additional factors are required for expression of the disease phenotype (Horvath *et al.,* 2006b).

1.3.3.2 The canonical Wnt signalling pathway in normal adrenal and in PPNAD

Wnt signalling molecules bind to cell-surface frizzled receptors and to lipoprotein receptor-related protein (LRP) co-receptors (Figure 1.12) (Schinner *et al.,* 2009). Wnt signals are transmitted by the association between Wnt receptors and the intracellular protein, Dishevelled (Dvl); which disrupts a multiprotein complex comprised of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3β (GSK3β), casein kinase I (CKI) and β-catenin. Disruption of the protein complex inhibits the degradation of β-catenin (Jin *et al.,*

2008). Accumulated β-catenin translocates to the nucleus and activates T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors of canonical Wnt target genes (Figure 1.12) (Gordon and Nusse, 2006). In the absence of Wnt signalling, β-catenin levels are low: β-catenin is phosphorylated and then degraded by the ubiquitin-proteasome system (Kikuchi, 2003).

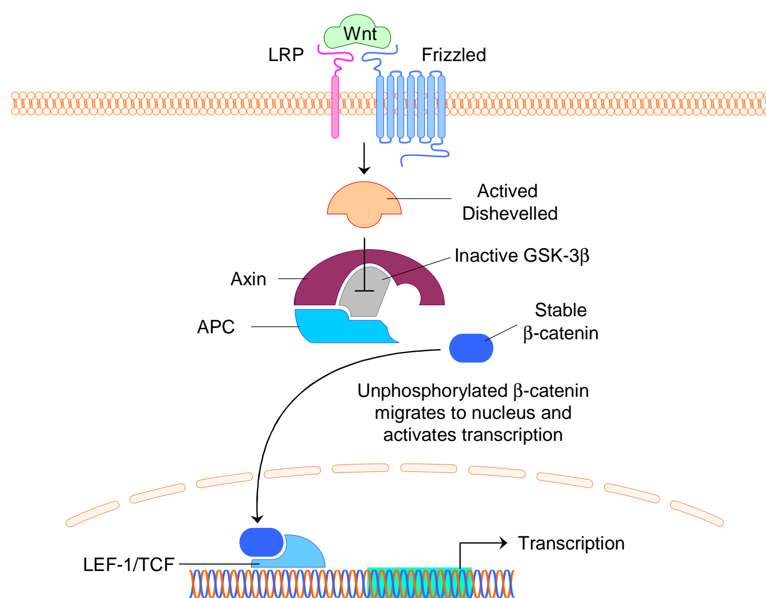


Figure 1.12. The canonical Wnt signalling pathway.
APC – adenomatous polyposis coli; GSK-3β - glycogen synthase kinase-3β; LEF-1/TCF - lymphoid enhancer factor/T-cell factor; LRP - lipoprotein receptor-related protein

The Wnt signalling pathway is involved in organogenesis - the restricted tissue expression of individual Wnt genes suggests specificity in organ development (Lako *et al.,* 1998). The presence of nuclear β-catenin in glomerulosa cells of the fetal adrenal cortex, suggests involvement of canonical Wnt signalling in adrenal development (Eberhart and Argani, 2001).

Somatic β-catenin (*CTNNB1*) mutations were detected in adrenal macronodules from two patients with PPNAD due to CNC; one of whom had a germline *PRKAR1A* mutation (Tadjine *et al.,* 2008b). Another study also found somatic *CTNNB1* mutations in two macronodules

occurring on a histological background of PPNAD (Gaujoux, Tissier *et al.,* 2008). The data suggest "crosstalk" between PKA and Wnt/β-catenin pathways. This might involve PKA-mediated phosphorylation of the multiprotein complex or β-catenin, resulting in β-catenin accumulation (Fang *et al.,* 2000; Taurin *et al.,* 2006; Hino *et al.,* 2005).

1.3.4 ACTH-independent Macronodular Adrenal Hyperplasia

1.3.4.1 Introduction

ACTH-independent Macronodular Adrenal Hyperplasia (AIMAH) is a rare cause of primary adrenal CS (< 1% of endogenous CS) (Lacroix, 2009). It is characterized by massive bilateral adrenal enlargement and relatively mild adrenocortical hormone hypersecretion. AIMAH has been regarded a sporadic disease, although several reports of familial AIMAH suggest it may be inherited; the genetic basis of familial AIMAH is presently unknown. In this thesis, I will discuss the clinical and genetic studies we have performed in four AIMAH families, aiming to elucidate the genetic basis and molecular pathogenesis of familial AIMAH.

AIMAH was first reported in a 40 year old woman with longstanding classic CS in whom retroperitoneal air insufflation revealed bilateral suprarenal masses (Kirschner *et al.,* 1964). The resected adrenal glands together weighed 94gm (normal 8-12gm), and each was comprised of multiple, bright yellow nodules (0.2 to 3.5cm in diameter) which completely distorted the external configuration of the gland; no undistorted cortex was seen microscopically. She remained well at least 30 years postoperatively, with no evidence of disease recurrence (Doppman *et al.,* 1991).

An intriguing feature of AIMAH is that despite massively enlarged adrenal glands, hypercortisolism is relatively mild. The clinical implication is that hypercortisolism and the associated clinical features, which may be atypical or cyclical, develop insidiously. In one series, clinical history suggested an average delay in diagnosis of 7.8 years (Swain *et al.,*

1998). Accordingly, preclinical AIMAH likely antedates the onset of CS by many years. One of the aims of our studies has been to define these preclinical forms.

In Chapter 2 of this thesis I will present the results of screening two kindreds with familial AIMAH and a third kindred, the children of an apparently sporadic AIMAH case, for preclinical disease. Our data reveal that preclinical AIMAH is detectable using conventional endocrine and adrenal imaging testing; furthermore we show that relatives of apparently sporadic cases may have preclinical AIMAH, which would remain undetected without screening. The identification of preclinical AIMAH in individuals could assist in clinical care, since it would allow careful assessment for early features of CS, thereby aiding in decision-making regarding the optimal timing of treatment. This is especially important in AIMAH, where clinical features develop insidiously and thus may not be recognised until the disease is clinically advanced and atrophic features (proximal myopathy, easy bruising, and osteoporosis and fragility fractures) have developed.

1.3.4.2 Clinical presentation

CS is the most frequent presentation. A minority of patients presents during the first year of life; this may be associated with MAS (Kirk *et al.,* 1999). More frequently, AIMAH presents in the 5th and 6th decades, and affects males and females equally (Lieberman *et al.,* 1994). This is in contrast to Cushing's disease, which presents earlier (4th decade) and preferentially affects women (Lindholm *et al.,* 2001).

Hyperaldosteronism, feminization and virilisation have been reported due to concurrent aldosterone or oestrone, or pure androgen, secretion (Godbout *et al.,* 2008; Staermose *et al.,* 2008; Goodarzi *et al.,* 2003; Malchoff *et al.,* 1989). AIMAH may be an incidental finding on abdominal imaging (Nemoto *et al.,* 1995). Many patients have evidence of cortisol (or other hormone) secretory abnormality upon testing or long-term follow-up, although these evolve

slowly (Bourdeau *et al.,* 2001; Ohashi *et al.,* 2001). Adrenal enlargement also progresses slowly (Lacroix *et al.,* 1997a; N'Diaye *et al.,* 1999). That hormone secretory abnormalities may develop, underscores the need for long-term surveillance of affected individuals to allow optimal timing of treatment.

Thyroid, parathyroid, parotid and uterine leiomyomatous tumours may occur in patients with AIMAH and their relatives (Nagai *et al.,* 1999; Hsiao *et al.,* 2009; Sato *et al.,* 2006). Two siblings with AIMAH had meningiomas (Lee *et al.,* 2005). Whether AIMAH and these other tumours have a common pathogenesis, or occur coincidentally, is uncertain.

1.3.4.3 Diagnosis

The clinical presentation and the characteristic imaging findings are pathognomonic; the histopathology (discussed later) diagnostic, for AIMAH (Doppman *et al.,* 2000). Urinary 17-hydroxycorticosteroids (17-OHCS) are highest in AIMAH, even when urinary free cortisol is within or near the normal range, and appear to most clearly distinguish AIMAH from other adrenocortical tumours (Hsiao *et al.,* 2009). Hence this has recently been proposed to be a sensitive screening test in patients with suspected early or atypical CS and AIMAH. 17-OHCS represents the fraction of corticosteroids possessing a hydroxyl group at position 17 of the steroid structure and includes cortisol, cortisone and other metabolites (Hsiao *et al.,* 2009; Hoshiro *et al.,* 2006). These data suggest that glucocorticoid metabolites not measured by the urinary free cortisol assay may be excreted in AIMAH.

On computed tomography scanning, the adrenal glands are massively enlarged and consist of multiple nodules (some greater than 5cm in diameter), which distort and completely obscure the normal glandular architecture (Wada *et al.,* 1996; Doppman *et al.,* 2000). Adrenal scintigraphy usually shows bilateral uptake, consistent with bilateral adrenal hyperfunction (Doppman *et al.,* 1991; Doppman *et al.,* 2000).

1.3.4.4 Histopathology

AIMAH is a cytologically benign process that is not known to metastasize, or undergo malignant transformation, and, histologically, is comprised of two cell types. *Clear cells* have lipid-rich cytoplasm, and form cordon nest-like structures, whilst *compact cells* are relatively devoid of cytoplasm and form nests or island-like structures (Aiba *et al.,* 1991).

AIMAH can be classified based on the characteristics of the internodular cortical tissue: *type 1* - internodular cortical atrophy and *type 2* - internodular cortical hyperplasia (Stratakis and Boikos, 2007). The biological basis for the two histologies is unknown; they could represent different genetic or paracrine factors acting on the adrenal (Stratakis and Boikos, 2007). Alternatively, they could represent different stages of disease – e.g., type 1 could reflect advanced disease – where the hyperplastic internodular tissue has been compressed by expanding adrenal nodules. Alternatively, type 2 could be the hallmark of familial disease – with diffuse hyperplasia of nodular and internodular cortex reflecting the presence of the germline mutation from the initiation of adrenal organogenesis. However in a report of three familial AIMAH cases, all were of type 1 histology (Hsiao *et al.,* 2009).

1.3.4.5 Inefficient steroidogenesis

Immunohistochemical and enzyme immunoreactivity studies have shown that 3β-hydroxysteroid dehydrogenase (3βHSD) is expressed exclusively in clear cells; whilst 17α-hydroxylase (CYP17A1) is expressed predominantly in compact cells (Sasano *et al.,* 1994; Wada *et al.,* 1996; Aiba *et al.,* 1991). This differential enzyme localisation renders the cells codependent for steroidogenesis - progesterone produced by clear cells must reach compact cells for cortisol production (Sasano *et al.,* 1994; Wada *et al.,* 1996; Aiba *et al.,* 1991). This pattern of enzyme expression is unique to AIMAH and may account for the discordance between the macronodular adrenal glands and the relatively mild adrenocortical hormone hypersecretion. Thus, hormone hypersecretion is a result of a massively increased total

adrenocortical cell mass, rather than augmented steroidogenesis per cell (Aiba *et al.,* 1991; Sasano *et al.,* 1994). Immunohistochemical studies of other steroidogenic enzymes have been inconsistent, but include reduced 21-hydroxylase and CYP11A1 expression in some studies (Sasano *et al.,* 1994; Koizumi *et al.,* 1994; Wada *et al.,* 1996; Morioka *et al.,* 1997). Thus, inefficient steroidogenesis is a *sine qua non* of AIMAH; in Chapter 3, I will present the results of basal and ACTH-stimulated levels of steroid hormones and their intermediates, in two patients from the first AIMAH family (AIMAH-01) we have studied.

1.3.4.6 Pathogenesis of AIMAH

Whilst the pathogenesis is unknown, the prevailing theory is that AIMAH is due to an intrinsic, ACTH-independent increase in adrenocortical cell proliferation, since cultured AIMAH cells grow much more rapidly than normal human fetal adrenal or adrenal adenoma cells *in vitro* (Cheitlin *et al.,* 1988). It was postulated that a circulating humoral factor might stimulate cellular proliferation; but no such factor has been identified (Aiba *et al.,* 1991). The adrenal glands contain polyclonal and monoclonal lesions; monoclonal nodules, even from the same adrenal, may not be from the same progenitor cell (Gicquel *et al.,* 1994; Beuschlein *et al.,* 1994). Different stages of a common multistep tumoral process may be simultaneously present in different locations in AIMAH.

Bilateral macronodular adrenal hyperplasia develops in patients with longstanding ACTH-dependent hypercortisolism in Cushing's disease, ectopic ACTH production, and in poorly controlled congenital adrenal hyperplasia (Aron *et al.,* 1981; Smals *et al.,* 1984; Doppman *et al.,* 1988). Thus, it has been postulated that AIMAH may develop from longstanding excess ACTH stimulation of the adrenals; the transition from ACTH-dependence to ACTH independence is due to hypercortisolaemia with adrenal autonomy and suppression of an ACTH-secreting pituitary adenoma (Hermus *et al.,* 1988). However, if this were the mechanism in AIMAH, Nelson's syndrome - rapid growth of an ACTH-secreting pituitary

tumour, high ACTH levels and pigmentation - should develop after bilateral adrenalectomy. Nelson's syndrome has not been reported in the long-term (up to thirty years) follow-up of treated patients (Doppman *et al.,* 1991; Swain *et al.,* 1998; Doppman *et al.,* 2000).

The absence of pituitary lesions and restoration of normal HPA axis dynamics post-operatively also do not suggest a primary hypothalamic or pituitary defect (Wada *et al.,* 1996; Aiba *et al.,* 1991; Doppman *et al.,* 1991; Koizumi *et al.,* 1994). These clinical observations, together with data from xenotransplantation models (discussed later) provide compelling evidence for the ACTH-independence of AIMAH. The expression of several ACTH receptor pathway genes was reduced in one study of AIMAH; although this does not preclude earlier ACTH-dependence (Antonini *et al.,* 2006).

Molecular studies suggest that the PKA and Wnt pathways may be involved in AIMAH. Gene expression studies (described in Chapter 4) have identified altered expression of genes involved in the canonical Wnt signalling pathway (Bourdeau *et al.,* 2004; Lampron *et al.,* 2006; Almeida *et al.,* 2011). Chromosomal changes of 17q (the *PRKAR1A* locus) were found in 73% of AIMAH tumours studied and were correlated with decreased *PRKAR1A* expression and exaggerated cAMP-stimulated PKA activity (Bertherat *et al.,* 2003; Bourdeau *et al.,* 2006). In Chapter 4, I present the gene expression studies we have performed of AIMAH tumours from three siblings, with the aim of elucidating the mechanisms involved in the pathogenesis of familial AIMAH.

1.3.4.7 Aberrant G-protein coupled receptors in AIMAH

In AIMAH and in other adrenocortical tumours, and in contrast to the previous notion of "autonomous hormone production", cortisol secretion may be regulated by ectopically or eutopically overexpressed G-protein coupled receptors (GPCR) that are coupled to the steroid synthetic pathway (Figure 1.4) (Lacroix *et al.,* 2001). In such cases, cortisol secretion

becomes driven by a hormone that escapes cortisol-mediated negative feedback. The binding of a ligand to its specific receptor results in stimulation of the Gs subunit of the G protein, stimulating adenylate cyclase, cAMP production and steroidogenesis, analogous to the normal function of the ACTH receptor (Figure 1.4) (Lacroix *et al.,* 2004). This can be demonstrated by modulating circulating levels either through its exogenous administration or by providing a physiological stimulus to endogenous secretion, and measuring serial ACTH and cortisol levels (Lacroix *et al.,* 1999b). Pre-treatment with dexamethasone suppresses endogenous ACTH; ensuring the cortisol response is ACTH-independent, i.e., aberrant. Lacroix *et al.,* developed a protocol to systematically screen for the presence of aberrant receptors (Figure 1.13) (Lacroix *et al.,* 1999b). Aberrant cortisol responses are frequent in AIMAH (87%) (Libé *et al.,* 2010).

Aberrant receptor expression in AIMAH has been the most extensively studied phenomenon over the past 20 years, after the serendipitous observation of food-dependent cortisol secretion in two patients with AIMAH, which was subsequently shown to be regulated by gastric-inhibitory polypeptide (GIP) (Lacroix *et al.,* 1992; Reznik *et al.,* 1992). The GIP receptor is a GPCR; once ligand bound, adenylate cyclase is activated and cAMP produced (Figure 1.4). The *in vivo* and *in vitro* cortisol responses to GIP were consistent with coupling of the ectopic receptor to the cAMP pathway and steroidogenesis (Figure 1.4) (Groussin *et al.,* 2002c).

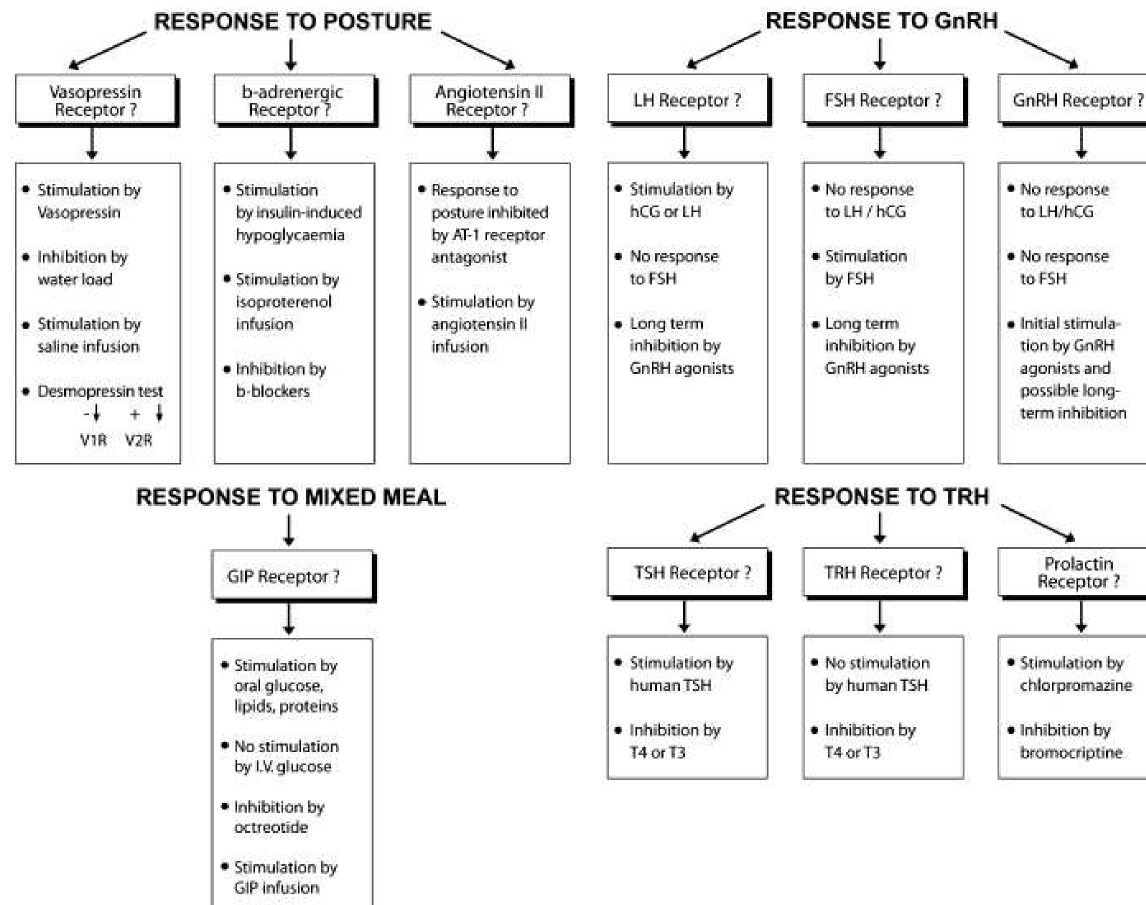


Figure 1.13. Aberrant receptor screening protocol. (Lacroix *et al.*, 1999b).

Whilst VP-sensitive AIMAH will be discussed, a full discussion of all the aberrantly expressed receptors in AIMAH is beyond the scope of this thesis. However, they include luteinizing hormone/human chorionic gonadotropin (LH/hCG), β-adrenergic, serotonin, angiotensin and glucagon receptors (Lacroix *et al.,* 1999a; Goodarzi *et al.,* 2003; Lacroix *et al.,* 1997a; Cartier *et al.,* 2003; de Miguel *et al.,* 2010). Anecdotally, specific antagonists of the LH/hCG (leuprolide), β-adrenergic (propranolol) and glucagon (octreotide) receptors, have been used for the medical management of hormone hypersecretion syndromes in AIMAH, in cases when hormone secretion was shown to be regulated by a specific receptor ligand (Lacroix *et al.,* 1999a; Goodarzi *et al.,* 2003; Lacroix *et al.,* 1997a; de Miguel *et al.,* 2010). Recently, expression of novel GPCR has been identified: α2A adrenergic, motilin and γ-aminobutyric acid (GABA) receptors (Assie *et al.,* 2010).

The biological basis of aberrant expression of non-mutated GPCR is not known. Mutations in the GPCR gene promoters (which regulate gene expression) have not been found, and transcription factors regulating GPCR gene expression are not altered; although only the GIP receptor has been studied (N'Diaye *et al.,* 1998; Baldacchino *et al.,* 2005; Antonini *et al.,* 2004). The role of aberrant receptors in the pathogenesis of AIMAH is not known. Their expression could represent an epiphenomenon of de-differentiation during cellular proliferation and tumorigenesis; that aberrant receptors are also expressed by non-AIMAH adrenocortical tumours supports this postulate.

However, the potential for ectopic GIP receptor expression to primarily stimulate adrenocortical cell proliferation and tumorigenesis is evidenced by GIP stimulation of DNA synthesis in GIP-sensitive adrenal adenomas (Chabre *et al.,* 1998a; Chabre *et al.,* 1998b). Furthermore, in a xenotransplantation murine model (transplanted with bovine adrenocortical cells genetically engineered to express the GIP receptor), ectopic GIP receptor expression was

sufficient to generate GIP-responsive cortisol secretion, a Cushingoid phenotype and adrenocortical hyperplasia, reminiscent of human AIMAH (Mazzuco *et al.,* 2006b).

The ACTH-independence of tumorigenesis was shown in a substudy of mice chronically perfused with dexamethasone (to suppress plasma ACTH) from the onset of transplantation (Mazzuco *et al.,* 2006b). Whilst the transplant was atrophic in control mice, the GIP receptor transplants developed to a similar size as they had in the presence of ACTH (Mazzuco *et al.,* 2006b). These data suggest that physiological GIP may act as a trophic factor *via* activation of the adrenal ectopic GIP receptor, analogous to the state of ACTH excess, in which adrenal hypertrophy and hyperplasia are stimulated (Dallman, 1984). The data also suggest that ectopic GIP receptor expression is likely to occur early, if it is not directly causative, in the pathogenesis of AIMAH. Similar findings were obtained in a xenotransplantation murine model of LH-sensitive AIMAH (Mazzuco *et al.,* 2006a). Thus if ectopic receptor expression is primary in the pathogenesis of AIMAH or other adrenocortical tumours, the phenotype may be determined by the timing of the genetic mutation (e.g., AIMAH – germline or very early in embryogenesis; adenoma – somatic).

1.3.4.8 Vasopressin-sensitive AIMAH and other adrenocortical tumours

Normally, VP regulates HPA axis function primarily as an ACTH secretagogue. However, since VP is found in the adrenal medulla and AVPR1A is expressed on normal adrenal cortex, then VP may be involved in the paracrine regulation of cortisol secretion (Perraudin *et al.,* 1993; Guillon *et al.,* 1995). However, this is believed to be a minor effect.

In contrast, VP may directly regulate cortisol secretion in sporadic and familial AIMAH and sporadic adrenal adenomas; operating via eutopic AVPR1A overexpression or ectopic expression of the adrenocorticotropic VP receptor, AVPR1B, coupled to the steroidogenic pathway (Figure 1.4) (Horiba *et al.,* 1995; Lacroix *et al.,* 1997b; Arnaldi *et al.,* 1998;

Miyamura *et al.,* 2002; Lee *et al.,* 2005). In VP-sensitive AIMAH, changes in cortisol secretion are concordant with physiological modulation (water-loading – suppression; saline infusion – stimulation) of endogenous VP secretion (Bourdeau *et al.,* 2001). Intra-adrenal VP has been detected in VP-sensitive AIMAH, suggesting that VP may function as a paracrine or autocrine regulator of steroidogenesis (Bertherat *et al.,* 2005). In one study of VP-sensitive adrenocortical adenomas, VP receptors were not overexpressed; increased receptor coupling efficiency to the steroidogenic pathway or an indirect action of VP were suggested alternative mechanisms of VP sensitivity (Joubert *et al.,* 2008).

The dose of VP administered in the evaluation of VP sensitivity has varied, although 10 I.U. IM has been the conventional dose (Lacroix *et al.,* 1999b). This dose is associated with hypertension, nausea, tachycardia, chest discomfort and abdominal cramps, due to the vasoconstrictor properties of VP; adverse effects which may preclude evaluation (Lacroix *et al.,* 1997a). Our studies in familial AIMAH began after the serendipitous discovery of a family with CS due to VP-sensitive AIMAH. In our studies, we administered "physiologic-dose VP" (PD-VP); i.e., 1I.U./70kg body weight IV, a dose which had been previously shown to stimulate the normal HPA axis and was associated with minimal side-effects (Torpy *et al.,* 1994). We found that this dose was well-tolerated and was sufficient to detect an aberrant response in VP-sensitive AIMAH (Chapter 2). We also evaluated the ACTH-cortisol response to PD-VP in patients with functioning and nonfunctioning solitary adrenal adenomas (Chapter 7).

1.3.4.9 Familial AIMAH

AIMAH has been predominantly regarded a sporadic disease; however early reports of affected sibling or parent-child dyads presenting with CS due to AIMAH suggested it could be familial (Findlay *et al.,* 1993; Minami *et al.,* 1996; Miyamura *et al.,* 2002). Findlay *et al.,* first reported familial AIMAH affecting two generations of one family (Findlay *et al.,* 1993). They

reported an African-American mother and daughter each presenting with CS at the age of 38 (Findlay *et al.,* 1993). Minami *et al.,* reported a brother and sister (proband), aged 59 and 60 years at diagnosis, respectively, with CS; the siblings had another sister (64 years) and brother (54 years) with biochemical and imaging abnormalities consistent with SCS and two unaffected sisters (61, 72 years) (Minami *et al.,* 1996). The proband's son (45 years) and daughter (40 years) were phenotypically normal (Minami *et al.,* 1996). Miyamura *et al.,* reported on a mother with CS and her son, who had macronodular adrenal glands, hypertension and obesity, although was not hypercortisolaemic (Miyamura *et al.,* 2002).

Several other kindreds with multiple individuals with overt or subclinical CS due to AIMAH have since been reported, providing compelling evidence that AIMAH may be inherited (Nies *et al.,* 2002; Lee *et al.,* 2005; Vezzosi *et al.,* 2007; Staermose *et al.,* 2008). Nies *et al.,* reported on a family with CS predominantly affecting female family members (Nies *et al.,* 2002). The proband was a 50 year old female with florid CS (Nies *et al.,* 2002). Her sister had had a bilateral adrenalectomy for CS due to "bilateral adrenocortical adenomas" at age 34 (Nies *et al.,* 2002). A female cousin had undergone the same operation for the same diagnosis (bilateral adrenocortical hyperplasia) at age 38 (Nies *et al.,* 2002). The cousin's mother and another of the proband's aunts had both died at age 54 of unknown cause; although both had clinical features of CS (Nies *et al.,* 2002). The proband's paternal grandmother also had typical Cushingoid features and died at the age of 35, cause unknown (Nies *et al.,* 2002). The proband's father, aged 79 years, did not have CS, but demonstrated cortisol secretory autonomy (nonsuppression to dexamethasone) and had bilateral nodular adrenal hyperplasia (Nies *et al.,* 2002). In this family, AIMAH had a predilection to manifest clinically in only female family members; perhaps due to an effect of oestrogen on adrenocortical cell proliferation (Nies *et al.,* 2002). This family is AIMAH-04, the fourth family in whom we performed linkage analysis studies (described later) in an attempt to identify the genetic basis of familial AIMAH (Chapter 5).

Lee *et al.,* reported on two Asian sisters (46 and 58 years) with CS; more extensive familial screening was not performed, although it was noted that another sister had diabetes and their brother, hypertension (Lee *et al.,* 2005). Vezzosi *et al.,* reported on a father, son and two daughters with AIMAH (Vezzosi *et al.,* 2007). Two siblings (females; 54 and 56 years) had presented with mild CS and bilateral macronodular adrenal enlargement (Vezzosi *et al.,* 2007). Their father (81 years) had central obesity, T2DM, hypertension, easy bruising and dorso-cervical fat pads (Vezzosi *et al.,* 2007). The siblings' brother (57 years) was overweight and bruised easily (Vezzosi *et al.,* 2007). Both the father and brother had biochemical abnormalities consistent with subtle hypercortisolism and bilaterally thickened adrenal glands on imaging (Vezzosi *et al.,* 2007).

Two families with autonomous secretion of cortisol and aldosterone have also been reported (Staermose *et al.,* 2008). The proband of the first family was a 46 year old female who presented with hypokalaemia, suppressed renin and bilateral adrenal masses (Staermose *et al.,* 2008). She had autonomous cortisol and aldosterone secretion demonstrated by nonsuppression to oral dexamethasone and intravenous saline infusion, respectively (Staermose *et al.,* 2008). Her mother had had bilateral adrenalectomy for hyperaldosteronism; her aunt had bilateral macronodular adrenal glands and SCS; and her sister had an adrenal macronodule (Staermose *et al.,* 2008). The proband of the second family was a 46 year old male with hypertension and bilateral adrenal masses, who also demonstrated autonomous secretion of aldosterone and cortisol; he underwent resection of two large macronodular adrenal glands (Staermose *et al.,* 2008). A female cousin had hyperaldosteronism due to bilateral macronodular adrenal hyperplasia (Staermose *et al.,* 2008).

These reports of AIMAH occurring in multiple generations within a single family provide evidence that it may be inherited as a Mendelian disorder. In none of these families was consanguinity reported. The segregation of the phenotype favours autosomal dominant

inheritance, although the precise genetic mechanisms are not known. In Chapter 2 of this thesis, I will report the phenotyping of three families, in whom, together with AIMAH-04, we have attempted to elucidate the genetic basis of inheritance of familial AIMAH.

1.3.4.10 Genetics of AIMAH tumours

In isolated cases, germline or somatic mutations have been identified in sporadic AIMAH; that mutations in different genes have already been observed is evidence for the genetic heterogeneity of AIMAH. These mutations have been identified by sequencing candidate genes selected based on their known involvement in adrenal or other tumours (endocrine and non-endocrine) and/or CS.

Somatic point mutations in the *GNAS* gene affecting codon 201 were found in four unrelated sporadic AIMAH cases that were studied for such mutations (Fragoso *et al.,* 2003; Hsiao *et al.,* 2009). One person had a point mutation (single base change) in exon 8 resulting in a substitution of serine (AGT) for arginine (CGT) at codon 201 in both adrenal tissues (Fragoso *et al.,* 2003). The other cases had a somatic point mutation at codon 201, resulting in a change to histidine (CAT) (Fragoso *et al.,* 2003; Hsiao *et al.,* 2009). These mutations were not present in germline DNA. The somatic mutations had been previously reported in MAS; other manifestations of MAS in the AIMAH patients were absent. Missense mutations of *GNAS* affecting codon 201 result in constitutive activation of the Gsα protein, increasing intracellular cAMP and stimulating cellular proliferation, nodule formation and autonomous cortisol secretion.

A patient with sporadic AIMAH was homozygous for a germline missense mutation in the ACTH receptor (melanocortin 2 receptor; *MC2R*) that resulted in a substitution of phenylalanine by cysteine (F278C) in the C-terminal tail (Aloi *et al.,* 1995). The mutation was associated with reduced receptor desensitization and impaired internalization (Swords *et*

al., 2002). Elevated basal cAMP accumulation suggested constitutive activation of the mutated receptor (Swords *et al.,* 2002).

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal dominant disorder manifested by smooth muscle tumours of the skin or uterus, and/or papillary renal cell carcinoma, and caused by mutations in the fumarate hydratase (*FH*) gene (Launonen *et al.,* 2001). FH is an enzyme which catalyses the conversion of fumarate to malate in the tricarboxylic acid cycle. In HLRCC, *FH* acts as a tumour suppressor gene because all patients are heterozygote carriers and there is loss of the normal allele (loss of heterozygosity; LOH) in the tumours. A patient from a family with HLRCC, herself affected with cutaneous and uterine leiomyomas and with a germline mutation in *FH* (7 base pair deletion resulting in a premature stop codon - c.782_788del7bp), was found to have AIMAH (Matyakhina *et al.,* 2005). Implicating *FH* in the pathogenesis of AIMAH was LOH of the *FH* locus in the adrenal tumour tissue (Matyakhina *et al.,* 2005). Only one copy of the *FH* gene, the mutant allele, was retained – i.e., there was loss of the normal copy in the tumour tissue. This is consistent with LOH of this locus in other HLRCC-associated tumours, and with *FH* acting as a tumour suppressor gene, where loss of both normal copies is required for disease expression.

A patient with a history of colonic polyps, desmoid tumours and AIMAH was heterozygous for a germline mutation in the adenomatous polyposis coli (*APC*) gene (c.4393_4394delAG) (Hsiao *et al.,* 2009). This mutation had been reported in patients with familial adenomatous polyposis and was predicted to produce a truncated protein (Hsiao *et al.,* 2009). Inactivation of APC leads to accumulation of β-catenin and canonical Wnt pathway activation, a potential mechanism of adrenal tumorigenesis (Figure 1.12). A patient with hyperparathyroidism and AIMAH had a germline *MEN1* mutation (p.P494L) (Hsiao *et al.,* 2009).

The genetics of familial AIMAH have mostly not been studied, although mutations in the coding regions of the *MEN1* gene and genetic analysis of hot spot regions of *GNAS* were not found in a large AIMAH kindred (Nies *et al.,* 2002). Another familial AIMAH case was a carrier of the arginine 867 glycine (R867G) *PDE11A* polymorphism, which although a common variant, occurs in a highly conserved region of the gene and affects enzyme activity *in vitro* (Hsiao *et al.,* 2009). Other family members were not studied.

1.3.4.11 Treatment

Bilateral adrenalectomy is the mainstay of treatment of adrenocortical hormone hypersecretion due to AIMAH. Although curative, patients then have a life-long dependence on steroid replacement and are at risk of adrenal crisis. Hence, alternative treatments for AIMAH have been sought. Since hypercortisolism is due to an increased total adrenocortical cellular mass and individual cellular cortisol production is inefficient, remission might be achieved by sufficiently reducing cellular mass, such that endogenous hormone production is preserved. Unilateral adrenalectomy has been successful in selected (mild or moderate CS; dominant adrenal gland on imaging and/or scintigraphy) patients (Lamas *et al.,* 2002; Iacobone *et al.,* 2008).

Steroidogenic enzyme inhibitors and adrenolytic agents have been used in cases where there are surgical contraindications or to reduce cortisol levels as part of the preoperative optimisation of comorbidities (Nagai *et al.,* 1999; Omori *et al.,* 2001). As discussed, specific receptor antagonists have been used as an alternative or adjunctive treatment (Reznik *et al.,* 1992; Lacroix *et al.,* 1997a; Lacroix *et al.,* 1999a).

In this thesis, I will discuss the studies we have performed aiming to: (1) delineate the phenotype of inherited VP-sensitive AIMAH in three families (Chapter 2); (2) delineate the *in vivo* steroidogenic defects in two siblings with familial AIMAH (Chapter 3); (3) elucidate the

molecular mechanisms involved in familial VP-sensitive AIMAH using genome-wide gene expression studies (Chapter 4); (4) identify the presumed monogenic basis of familial AIMAH using linkage analysis and next-generation sequencing (Chapters 5 and 6) and (4) determine the prevalence of VP-sensitivity in sporadic adrenocortical tumours, utilizing PD-VP (Chapter 7). The genetic principles pertaining to aims 3 and 4 are discussed next.

1.4 Approaches to discovery of the genetic basis of Mendelian disease

1.4.1 Introduction

An understanding of the heritability of human disease had begun long before Watson and Crick described the structure of deoxyribose nucleic acid (DNA) (Watson and Crick, 1953). Aristotle first noted that "children often inherit anything that is peculiar in their parents" (Aristotle, 350BC). In the 19th century, Gregor Mendel's observations from experiments with common garden peas provided the foundation for understanding the propagation of genetic information to progeny (Mendel, 1866). Later, whilst studying sex-linked traits in Drosophila melanogaster (*Drosophila*), Morgan proposed the concept of linkage, which pertains to the tendency for two "factors" lying close together on a chromosome to be inherited together; this is the fundamental concept on which modern linkage analyses performed for discovery of causes of monogenic, inherited disease, are based (Morgan, 1911). Subsequently, it was realised that if Morgan's postulate was correct, then it should be possible to construct a linear gene map; two years later this was completed for *Drosophila* (Sturtevant, 1913).

The human genome consists of double-stranded DNA, organised linearly into 23 pairs (diploid) of chromosomes, in most cells of the human body (Thompson *et al.,* 1991). Twenty-two of these pairs are autosomes; the 23rd pair is comprised of the sex chromosomes which consist of two X chromosomes in normal females, and an X and a Y chromosome in normal males. Exceptions to these are gametes, which are haploid (23 single chromosomes): oocytes contain 22 autosomes and an X chromosome; and spermatogonia contain 22 autosomes and

either an X or Y chromosome – the union of two gametes restores the full chromosomal complement of 23 pairs in the embyro. Thus, for each chromosomal or homologous pair in diploid cells, one chromosome has been maternally inherited, whilst the other has been paternally inherited.

Genes are the physical unit of inheritance, and are found along each of the chromosomes. The unique position of a gene along a chromosome is its *locus*. *Alleles* refer to one or more versions of a genetic sequence at a particular location in the genome; thus for the same locus on a pair of chromosomes, the alleles may be the same (homogeneous) or different (heterogeneous). The alleles present at a locus constitute the *genotype* of the locus.

1.4.2 Meiosis

The genetic constitution of an individual (offspring) is inherited from their parents; whilst there are similarities to the parental genetic constitution, there are also important differences. These differences are determined by the events that have occurred in each parent, during meiosis, the specialised form of cell division specific to gametes (oocytes, spermatogonia).

Meiosis begins in oocytes during fetal life; whilst in males it begins for a particular spermatogonial cell following puberty. The processes involved in meiosis are shown in Figure 1.14; the salient features are summarised here (Brown, 2002). Meiosis is preceded by DNA replication; thus at the commencement of meiosis there are 46 chromosomes, each of which has replicated to consist of two chromatids. In the first phase of cell division, homologous chromosomes pair together, and may undergo crossing over and exchange of genetic material (recombination).

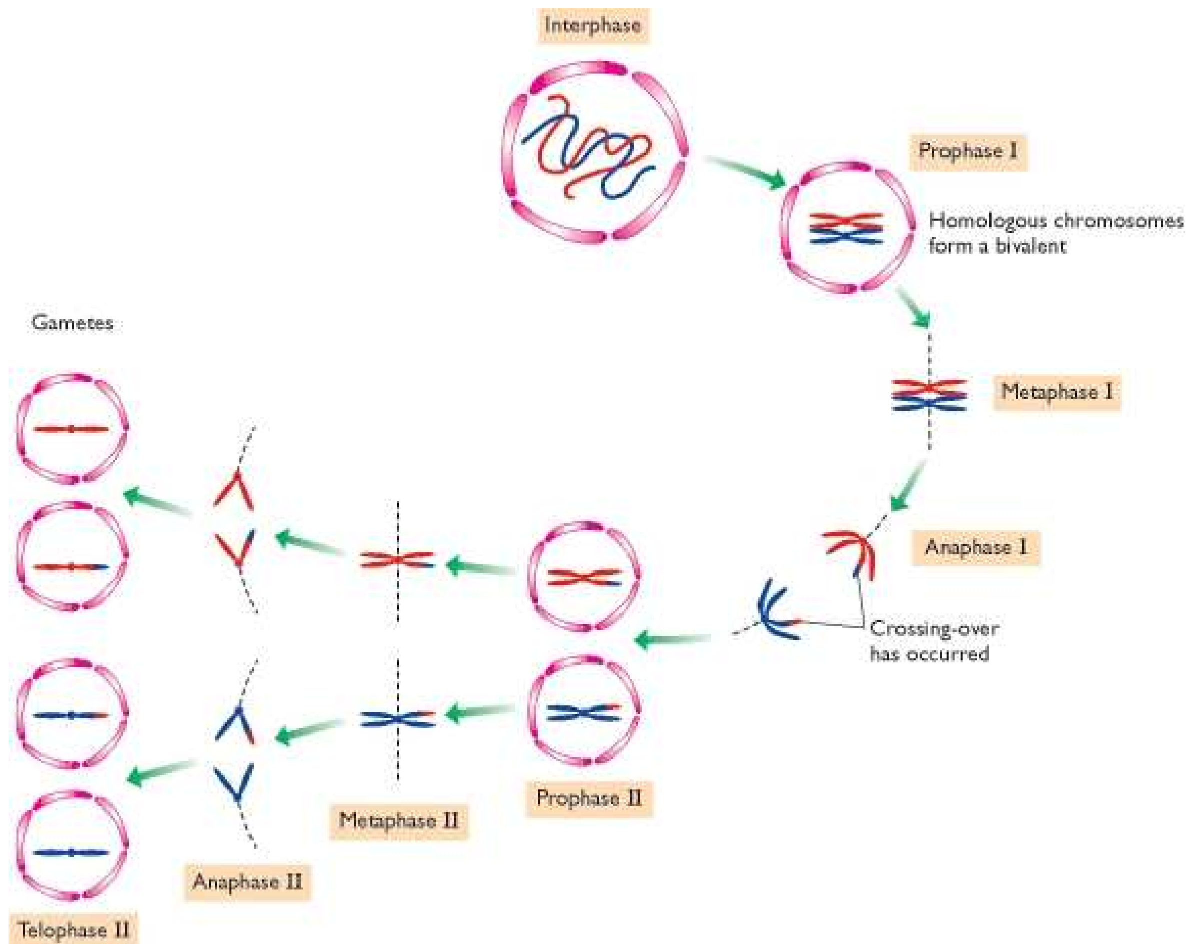


Figure 1.14. Meiosis. The events involving one pair of homologous chromosomes are shown; one member of the pair is red, the other is blue. At the start of meiosis the chromosomes condense and each homologous pair lines up to form a bivalent. Within the bivalent, crossing-over might occur, involving breakage of chromosome arms and exchange of DNA; the process of recombination. Meiosis then proceeds by a pair of mitotic nuclear divisions that result initially in two nuclei, each with two copies of each chromosome still attached at their centromeres, and finally in four nuclei, each with a single copy of each chromosome. These final products of meiosis, the gametes, are therefore haploid. (Brown, 2002).

Recombination involves exchange of DNA between two homologous chromosomes and results in a reorganisation of the alleles on the recombined chromosomes (Figure 1.15). The first meiotic division is completed as the homologous chromosomes separate and two daughter cells result. Each daughter cell contains 23 chromosomes, although each chromosome is comprised of two chromatids. The cells then undergo a second round of division. Haploid gametes result. Thus meiosis has two functions: it results in the production of haploid gametes and provides opportunity for recombination, which contributes to the unique genetic constitution of each individual. Since individual chromosomes assort

randomly and independently, and recombination occurs between homologous chromosomes, the capacity for variation from the parental genetic constitution is enormous.

NOTE:
This figure is included on page 47
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.15. **Recombinant and nonrecombinant gametes**.
An individual has haplotypes *AB* and *ab*, meaning that alleles *A* and *B* were inherited together on a single chromosome from one parent, while alleles *a* and *b* were inherited together on a single chromosome from the other parent. If no recombination events (crossovers) occur between the two loci during a meiosis, then the meiosis yields four gametes with nonrecombinant (parental) haplotypes: two *AB* and two *ab* haplotypes. If a single recombination event occurs, then the meiosis produced two recombinant gametes with haplotypes *Ab* and *aB*, plus two nonrecombinant gametes with haplotypes *AB* and *ab*. Meiosis results in four gametes because chromosomes duplicate prior to gamete formation. (Griffiths *et al.,* 1999).

Recombination events between alleles depend on their proximity along a chromosome. Thus, for two alleles that are far apart, recombination events between them during meiosis are likely, and the inheritance of each allele is therefore independent of the other; this is Mendel's Law of Random Independent Assortment (Mendel, 1866). Conversely, for two alleles that are close together, recombination events between them are less likely, and thus these alleles are more likely to be inherited together ("linked"). Genetic linkage can be defined as the tendency for alleles close together on the same chromosome to be transmitted together as an intact unit, through meiosis, than would be expected under Mendel's Law of Random Independent Assortment (Mendel, 1866). This is the fundamental principle on which linkage analysis is based.

1.4.3 Monogenic vs polygenic inheritance

If a person has inherited a disease for which a single gene is necessary and sufficient for the *phenotype* (disease manifestation), then the disease is monogenic or Mendelian. Mendelian inheritance may be: autosomal or X-linked, dominant (one disease allele is necessary for the disease phenotype) or recessive (one normal allele is required for a normal phenotype); Y-linked (very rare). Examples of the distribution (segregation) of the phenotype within pedigrees (diagrammatic representation of a family, showing the genetic relationships between individuals) with these types of Mendelian inheritance are shown in Figure 1.16.

The penetrance of a disease allele is the probability that a person with the allele will display the phenotype; this suggests that the allele itself, whilst possibly predisposing, requires enabling factors, e.g., other genes or environmental factors, for disease expression. Incomplete penetrance (carriers of the disease allele and a normal phenotype) can confound assessment of the mode of inheritance, and also impede discovery of the genetic basis of an inherited condition due to misleading phenotype-genotype data, which are a crucial component of one gene-finding methodology, linkage analysis, discussed shortly.

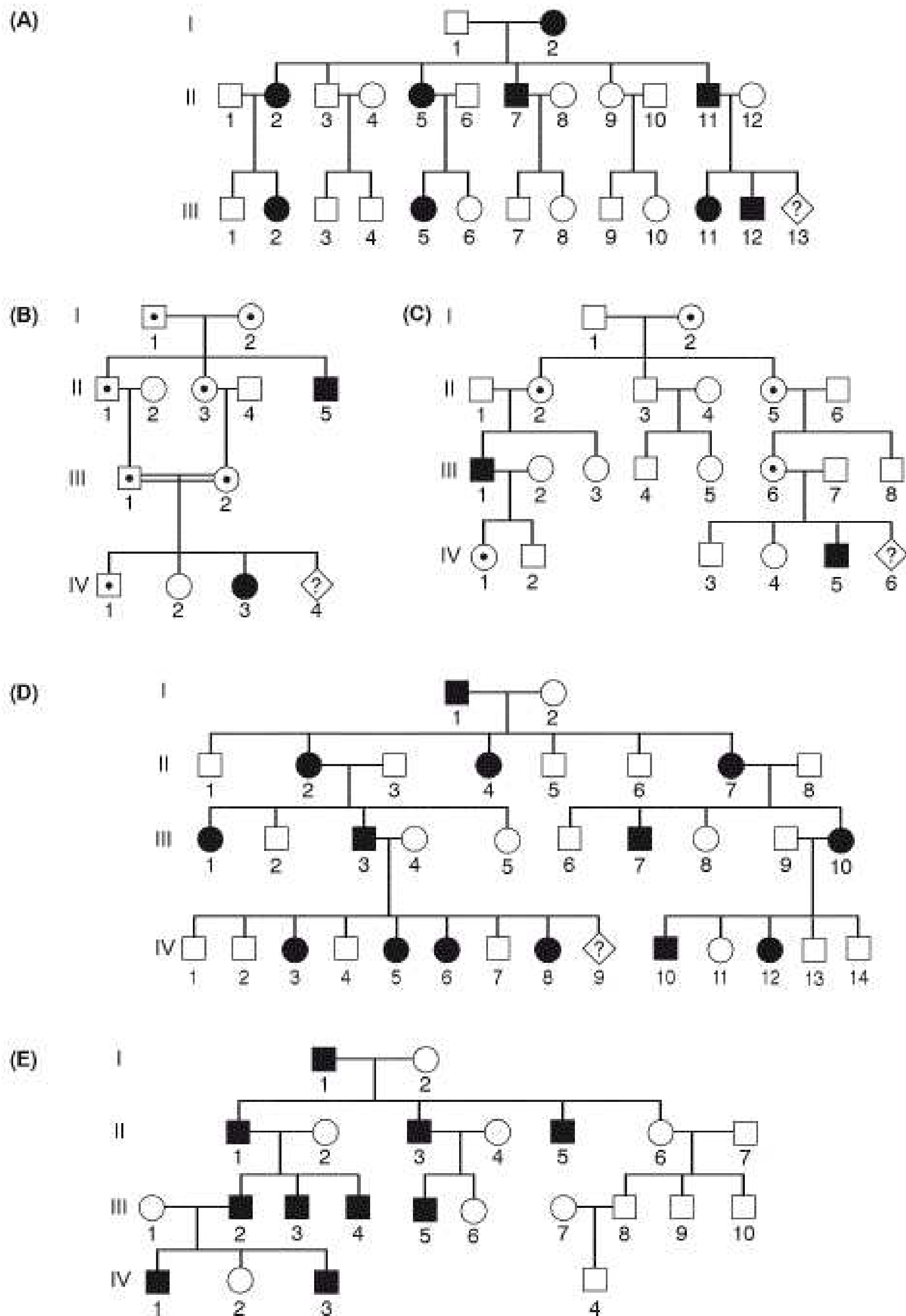


Figure 1.16. Patterns of Mendelian inheritance.
A: Autosomal dominant; B: Autosomal recessive; C: X-linked recessive; D: X-linked dominant; E: Y-linked; □ unaffected male, ■ affected male, ○ unaffected female, ● affected female (Strachan and Read, 1999).

Most diseases, even those appearing to be monogenic, are governed by genes at more than one locus, i.e., they are polygenic. The presence of disease may be determined by: (1) multiple genes, each of which has a small effect; or (2) one gene with a major effect on phenotype, the effect of which is modified by multiple genes at other loci (Strachan and Read, 2011). Furthermore, environmental factors may modulate the effect of genetic loci, e.g., T2DM, a polygenic disorder whose expression is at least modulated by obesity, physical inactivity and dietary intake of fat (McCarthy, 2010; Wareham *et al.,* 2002). In addition, diseases that appear to be monogenic may have an entirely non-genetic primary aetiology. Children not only share genetics in common with their parents and siblings, they also frequently share a common environment, which may be the underlying basis for a disease; unravelling the effect of environment *vs* genetics can be difficult.

1.4.4 Methods of Mendelian disease gene discovery

Until recently, linkage analysis and positional cloning studies have been the foundation for the discovery of monogenic, inherited disease genes (Strachan and Read, 2011). In positional cloning, the gene is identified based only on its approximate chromosomal location; this strategy has often been used after a linkage analysis has been performed. An example in endocrinology where this approach led to gene discovery was in identification of the causative *MEN1* gene in the MEN1 syndrome. Linkage studies in several families suggested the gene was one of a number of candidates within the 11q13 locus (Larsson *et al.,* 1988; Bale *et al.,* 1989; Larsson *et al.,* 1992; Fujimori *et al.,* 1992; Thakker *et al.,* 1993). Positional cloning of genes within the locus led to gene discovery, after 12 different mutations in the *MEN1* gene were found in 14 probands from 15 families (Chandrasekharappa *et al.,* 1997).

1.4.4.1 Linkage analysis

Linkage analysis is used to identify a region(s) within the genome that might contain a monogenic disease gene in a family, in the absence of a previous biologically-based

hypothesis. The first step in linkage analysis is to assign a phenotype to each person – this is specific to the disease being studied; the simplest classification may be “affected” or “unaffected”. DNA from each person is then “genotyped”, using genetic markers with a known sequence and a known location within the genome.

A genetic linkage map is a chromosomal map with genes or markers that are aligned linearly, and with intervening distances that reflect the recombination frequency between adjacent genes or markers (Sturtevant, 1913). A genetic map distance of 1 centiMorgan (cM) denotes a recombination event of 1% (i.e., 1 recombinant event per 100 meioses); such a genetic distance therefore signifies two loci that are relatively close together. 1cM corresponds approximately to 1 million base pairs, although this varies across the genome, because of “recombination hotspots” – regions in the genome that exhibit highly elevated rates of recombination (Strachan and Read, 2011).

The genetic markers used in linkage analysis have evolved in parallel with our knowledge of the structure of the genome. The markers chosen are generally polymorphic, i.e., there are at least two or more alternative genotypes in the normal population, with the rarer allele having a frequency of at least 1%. In the 1980s, Botstein proposed that restriction fragment length polymorphisms (RFLPs) were sufficiently ubiquitously distributed throughout the human genome to provide a usable map (Botstein *et al.,* 1980). For the first time, this gave geneticists the means to tag the genome with uniformly distributed and highly polymorphic markers, permitting more detailed linkage studies. Subsequently, microsatellites were reported and became the standard marker for linkage studies because they were more abundant, informative and technically easier to genotype than RFLPs (Weber and May, 1989).

Single nucleotide polymorphisms (SNPs) are single base substitutions which occur in the genome on average every 100-300 base pairs, and are a common (population frequency at

least 1%) form of human genetic variation (Brookes, 1999; Ensenauer *et al.,* 2003; Chorley *et al.,* 2008). Linkage analysis requires heterozygosity at the locus of both the unknown gene and the genetic marker (to enable detection of recombination) (White *et al.,* 1989). SNPs are mostly diallelic and hence have limited heterozygosity, which constrains their ability to detect recombination; however their uniformity and frequency throughout the genome make them ideal markers for linkage studies (Wang and Moult, 2001; Burton *et al.,* 2005).

An important aspect of SNPs is that they can be inherited together in haplotype blocks. These blocks consist of SNPs where little historical recombination has occurred, i.e., they are in "linkage disequilibrium" with each other. Therefore, genotypes of SNPs in the same block tend to be correlated and not all SNPs in a block need to be directly assayed. Given this property of SNPs, the number of variants which need to be typed in order to comprehensively cover the genome is reduced. In regions of the genome where haplotype blocks do not exist, a proportionately higher number of variants need to be assayed.

Linkage analysis studies examine the joint inheritance of presumed underlying disease genotypes and genotypes of markers whose position on a chromosome is known, in conjunction with the phenotype status of the individuals. The closer the unknown disease locus is to a genetic marker, the more likely it is to be inherited with the genetic marker, because recombination events during meiosis are less likely, than if the two loci were distant. Thus, in linkage analysis, the segregation of disease and genetic markers is examined – if the disease and genetic marker(s) do not segregate independently, then this suggests that the disease locus and genetic marker(s) also do not segregate independently. Hence, the two loci must be in physical proximity to each other. This approach aims to identify a chromosomal locus (or loci) which could contain the disease gene.

There are two methods of linkage analysis: parametric and nonparametric. Parametric linkage analysis requires specification of a genetic model: the mode of inheritance, the allelic (marker and disease) frequency in the normal population and the penetrance of the disease allele (the probability of the disease phenotype given the disease allele is present). It is the more powerful method, providing that the disease model specified is correct. A test of linkage using a parametric approach is a test of all the assumptions of the disease model of which linkage is but one; failure to find linkage could be due to misspecification of any of these parameters; it does not prove lack of linkage.

The parametric logarithm of the odds (LOD) score, a concept first introduced by Morton, is a likelihood-based statistical measure which quantifies the degree of linkage in a pedigree (Morton, 1955). The odds ratio is the probability of observing the specific genotypes in the family given linkage *vs* the same probability computed conditional on independent assortment (no linkage). High values of the odds ratio favour the linkage hypothesis, whilst values close to 1 favour independent assortment. A LOD score of 3, by convention, is taken as statistically significant evidence for linkage; this means that the linkage hypothesis is 1000 times more likely than the hypothesis that the two loci are not linked. Conversely, a LOD score of -2 or less is evidence against linkage (Morton, 1955).

Nonparametric linkage (NPL) analysis is required when the genetic model can not be specified with any confidence. NPL methods look for alleles or chromosomal segments that are shared by affected individuals more often than random Mendelian segregation would predict (Strachan and Read, 2011). Affected sib pairs analysis is one form of NPL, although any two affected relatives can be used. In this case, a nonparametric LOD score is calculated; because NPL is less powerful than parametric linkage, LOD score thresholds of 3.6 have been suggested (Strachan and Read, 2011).

Gene discovery by linkage analysis can be impeded by: (1) incomplete penetrance – unaffected carriers; (2) too few affected individuals in a family to have the statistical power to detect linkage; (3) subtle (or atypical) phenotypes – thus identifying "affected" and "unaffected" individuals can be difficult; (4) genetic heterogeneity - the pattern of disease in families being consistent with a strong major genetic component does not necessarily imply that only one gene is involved - different genotypes may produce the same clinical phenotype; (5) locus heterogeneity (same disease, different gene) between families; and (6) misspecification of the mode of inheritance and genetic model parameters which reduces the power of parametric linkage analysis. With these considerations in mind, genome-wide linkage analysis studies have comprised our first approach to the discovery of the genetic basis of familial AIMAH (Chapter 5).

1.4.4.2 Loss of heterozygosity studies

By allowing refinement of critical regions identified by linkage analysis, the study of loss of heterozygosity (LOH) in tumours has been vital in identifying tumour suppressor genes. The principle of LOH is based on the two-hit hypothesis of tumorigenesis, which was first proposed by Alfred Knudson in the context of autosomal dominantly-inherited retinoblastoma (Knudson, Jr, 1971). Knudson proposed that retinoblastoma could be due to an inherited germline mutation; the second mutation was somatic, and tumorigenesis did not develop in the absence of the second mutation (Knudson, Jr, 1971). The concept of LOH in familial tumours is illustrated in Figure 1.17, panel B. A germline mutation in a gene is inherited (the "first hit"); there is heterozygosity for the normal allele in the germline DNA. The "second hit" occurs in the tumour tissue, resulting in retention of only the disease allele; therefore the tumour tissue shows LOH for the normal allele. The converse is also true; regions of LOH shared between familial tumours of unknown genetic basis may reveal the locus of a disease-causing allele.

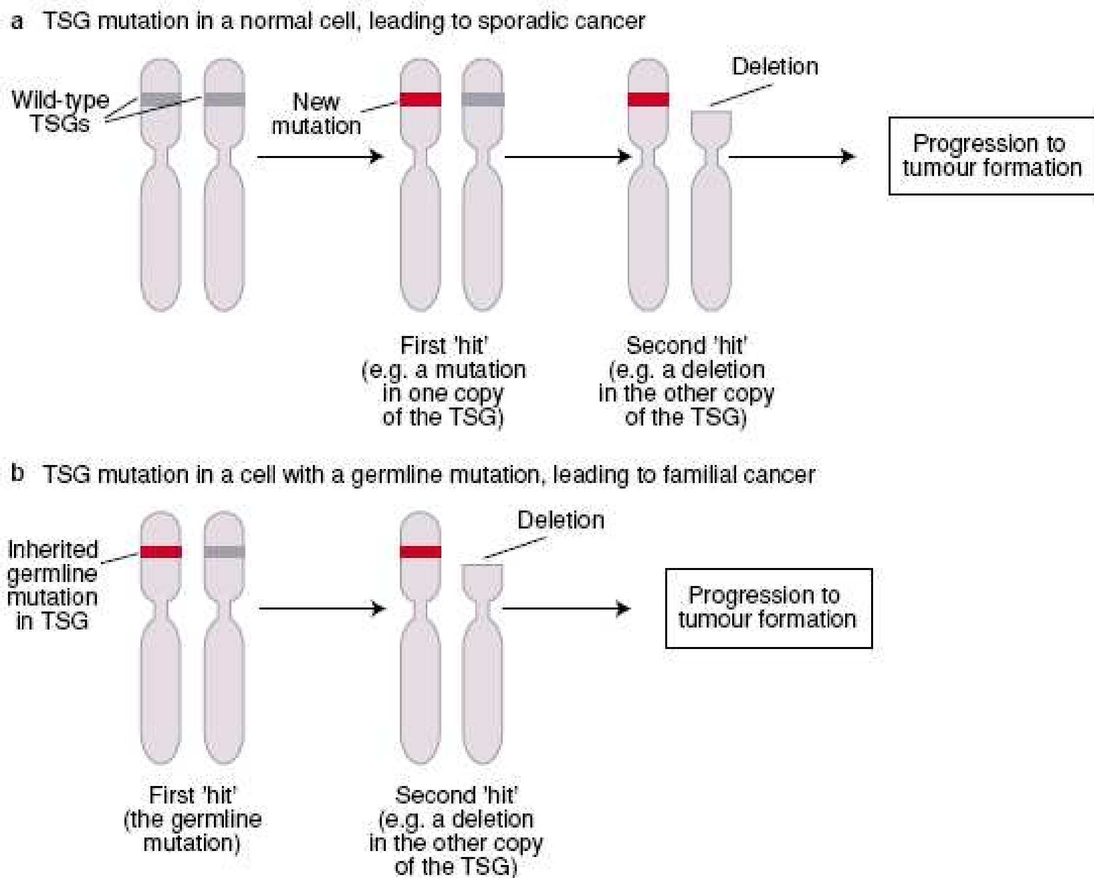


Figure 1.17. Knudson's two hit hypothesis of tumorigenesis.
a: Sporadic cancer due to a tumour suppressor gene – somatic mutations in both are required for tumorigenesis.
b: Familial cancer due to a tumour suppressor gene – a germline mutation in a tumour suppressor gene is inherited – the "first hit". Thus, the germline is heterozygous for (has only one copy of) the normal allele. The "second hit" is a somatic mutation in the normal copy of the gene and is necessary for tumorigenesis to occur – there has now been loss of heterozygosity for the normal allele. (Richards, 2001).

LOH was found in the region of the *MEN1* gene in familial tumours associated with the MEN1 syndrome, consistent with this gene operating as a tumour suppressor (Thakker *et al.,* 1989; Friedman *et al.,* 1989). An example where LOH studies have led to gene discovery is in identification of the causative gene, protein kinase A regulatory subunit 1α (*PRKAR1A*), in some families with Carney complex. Linkage analysis studies had mapped to 17q22-24, suggesting this could contain the disease gene (Casey *et al.,* 1998). Tumour studies from affected individuals revealed LOH of 17q22-24 around or within the *PRKAR1A* gene, in which germline mutations were later identified (Kirschner *et al.,* 2000b).

1.4.4.3 Somatic copy number variation analysis

In its simplest form, copy number variation (CNV) in the genome refers to a segment of at least 1000 bases in length that is present in a variable number of copies in the genome (deletion, insertion or duplication). CNVs are less frequent than SNPs, but because they comprise much larger segments of DNA, they are a greater source of normal human genetic variation (Rotimi and Jorde, 2010). However, by disrupting genes and altering gene dosage, CNVs may also be associated with disease (Iafrate *et al.,* 2004; Sebat *et al.,* 2004). Germline CNVs are a cause of monogenic disorders (e.g., a CNV at the alpha-globin locus is a cause of alpha-thalassaemia) (Goossens *et al.,* 1980). Somatic CNVs are frequent in solid tumours, may be associated with the behaviour of the disease process (advanced stage; recurrence) and frequently concur with gene expression patterns (Cancer Genome Atlas Research Network, 2008). The mechanisms of tumorigenesis in association with CNVs are not fully understood but possible explanations include: (1) Knudson's two hit hypothesis of tumorigenesis – tumour suppressor genes may be lost as a consequence of a homozygous deletion, leading directly to cancer susceptibility; (2) heterozygous deletions may harbour tumour candidate genes that become unmasked when a functional mutation arises in the other chromosome, resulting in tumorigenesis; and (3) gains of chromosomal regions may result in increased expression of one or more oncogenes.

We have also performed LOH and somatic CNV studies in resected adrenal tumours from two siblings with familial AIMAH, in an attempt to identify common regions of LOH or CNV, since these might harbour the disease-causing allele (Chapter 5).

1.4.4.4 Next-generation sequencing

Linkage analysis and LOH studies have been the traditional approaches undertaken for the discovery of novel familial disease genes. When successful, these studies have resulted in a subset of possible candidate genes, within linkage and/or LOH regions, for further study. The

limitations of these analyses have been discussed: the critical limitation of linkage analysis for discovery of rare Mendelian disease genes is having enough affected individuals in a family to have the statistical power to detect linkage, or being able to be certain of locus homogeneity to enable data from unrelated families to be combined. Likewise, LOH studies are only yielding if the genetic defect is in a tumour suppressor gene.

Further studies following on from linkage/LOH studies would primarily involve direct sequencing of candidate genes, frequently in succession, and utilizing Sanger biochemistry, the dideoxynucleotide chain termination method of DNA sequencing (Figure 1.18) (Sanger *et al.,* 1977). Sequencing is performed after polymerase chain reaction (PCR) amplification of the DNA to be sequenced ("target region"). The sequencing reaction involves primer annealing to and extension of single-stranded DNA. Primer extension is randomly terminated by incorporation of fluorescently-labelled dideoxynucleotides. The result is a mixture of extension products of various lengths – the sequence can be determined by the length of (position), and fluorescence emitted by (base call), each product.

The approach to the study of rare Mendelian disorders has changed substantially in recent times. In part, this has been motivated by the development and availability of inexpensive and large-scale sequencing methods in association with the sequencing of the human genome (Venter *et al.,* 2001). Now, large compartments of the human genome (targeted exon, whole exome or whole genome) can be selected and resequenced; this is next-generation sequencing (NGS) (Albert *et al.,* 2007; Porreca, Zhang *et al.,* 2007; Okou *et al.,* 2007; Hodges, Xuan *et al.,* 2007; Wheeler, Srinivasan, Egholm, Shen *et al.,* 2008).

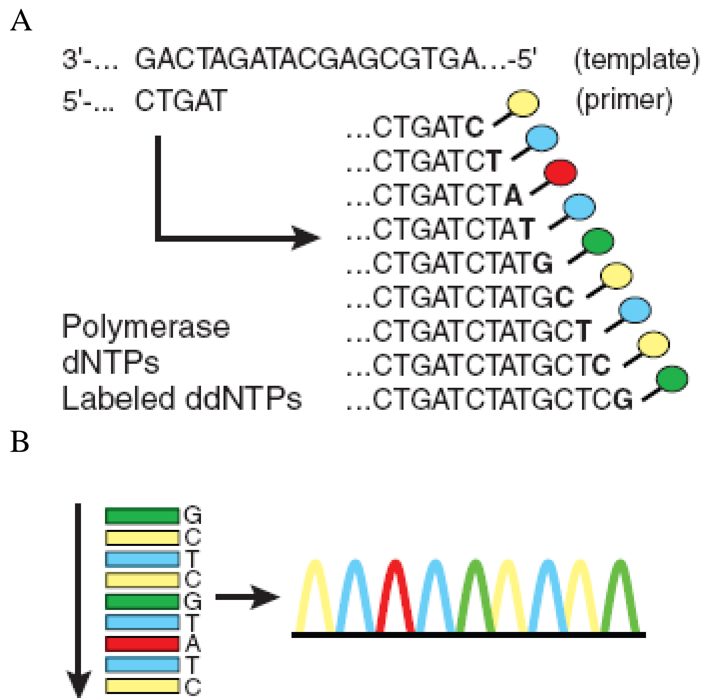


Figure 1.18. The Sanger sequencing method.
Panel A - cycle sequencing; Panel B - electrophoresis and a Sanger sequence trace. (Shendure and Ji, 2008).

Briefly, the steps involved in NGS are: (1) fragmentation of DNA (for some methods only); (2) array-based or liquid phase capture of target DNA (targeted exons, whole exome or whole genome) and enrichment by PCR, resulting in the generation of multiple short-sequence nucleotide segments (reads); (4) sequencing and visualisation of the data; (5) data preprocessing to remove low-quality sequences; and (6) detection of variants by alignment to the reference sequence. An example is shown in Figure 1.19.

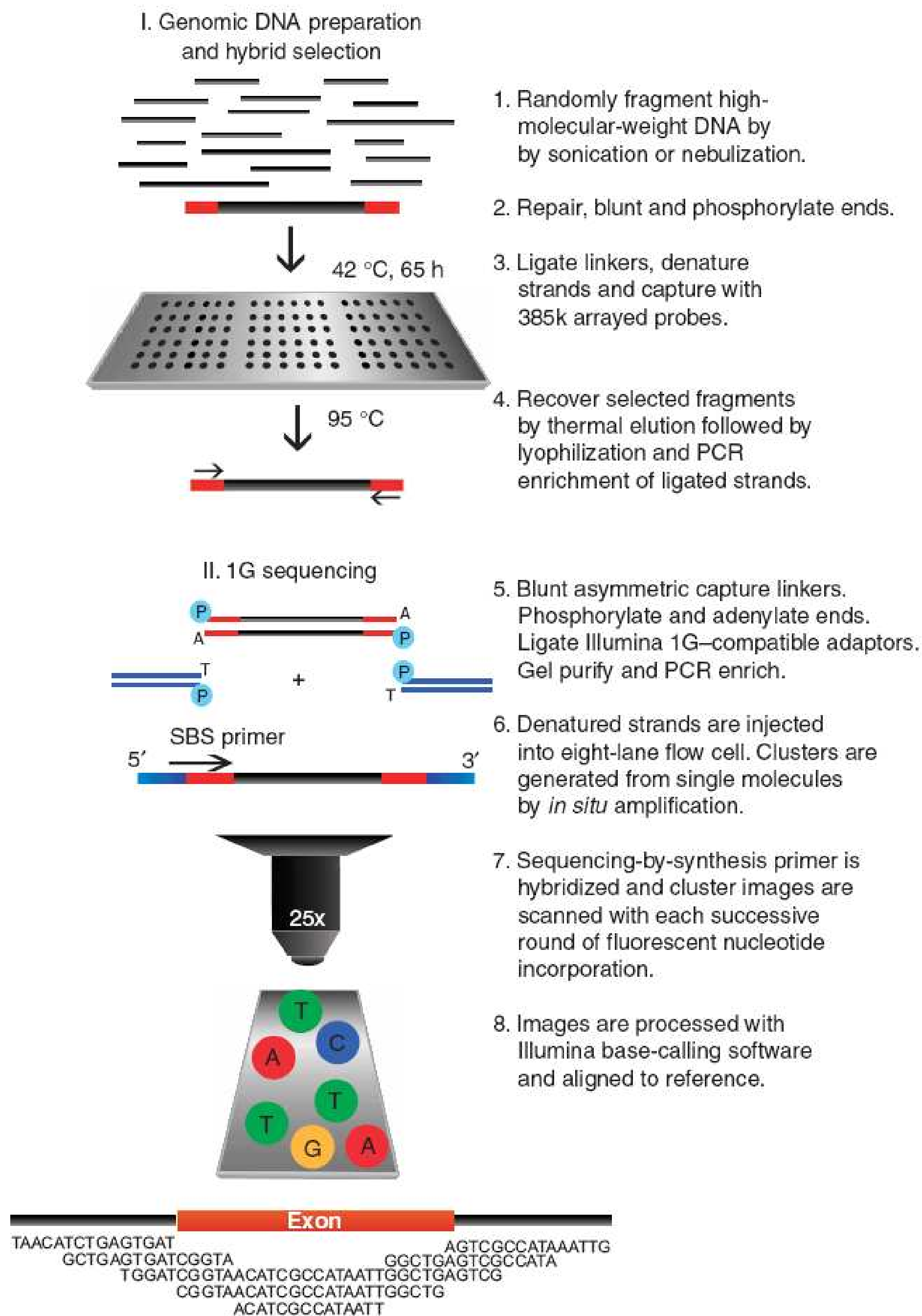


Figure 1.19. Next-generation sequencing. Array-based exon selection scheme followed by sequencing is shown. (Illumina IG sequencer). Human genomic DNA was randomly fragmented to an average size of 500 base pairs. Fragmented DNA was then hybridised to exon arrays, after which eluted material was ligated with Illumina 1G-compatible linkers and enriched by PCR. The enriched material was added to one lane of an eight-chamber flow cell, and sequence clusters were generated from single molecules. For each base-incorporation cycle, an image was read and a base called. The sequence reads were filtered for quality and image mapping, and then aligned to the reference sequence. (Hodges, Xuan *et al.,* 2007).

The technology enables analysis of genetic sequence variation on a large-scale. It overcomes problems which previously impeded novel gene discovery in individuals with rare Mendelian disorders; specifically, the lack of multiplex families with sufficient power for linkage analysis and the relatively low number of (even unrelated) affected cases. Comparing all variants within a genomic compartment (exome, genome) from a few (related or unrelated) affected individuals has already led to novel gene discovery for rare Mendelian disorders (Ng *et al.,* 2009; Ng, Bigham *et al.,* 2010; Ng, Buckingham *et al.,* 2010; Gilissen, Arts, Hoischen *et al.,* 2010).

The end result of NGS is a list of sequence variants, of which one may be pathogenic. Determining which this is requires bioinformatics analyses during which various assumptions are made in order to prioritise the variants for validation (e.g., by Sanger sequencing). If any of these assumptions are incorrect then the pathogenic variant may be mistakenly excluded. The first assumption is that the pathogenic variant is within the region that has been resequenced. Other assumptions include: (1) for a rare Mendelian disease, the variants may be compared against publicly available databases, and known variants excluded; (2) for a presumed recessive disease, only variants occurring in homozygosity may be retained; likewise, for a presumed dominantly-inherited disease, only variants occurring in heterozygosity may be retained; (3) prediction programs (e.g., PolyPhen) can be used to predict the pathogenicity of sequence variants, and all "benign" variants excluded (Sunyaev *et al.,* 2001); and (4) data from related or unrelated individuals with the same disease may be compared, and all variants or genes not shared by all, or subsets of, affected individuals excluded from further analysis, which assumes at least a degree of allelic or genetic homogeneity. This last assumption, however, is a powerful method to substantially reduce the number of variants to be considered.

Confirmation of the pathogenicity of the variant might involve demonstrating the variant: (1) segregates with the phenotype; (2) is present in unrelated, affected individuals; (3) is not present in the normal population; or (4) in an animal model, is sufficient for an analogous disease phenotype. Of course, if the variant is not found in other unrelated, affected individuals, then this may be because of locus heterogeneity (different genetic cause) or allelic heterogeneity (same gene, different allele). To exclude allelic heterogeneity, screening of the entire gene in unrelated, affected individuals may be necessary. As discussed earlier with regards to germline *PDE11A* variants in patients with micronodular adrenal hyperplasia, the presence of the variant in the normal population does not necessarily exclude that the variant is pathogenic, i.e., the disease allele may have low penetrance (Horvath *et al.,* 2006b).

Despite the success of this technology, it does have limitations. These include: (1) incomplete coverage of the target region – in no study has 100% coverage been achieved; (2) PCR errors; (3) sequencing errors; (4) variable depth of sequencing (relates to the number of "reads" obtained and thus to the confidence that can be placed in the obtained sequence); (5) "read" length – there is increasing potential for error with increasing length of the read, however short reads may align to multiple regions in the target region; (6) there is usually a trade-off in the technology regarding depth of sequencing and read length such that for a greater depth of sequencing, shorter reads are usually obtained; and (7) there may be errors in the reference genome (or part there-of) to which the sequence data are aligned. With these issues in mind, and having performed the linkage studies, we next proceeded to initially targeted exon capture, and then whole exome capture and NGS of germline DNA of two siblings from the first AIMAH family (Chapter 6).

1.4.5 Gene expression studies

1.4.5.1 Introduction

A pathogenic genetic variant (mutation) may alter the function of the gene product (protein): the most extreme changes are a complete loss-of-function or a gain-of-function. Thus a genetic mutation may alter the interaction of a protein with molecular signalling pathways. Therefore, whether or not the causative genetic mutation is known, gene expression profiling may provide insight into the molecular mechanisms involved in a disease process. This is achieved by identifying genes which are differentially expressed in a diseased tissue (e.g., tumour) compared with normal tissue (from the same individual or not). Changes in gene expression may occur as a part of the primary pathogenesis of a disease or secondary to other cellular processes involved in the pathophysiology of the disease.

Genome-wide gene expression (transcriptome) profiling has already been used for tumour classification and prognosis assessment in solid (breast, lung) tumours (Wang *et al.,* 2005; Potti *et al.,* 2006). Thus, in addition to their utility as a research tool, by refining the diagnosis and classification of tumours and assisting in ascertainment of prognosis, gene expression profiling may also have a clinical application. This is particularly applicable to those conditions for which the current, conventional methods have obvious deficiencies. In the field of endocrinology, adrenocortical carcinoma is an example where, although much work still remains to be done, early results from transcriptome profiling suggest that it might ultimately assist in the clinical care of the patient.

Apart from certain radiological features, the presence of locoregional invasion or distant metastases at presentation, the diagnosis of an otherwise circumscribed adrenal mass as an adenoma or a carcinoma is based on histological criteria (Weiss score). Whilst tumours can generally be classified clearly as adrenocortical adenoma (Weiss score 0 or 1) or carcinoma (Weiss score 4+), tumours with intermediate histological scores can not be unambiguously

classified (Pohlink *et al.,* 2004). Transcriptome profiling of adrenocortical tumours was able to distinguish benign from malignant tumours, and within the malignant tumour group, tumours with a benign course, could be distinguished from those with an aggressive, malignant (metastases, recurrence or relapse, death) course (de Reyniès *et al.,* 2009). Ultimately, refining the classification of adrenocortical tumours might assist in guiding treatment. Moreover, molecular classification of adrenocortical tumours might also enable development of specific targeted therapies.

A microarray is an orderly arrangement (the coordinates are known) of a rectangular grid of cells or features on a chip (Figure 1.20). Microarray technology allows expression profiling of thousands of genes in parallel in a single experiment and across numerous conditions (e.g., time, treatment groups). The experimental design is conducive to the identification of novel mechanisms involved in disease processes, since the experiment is conducted without bias from existing biological knowledge and gene expression is studied on a genome-wide scale. The general principles pertaining to microarrays and the data analysis will be introduced here. Since there are many different array platforms available, and we used the Affymetrix GeneChip® Human Gene 1.0 ST array (Affymetrix Inc, Santa Clara, CA, USA) in our studies, the discussion pertaining to platforms will focus on this gene array.

NOTE:
This figure is included on page 64 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.20. Example of an Affymetrix GeneChip®.

The Affymetrix GeneChip® Human Gene 1.0 ST array interrogates 28,869 well-annotated genes with 764,885 distinct probes; amongst the most comprehensive of the commercially available platforms when we designed our study (Affymetrix, 2007a). The platform is based on the March 2006 human genome sequence assembly; University of California, Santa Cruz (UCSC), hg18, National Centre for Biotechnology Information (NCBI) build 36. The array uses perfect-match probes (25 bases; 25-mer) which have been synthesised *in situ* on the array (Affymetrix, 2007a). The target gene transcript (cDNA synthesised from mRNA which has been extracted from the tissues) hybridises to the probe(s) by complementary base-pairing. The probes are designed to be sensitive, unique and sequence-specific detectors.

Probe design has been based upon complementarity to the selected gene transcript, sequence uniqueness relative to other genes, and an absence of near-complementarity to other RNA that may be highly abundant in the sample (e.g., ribosomal RNA) (Lipshutz *et al.,* 1999). Furthermore, probes have been chosen based on a set of empirically derived, composition-dependent design rules, which assist in selecting probes that will hybridize with high affinity and specificity (Lockhart *et al.,* 1996; Wodicka *et al.,* 1997).

Each transcript is represented by multiple probes (a probe set; "probe redundancy"). Each probe hybridizes to a different region of the transcript; the overlap between probes for a specific transcript is minimal and the probes interrogate the full length of the transcript (Lipshutz *et al.,* 1999). Probe redundancy improves the signal-to-noise ratio and the accuracy of RNA quantitation, mitigates effects of non-specific hybridization and reduces the rate of false-positives or miscalls; allowing the distinction between real and nonspecific signals (Lipshutz *et al.,* 1999). Genome-wide representation is ensured by preparing probes using gene-specific sequences available from public databases (NCBI). Background and control probes provide quality control measures of the integrity of the experiment.

After hybridisation of cDNA to the arrays, the arrays are washed and scanned. The amount of hybridisation (measured as the intensity of the emitted signal) provides a quantitative measure of the abundance of a particular sequence in the sample studied. The intensity information from the values of each of the probes in a probe set are combined together to obtain an expression measure. Comparison of hybridisation patterns enables the identification of mRNAs that differ in abundance between target samples.

1.4.5.2 Experimental design

Replication is a necessary component of microarray experiments, allowing assessment of which results might be verified in another experiment, and which observations are spurious. There are two types of replication: (1) technical replication – where the same RNA is hybridised on separate occasions – this determines differences due to technical factors; and (2) biological replication – where RNA from independent samples is processed – this will help to detect those changes that are reproducible and relevant (Breitling, 2006).

Principles of microarray data analysis

1.4.5.3 Normalisation

The need for normalisation arises from the use of multiple arrays in an experiment. Normalisation is intended to contend with the variation which is due to technical factors arising from the conduct of the experiment and unrelated to the biological process being studied, the "obscuring variation"; so that variation due to the biological process, the "interesting variation", can be identified (Bolstad *et al.,* 2003; Smyth and Speed, 2003).

Sources of "obscuring" variation may be introduced during: (1) sample preparation – tissue collection, RNA extraction, other sample processing prior to hybridisation; (2) hybridisation of the sample on the array – amount of sample applied, amount of target hybridised to the probe (this may be influenced by the reagents being used, the temperature and duration of the hybridisation reaction, probe saturation); or (3) after array hybridisation – optical measurements, fluorescence intensity computed from the scan image (Hartemink *et al.,* 2001).

1.4.5.4 Array quality

Even after normalisation, variation in data quality will remain. Therefore, an assessment of array quality is needed prior to data analysis. Better quality arrays can be given a higher weighting; similarly poorer quality arrays can be given a lower weighting, or be excluded from the analysis (Ritchie *et al.,* 2006).

1.4.5.5 Statistical analysis

Various methods may be used to detect differential gene expression. Limma is a computer package for differential expression analysis of microarray data (Smyth, 2005). The central idea is to fit a linear model to the expression data for each gene. A moderated *t*-test using empirical Bayesian methods to borrow information across genes makes the analyses stable even for small experiments (Smyth, 2004).

The result of all statistical tests is a *p*-value for each gene, describing the likelihood of observing a particular differential expression by chance alone. A *p*-value < 0.05 is usually considered statistically significant, with only a 5% chance that the observation would occur by chance alone. With the multiple testing used in microarrays (where one tests the differential expression of thousands of genes simultaneously) a *p*-value of 0.05 is rarely significant and a multiple test correction or calculation of the false discovery rate (FDR) is necessary (Benjamini and Hochberg, 1995). This controls the expected number of false-positives in the list of results. The FDR estimates how many genes reported as differentially expressed are likely to be false positives. Within this list of genes, those with the smaller *p*-values will still be the most significant candidates. As any statistical test has the potential to reject true positive results (false negative rate (FNR)); optimal data interpretation requires a balance between the FDR and FNR (Norris and Kahn, 2006).

Another component of the statistical analysis is to determine the order of magnitude of differential gene expression (fold-change; FC). Although there is no consensus on the criteria for differential gene expression, a statistically significant *p*-value (after multiple test correction) together with an absolute FC meeting a predefined, though arbitrary, criterion, e.g., an at least two-fold increase or decrease in gene expression, are frequently utilised. The FC criterion is arbitrary, since a two-fold differential expression may be of no biological consequence. Furthermore, biological and statistical significance are not necessarily equivalent – i.e., statistically significantly differentially expressed genes by whatever criteria chosen, may not be biologically significant, and, likewise, biologically significant differences may not achieve statistical significance.

1.4.5.6 Biological interpretation of data

Once a differentially expressed gene list is obtained it can be analysed in a number of ways. There is limited utility in analysing a gene list at an individual gene level, since important

effects on pathways may not be readily apparent. There are several analytical methods that can be used. These include:

(1) Classification according to gene function – Gene ontology (http://www.geneontology.org/), Ingenuity® systems pathway analysis (IPA; http://www.ingenuity.com/) or Database for Annotation, Visualisation and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Ochs *et al.,* 2007; Huang *et al.,* 2009);

(2) Pathway analysis – Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) or IPA (Kanehisa and Goto, 2000);

(3) Comparison with existing microarray data obtained from the Gene Expression Omnibus (GEO) database (a gene expression repository; http://www.ncbi.nlm.nih.gov/geo/) using Gene Set Enrichment Analysis (GSEA; discussed in Chapter 4; http://www.broadinstitute.org/gsea/) (Subramanian *et al.,* 2005);

(4) Modelling gene expression changes in terms of transcription factors that are predicted to be changing their activity (Motif activity response analysis [MARA]; discussed in Chapter 4; http://test.swissregulon.unibas.ch/cgi-bin/mara) (The FANTOM Consortium and the Riken Omics Science Centre, 2009; Pachkov *et al.,* 2007; van Nimwegen, 2007).

Gene expression data can also be analysed in the context of other biological data. For example, regions of copy number amplification or deletion in tumours may concur with upregulated or downregulated genes, respectively, suggesting a mechanism for differential gene expression. Differentially expressed genes can also be studied in the context of linkage data, and may assist in prioritisation of candidate genes for sequencing. For example, a locus derived from linkage analysis may contain a gene which regulates a particular molecular pathway – if genes involved in that pathway are also differentially expressed, then that strengthens the case for that gene as a candidate in the molecular pathogenesis of the disease.

1.4.5.7 Validating microarray data

The requirement for data validation arises from issues surrounding the sensitivity, specificity and reproducibility of arrays; some of these issues have already been discussed. Since there are many steps in the process of generating microarray data, there are many points at which technical variability could confound the biological variation that the experiment is trying to detect. Furthermore, array platforms have their own limitations and biases based on the nature of the probe design (Rockett and Hellmann, 2004). These include: (1) false positive signals generated by cross-hybridisation with related, but different, genes; (2) many genes have splice and tissue variants which may bind to the same probe depending on the probe design (the probe may or may not be transcript-specific); and (3) concern that complex multiplex hybridisation reactions on such a small scale might create hybridisation kinetics leading to false-negative or false-positive results.

Microarray data are regarded as validated when another method of quantitating mRNA abundance (e.g., reverse transcription-quantitative polymerase chain reaction [RT-qPCR]) has also shown differential gene expression. The issue of which genes to select for validation is unresolved, since it is not practical to validate all differentially expressed genes (which may be hundreds). In general, a handful of genes that are purportedly the most differentially expressed are selected (Rockett and Hellmann, 2004).

1.4.5.8 Comparing microarray studies

With the availability of high-throughput expression arrays, there has been a large amount of microarray data deposited in public databases. It would be ideal to compare expression data with that made available by others. However, the conduct of experiments in different laboratories, and often, using different arrays, adds a complexity to such analyses.

Firstly, it has been suggested to be erroneous to compare absolute intensity values, rather than relative values, across platforms; because absolute values are adversely affected by probe-specific and platform-specific effects, whereas these have, to an extent, been accounted for by calculation of a relative value (Irizarry *et al.,* 2005). Differences in the algorithms used to "preprocess" array data and different statistical tests can also provide misleading results in the direct comparison of different arrays (Suárez-Fariñas and Magnasco, 2007). Laboratory influences and inconsistencies may adversely affect comparisons. Finally, agreement between platforms can be difficult because there is uncertainty surrounding the best way to identify common genes (Kuo *et al.,* 2002; Yuen *et al.,* 2002). Whilst sequence-matched probes could increase cross-platform comparison, this may be difficult to do, especially if multiple studies are being compared and there is a large number of genes involved.

We have used each of the aforementioned molecular techniques in order to identify: (1) the molecular mechanisms involved in familial AIMAH tumorigenesis and (2) the genetic mutation underlying the presumed monogenic inheritance of familial AIMAH. The data will be presented in Chapters 4, 5 and 6 of this thesis.

1.5 Screening for Subclinical Cushing's syndrome in Type 2 Diabetes Mellitus

1.5.1 Definition

Subclinical Cushing's syndrome (SCS) is conceptually defined as the presence of hypercortisolism in the absence of clinically apparent Cushing's syndrome (CS) (Mantero *et al.,* 1997). Operationally, it has been defined as the presence of at least two abnormal basal or dynamic tests of the HPA axis (Mantero *et al.,* 1997). There are, however, no international consensus definitions or diagnostic criteria for SCS.

1.5.2 The morbidity of subclinical Cushing's syndrome and treatment outcomes

Overt cortisol excess, as occurs in CS, predisposes to the development or worsening of hypertension, T2DM, visceral obesity and osteoporosis and is associated with an increased mortality (Newell-Price *et al.,* 2006). However, more subtle glucocorticoid excess may also be detrimental. This is suggested by data from patients treated for adrenal insufficiency, when glucocorticoid replacement doses were inadvertently ≈ 50% higher than physiological daily adrenal cortisol production. Glucocorticoid over-replacement has been associated with lower bone mineral density, glucose intolerance and increased intraocular pressure (Zelissen *et al.,* 1994; McConnell *et al.,* 2001; Li Voon Chong *et al.,* 2001). In addition, the higher mortality in hypopituitary patients, a proportion of which was due to increased cardiovascular deaths, may, in part, have been due to glucocorticoid over-replacement (Rosen and Bengtsson, 1990; Tomlinson *et al.,* 2001).

Additional data regarding the morbidity associated with subtle glucocorticoid excess come from patients with adrenal incidentaloma in whom hypertension (87.5%-91%), impaired glucose metabolism (25%-61%), dyslipidaemia (50%) and obesity (50%) are highly prevalent (Reincke *et al.,* 1992; Rossi *et al.,* 2000). SCS is also associated with increased carotid artery intima-media thickness and a higher prevalence of atherosclerotic plaques, both of which are surrogates for increased cardiovascular risk (Tauchmanovà *et al.,* 2002). Observational data suggest metabolic perturbations and obesity improve after adrenalectomy for SCS (Reincke *et al.,* 1992; Rossi *et al.,* 2000; Tauchmanovà *et al.,* 2002). Progression to overt CS is uncommon (< 3%), and thus is not motivation for detection of SCS (Barzon *et al.,* 1999).

There are inconsistencies in the reported morbidity of SCS. For example, metabolic perturbations (hypertension and T2DM) are not always more prevalent in those with SCS (Mantero *et al.,* 2000). One study reported a higher prevalence of central adiposity, hypertension, dyslipidaemia and glucose intolerance in patients with apparently non-

functioning adrenal incidentalomas, suggesting that adrenal incidentalomas *per se*, and independent of cortisol hypersecretion, may be associated with metabolic perturbations (Sereg *et al.,* 2009).

A significant limitation of the morbidity and treatment data is the lack of an international consensus on the definition and diagnostic criteria of SCS, due to the lack of a gold standard diagnostic test. Other limitations of the studies in this field include: small patient numbers, retrospective studies, absence of control groups, non-blinded treatment intervention, potential referral bias and that all studies can only suggest association between SCS and comorbidities.

Despite the observed, albeit inconsistent, associations, it has not been established that SCS is causative in either the metabolic or arterial changes, or is otherwise harmful; and nor is there evidence from randomised, double-blind, placebo-controlled clinical trials of a benefit of treatment; granted that ethical considerations preclude the conduct of such studies in humans. Notwithstanding these issues, the data have inspired prevalence studies of SCS specifically in patients with T2DM because: (1) in general, they are individuals in whom hypertension, dyslipidaemia and obesity are frequent; (2) studies suggest that treatment of SCS might ameliorate hyperglycaemia; and (3) they have a higher cardiovascular risk than non-diabetic individuals, and the potential additional contribution of SCS to enhancing their cardiovascular risk has provided additional momentum for prevalence studies.

We designed a study to screen ambulatory patients with T2DM attending two tertiary referral centres for SCS, using nocturnal salivary cortisol, a test with excellent diagnostic accuracy for CS. We selected overweight or obese individuals with poorly-controlled T2DM; hypothesising that these features might be indicative of, and thus might enrich our study cohort for, SCS. The data are presented in Chapter 8.

1.5.3 Screening tests for hypercortisolism

Biochemical screening for hypercortisolism may require several tests since no single test has sufficient sensitivity and diagnostic accuracy to be used alone. Traditionally, first-line screening strategies have used tests with high sensitivity. However, this may result in a large number of patients with apparently positive results submitted to further investigation, only to yield a very low number of true-positive results. Whilst this can be problematic for the diagnosis of CS, further investigations are guided by clinical suspicion. However this approach is particularly problematic for SCS, in whom clinical clues to the diagnosis are, by definition, lacking. Evidence-based clinical practice guidelines for the diagnosis of CS have been formulated; no such guidelines currently exist for SCS (Nieman *et al.,* 2008).

1.5.3.1 The 1mg dexamethasone suppression test (DST)

This test involves administration of one milligram of dexamethasone orally at 2300h and measurement of serum cortisol the next morning between 0800h and 0900h. In normal individuals an evening dose of dexamethasone negatively feeds back on the HPA axis to suppress the morning surge in ACTH, and hence, cortisol. In hypercortisolaemic states, there is a relative resistance to glucocorticoid negative feedback, and morning cortisol levels after dexamethasone are elevated. Although intraindividual variation is minimal, there is a large interindividual variability in glucocorticoid sensitivity in normal individuals (Huizenga *et al.,* 1998). The DST result may be erroneous in those taking cytochrome P450 enzyme-inducing drugs which metabolise dexamethasone (false-positives) and in those with hepatic or renal failure, in whom dexamethasone metabolism is reduced (false-negatives); measurement of dexamethasone levels may assist interpretation of results (Meikle, 1982; Terzolo *et al.,* 1995).

The 1mg DST is recommended as a first-line screening test for CS and has been validated in obese individuals (Nieman *et al.,* 2008; Ness-Abramof *et al.,* 2002). In mild or episodic hypercortisolism, which is clinically subtle and provides the greatest diagnostic dilemma,

cortisol suppression may still occur (Friedman, 2006). The reported false-positive rate of the DST is 1% in normal controls and 12-13% in obese controls and in those screened for suspected CS, depending on the cortisol threshold used (Cronin *et al.,* 1990; Fok *et al.,* 1991; Montwill *et al.,* 1994). It also has a high false-positive rate in major depression (43%), other psychiatric disorders (8-41%), alcoholism and other pseudo-Cushing's states (Murphy, 1991). The ever-decreasing cortisol threshold above which is considered non-suppression in order to increase the sensitivity of the DST has been at the expense of a reduced specificity (Arnaldi *et al.,* 2003). Ultimately, however, no single cortisol threshold reliably separates patients with SCS or overt CS from normal individuals.

Screening studies for SCS in T2DM have generally utilised the 1mg DST as the first-line screening test – although variable cortisol thresholds have been used to define adequate suppression, largely reflecting the changing thresholds recommended in the literature. In the T2DM screening studies, 20-30% of subjects had a positive DST (cortisol threshold of 50-60nmol/L); although SCS was not confirmed by any other investigation in 30-90% of cases (Catargi *et al.,* 2003; Chiodini *et al.,* 2005; Reimondo *et al.,* 2007; Newsome *et al.,* 2008). Thus, a positive DST was the catalyst for a series of investigations which ultimately refuted the diagnosis of SCS in the majority of subjects. This approach is neither feasible nor practical, since it leads to substantial unnecessary additional testing. Notably only 5% of subjects required re-evaluation when a higher cortisol threshold of 110nmol/L was used (compared with 32.3% for a threshold of 50nmol/L); although most (80%) of those re-evaluated did not have biochemical abnormalities confirmed (Reimondo *et al.,* 2007). Nevertheless, a false positive rate of 5% is still very high for practical purposes.

Thus, the DST has already been extensively evaluated as a screening test for SCS in T2DM. We believe its poor specificity is a major limitation to continuing to use the test in screening this patient cohort. As I will discuss, there are significant drawbacks to screening this patient

cohort using either urinary free cortisol or midnight serum cortisol, both of which have clinical utility in the diagnosis of CS. The aim of our screening study therefore was to determine the clinical utility (sensitivity and specificity) of nocturnal salivary cortisol in ambulatory patients with T2DM, obesity and poor glycaemic control. By detecting cases of SCS, a secondary aim of our study was to identify additional cases of adrenal incidentaloma in which we could study the ACTH-cortisol response to PD-VP.

1.5.3.2 Twenty-four hour urinary free cortisol (UFC)

For many years, measures of daily cortisol and cortisol metabolite excretion were used as an integrated measure of HPA axis function. The sequential introduction of urine steroid assays which reflect glucocorticoid secretion rates, such as 17-hydroxycorticosteroids, free cortisol by radioimmunoassay, high performance liquid chromatography assays, and most recently, liquid chromatography-mass spectrometry measurement of cortisol, have increased the specificity of testing for cortisol rather than its abundant metabolites. However, UFC may be normal in episodic or overt CS (Arnaldi *et al.,* 2003). In SCS, daily cortisol production may not be sufficiently increased to elevate UFC and qualitative measures of cortisol circadian variation may be more sensitive (Terzolo *et al.,* 1996; Valli *et al.,* 2001). UFC is unreliable in hepatic and renal disease, due to altered cortisol metabolism and excretion, respectively, and hyperglycaemia-induced polyuria in T2DM may increase urinary cortisol excretion, limiting the utility of UFC (Arnaldi *et al.,* 2003). Urinary collection is cumbersome and difficulties in obtaining an accurate 24 hour collection, limit the practical utility of the test.

1.5.3.3 Midnight serum cortisol

The midnight serum cortisol measurement has excellent sensitivity (92-100%) and specificity (96-100%) in patients with suspected CS and is able to distinguish CS from pseudo-Cushing's states (Papanicolaou *et al.,* 1998; Newell-Price *et al.,* 1995; Reimondo *et al.,* 2005). In SCS, midnight serum cortisol was positively correlated with fasting and 2 hour post-challenge

glucose levels and systolic blood pressure suggesting that it might be a useful marker of pertinent metabolic aberrations (Terzolo *et al.,* 2002; Terzolo *et al.,* 2005). Despite the excellent performance of this test, the need for standardized, stress-free blood collection requires hospitalisation and intravenous cannula insertion several hours prior to blood collection, making this test neither practical nor cost-effective for screening.

1.5.3.4 Nocturnal salivary cortisol

In plasma, cortisol exists in a protein-bound (corticosteroid-binding globulin – CBG and albumin) and unbound (free) state. It is the free cortisol, constituting 5% of the total circulating cortisol under basal conditions, which is biologically active. Salivary cortisol correlates with plasma free cortisol levels and is independent of salivary flow rates (Vining *et al.,* 1983). Since it reflects circulating free cortisol, salivary cortisol is unaffected by changes in CBG or albumin levels. The equilibrium between plasma and salivary cortisol occurs within minutes, and therefore salivary cortisol levels correlate well with, and are a convenient and accurate proxy for, free plasma cortisol (Read *et al.,* 1990).

The nocturnal salivary cortisol (NSC) test involves passive absorption of saliva into a cotton swab placed in contact with the buccal mucosa for three to five minutes. The swab is replaced into a centrifugation tube, stored in the refrigerator or at room temperature, and returned or posted back to the laboratory for assay. Salivary cortisol is extremely stable at room temperature (Chen *et al.,* 1992). Salivary cortisol levels may be falsely elevated by gingivitis or the stress of midnight awakening; collection of a sufficient quantity (50µl) of saliva for assay may be difficult in conditions associated with a dry mouth (Yaneva *et al.,* 2004). The advantages of NSC as a screening test are that it is non-invasive and can be collected at home.

NSC has been extensively validated as a screening test for CS: it has excellent sensitivity (92-100%), specificity (93-100%) and reproducibility; and is able to accurately distinguish CS

from simple obesity and other pseudo-Cushing's states (Raff *et al.,* 1998; Papanicolaou *et al.,* 2002; Yaneva *et al.,* 2004; Viardot *et al.,* 2005). In these studies "NSC" was collected between 2100h-0200h (but mostly from 2300h-2400h). In (obese) control subjects, and in those with subclinical, overt, cured or recurrent, CS, salivary cortisol levels were comparable regardless of the sampling conditions (inpatient *vs* outpatient; midnight *vs* bedtime) (Nunes *et al.,* 2009). Although a single salivary cortisol measurement may be normal in mild CS, repeated measurements over months may reveal elevated levels (Kidambi *et al.,* 2007). The convenience of salivary cortisol collection lends itself favourably to such repeated measurements, which may be required in early, intermittent or subclinical hypercortisolism.

In a study of elderly male veterans, 20% of all participants and up to 40% of individuals aged over 60 and with T2DM or hypertension had an elevated NSC in at least one of two samples collected, although no-one was diagnosed with CS (Liu *et al.,* 2005). However, screening was performed in a cohort of elderly veterans not suspected of having CS (Liu *et al.,* 2005). Furthermore the assay had not been validated in CS. It was suggested that an age- and perhaps comorbidity-adjusted reference range might be necessary (Liu *et al.,* 2005).

The overwhelming data suggest NSC compares favourably, in terms of both sensitivity and specificity, with other available diagnostic tests for CS. Thus NSC could represent a practical screening strategy in large high-risk cohorts such as selected T2DM, however its sensitivity and specificity in these cohorts needs to be evaluated.

1.5.4 Subclinical Cushing's syndrome in type 2 diabetes mellitus

Between 60-80% of patients with CS are glucose intolerant or have overt T2DM (Newell-Price *et al.,* 2006; Boscaro *et al.,* 2001). The prevalence of SCS in T2DM depends on the characteristics of the cohort studied, screening protocol and diagnostic criteria used. Those studies which recruited "consecutive" and therefore, relatively unselected, patients reported

the lowest (0-1%) prevalence of SCS (Caetano *et al.,* 2007; Reimondo *et al.,* 2007; Newsome *et al.,* 2008; Terzolo *et al.,* 2009). Studies which selected patients with features suggestive of subclinical hypercortisolism (poorly-controlled T2DM, hypertension, obesity) reported a prevalence of 2-9%, suggesting that rigorous selection criteria for screening are important in increasing the diagnostic yield (Catargi *et al.,* 2003; Chiodini *et al.,* 2005).

These studies have generally been performed in hospitalised patients and the prevalence data may not be applicable to ambulatory patients (Catargi *et al.,* 2003; Chiodini *et al.,* 2005; Reimondo *et al.,* 2007; Taniguchi *et al.,* 2008). In the two prevalence studies of SCS in ambulatory, overweight patients with T2DM, in none of the 103 and 171 people screened, respectively, was a diagnosis of SCS confirmed (e.g., by post-operative hypocortisolism) (Caetano *et al.,* 2007; Newsome *et al.,* 2008). However in neither study were the patients selected for features, apart from obesity, that might have increased the pretest probability of SCS (e.g., hypertension, poor glycaemic control).

Some of the previous studies of SCS in diabetes mellitus have included those with type 1 diabetes mellitus (Leibowitz *et al.,* 1996; Reimondo *et al.,* 2007). Since metabolic syndrome and insulin resistance are not major components of type 1 diabetes mellitus, one would expect the yield from screening such patients to be low – and accordingly, this group has been excluded from our study protocol. Conversely, the diagnostic yield could be expected to be increased if patients with features of the metabolic syndrome are selected: overweight or obesity (body mass index > 25kg/m^2), central adiposity (waist circumference > 80cm in females or > 94cm in males), hypertension and poor glycaemic control (glycosylated haemoglobin > 8%) (Alberti *et al.,* 2005). Our study has been the first to evaluate a clinically feasible screening strategy (NSC) for SCS in T2DM outpatients selected for poor glycaemic control and obesity. The results of our study have been published and are presented in Chapter 8 (Gagliardi *et al.,* 2010).

1.6 Continuous subcutaneous hydrocortisone infusion therapy in Addison's disease

1.6.1 Exogenous glucocorticoid replacement

Early studies suggested that cortisol production was 12-15mg/m^2/day (corresponding to a total daily dose of hydrocortisone of 30mg) (Kenny *et al.,* 1966; Petersen, 1980). However, glucocorticoid replacement doses of this magnitude resulted in Cushingoid features and growth retardation in children, suggesting that cortisol production rates had been overestimated. There had been considerable variation between the methods used to calculate cortisol production rates, and some studies using brief infusions of radiotracers did not account for the diurnal variation in cortisol production (Zumoff *et al.,* 1974). Revised estimates were in the order of 5-10mg/m^2/day (20mg hydrocortisone) (Esteban *et al.,* 1991; Kerrigan *et al.,* 1993).

These studies triggered a paradigm shift in the concept of "the glucocorticoid replacement dose". It was recognised that 30mg hydrocortisone (or equivalent) daily, overtreats the majority of patients and that 20mg hydrocortisone (or equivalent) daily, in divided doses, is generally sufficient. However, even lower doses of oral, intermittent glucocorticoid produce periods of cortisol excess (Figure 1.21) (Mah *et al.,* 2004). Adequacy of dosing is guided by clinical judgement (an absence of signs suggesting cortisol deficiency or excess); biochemical tests, including serum cortisol day curves are not helpful (Arlt *et al.,* 2006).

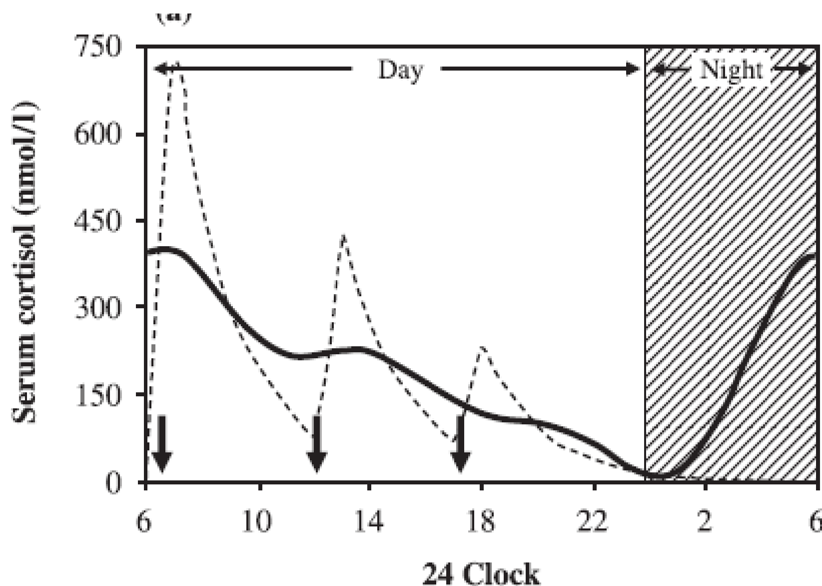


Figure 1.21. Serum cortisol in normal subjects and after thrice-daily hydrocortisone. Solid line = normal subjects; dotted line = thrice-daily hydrocortisone. Administration (10mg at 0600h, 5mg at 1200h and 2.5mg at 1800h, shown as solid arrows). (Mah *et al.,* 2004).

In patients receiving glucocorticoid replacement therapy, conventional (thrice-daily hydrocortisone) dosing does not achieve the physiological circadian or ultradian variations in cortisol (Mah *et al.,* 2004). Cortisol levels measured prior to the morning dose are consistently undetectable; they reach a supraphysiological peak one hour after the morning dose, and again reach almost undetectable levels six to eight hours after the peak, reflecting the rapid and near complete absorption and clearance of hydrocortisone (Figure 1.21) (Scott *et al.,* 1978; Feek *et al.,* 1981; Mah *et al.,* 2004; Derendorf *et al.,* 1991). Increasing glucocorticoid doses in order to attenuate the troughs in serum cortisol, inevitably results in hypercortisolism.

1.6.2 Mortality in Addison's disease

Before the availability of glucocorticoid replacement therapy in the 1950s, the one-year survival of patients with Addison's disease was less than 20% (Dunlop, 1963). Whilst glucocorticoid replacement has reduced mortality, it is uncertain whether the mortality rate returns to that of the background population (Mason *et al.,* 1968; Dunlop, 1963). Recent

studies report that the mortality rate of treated Addison's disease remains two-to-three fold increased; mainly due to cardiovascular, cancer and sepsis-related deaths (Bergthorsdottir *et al.,* 2006; Bensing *et al.,* 2008). The higher mortality is likely to be multifactorial, but may be due to glucocorticoid over-replacement, as well as under-replacement during periods of stress.

1.6.3 Subjective health status (health-related quality of life) in Addison's disease

Although glucocorticoid replacement has markedly reduced the mortality of Addison's disease, subjective health status remains impaired. Addison's disease has long been associated with "l'encephalopathie addisonienne": physical and mental fatigue, stress, anxiety, depression and impaired concentration; in contrast to early observations, these symptoms do not fully resolve with glucocorticoid replacement (Addison, 1855; Klippel, 1899). Instead, modern-day surveys reveal that subjective health status is consistently and severely impaired across various patient cohorts (Løvås *et al.,* 2002; Hahner *et al.,* 2007; Gurnell *et al.,* 2008; Bleicken *et al.,* 2008).

Self-reported (SF-36 health survey) general health and vitality were reduced; mental and physical fatigue and reduced stress tolerance were also reported (Løvås *et al.,* 2002; Hahner *et al.,* 2007; Gurnell *et al.,* 2008). Addisonian males (mean age, 39 years) reported impairments similar to those of men in the general population aged over 70 years; whilst females reported health scores similar to those of patients with congestive cardiac failure, which were much lower than for any healthy group (Løvås *et al.,* 2002). Furthermore, these impairments were associated with a working disability (26% *vs* 10%, normal population) (Løvås *et al.,* 2002).

Gastrointestinal symptoms were also frequently reported (Hahner *et al.,* 2007). Whilst gastrointestinal symptoms may be prominent prior to the diagnosis of adrenal insufficiency, or herald concomitant coeliac disease, anecdotal reports suggest that these symptoms may be due to inadequate glucocorticoid replacement (Tobin *et al.,* 1989).

1.6.3.1 Impaired subjective health status and DHEA

Poor subjective health status in Addison's disease has been attributed to adrenal androgen (dehydroepiandrosterone [DHEA]) deficiency. DHEA is the predominant androgen secreted by the zona reticularis of the adrenal cortex and is virtually absent in Addison's disease. Its sulphate ester, DHEAS, may have some biologic activity, although DHEA is largely considered to be a steroid precursor, with biological activity after peripheral conversion to oestrogen and testosterone. A DHEA receptor has never been identified, arguing against DHEA having intrinsic biological activity. *In vitro* and animal data suggest benefits of DHEA on cognition *via* neuronal protection (Li *et al.,* 2001; Kimonides *et al.,* 1998; Flood *et al.,* 1988). A DHEA dose of 50mg daily restores near physiological levels of DHEA, DHEAS and androgens in women with Addison's disease (Gebre-Medhin *et al.,* 2000).

The results of studies of the efficacy of DHEA on improving subjective health status in Addison's disease are conflicting. The first study, a randomized, double-blind, placebo-controlled, cross-over study (*n*=24; 14 primary adrenal insufficiency) suggested improved well-being and sexual functioning in women (Arlt *et al.,* 1999). However, androgenic side-effects were frequent (19/24 women) and could have "unblinded" the treatment to the participants and investigators. Another study reported improved subjective health status in males and females with Addison's disease (Hunt *et al.,* 2000). The result in males was unexpected because adrenal androgens contribute only 5% to total circulating androgens. Health status was not assessed prior to the second treatment, so a carryover effect can not be excluded. One study found a benefit only on emotional functioning in Addison's disease (Gurnell *et al.,* 2008). Other studies found no benefit on health status in primary and secondary adrenal insufficiency (Libè *et al.,* 2004; Løvås *et al.,* 2003). A meta-analysis of these and other trials found a small, possibly clinically trivial improvement in health status and depression with DHEA replacement in primary and secondary adrenal insufficiency (Alkatib *et al.,* 2009).

1.6.3.2 Impaired subjective health status and glucocorticoid replacement

Poor subjective health status in Addison's disease has also been attributed to the nonphysiological replacement of current, intermittent, oral glucocorticoid therapy. Current therapy reproduces neither the light-entrained circadian rhythm, nor the ultradian rhythm, of cortisol secretion. It also does not reproduce the cortisol-awakening response or stress-induced HPA axis activation. We, and others, have postulated that non-physiological glucocorticoid replacement may contribute to impaired health status in Addison's disease, since, as discussed earlier, cortisol has important neurocognitive and arousal effects (Debono *et al.,* 2009).

Health status remains impaired with conventional therapy regardless of the oral dosing regimen and no regimen is superior in this regard, although randomized, double-blinded studies have not been performed; notwithstanding this, thrice-daily dosing is currently considered "best practice" (Groves *et al.,* 1988; Alonso *et al.,* 2004). Attempts to improve well-being using increasing doses of glucocorticoids can be detrimental because of the catabolic effects of even subtle glucocorticoid over-replacement (Wichers *et al.,* 1999).

1.6.4 Circadian glucocorticoid replacement

With the consistently reported impairments in health status, together with evidence of a limited benefit of DHEA, and a biologically plausible postulate that nonphysiological glucocorticoid replacement in Addison's disease may be causative, efforts have turned to providing circadian glucocorticoid replacement. Circadian intravenous hydrocortisone infusions improved biochemical control of adrenal insufficiency (ACTH) and congenital adrenal hyperplasia (ACTH, 17-hydroxyprogesterone) compared with conventional treatment (Merza *et al.,* 2006). This provided the impetus for the development of an oral modified-release (delayed and sustained release) preparation of hydrocortisone (Chronocort™, Phoqus Pharmaceuticals Limited, West Malling, UK), which, in a proof-of-principle study, was

shown to mimic the normal cortisol circadian rhythm in dexamethasone-suppressed healthy volunteers (Figure 1.22) (Newell-Price *et al.,* 2008). A preliminary study of Chronocort™ in congenital adrenal hyperplasia reported improved nocturnal biochemical control of androgens (Verma *et al.,* 2010). A once-daily dual (immediate and sustained) release hydrocortisone, DuoCort™ (DuoCort Pharma AB, Helsingborg, Sweden), is also in development (Johannsson *et al.,* 2009). However, these formulations cannot mimic the ultradian and stress-associated secretion of cortisol.

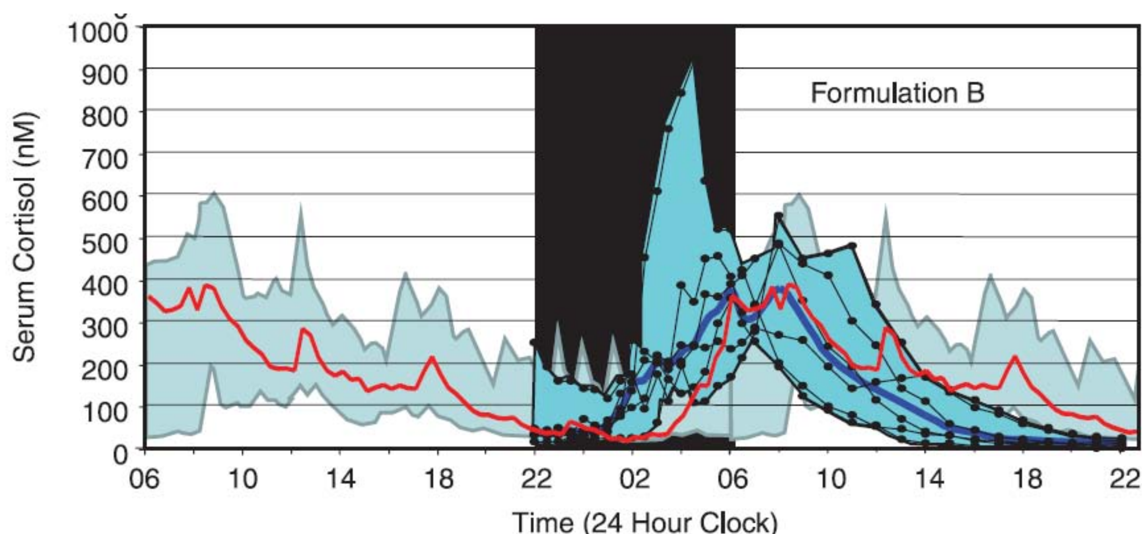


Figure 1.22. Serum cortisol-time profiles after delayed-release hydrocortisone.
Formulation B = delayed release hydrocortisone
The normal range of serum cortisol is indicated by the light blue area and the median value as the thick red line. Individual data following administration are shown as thin black lines and median values as the thick blue line together with the boundaries of observed cortisol concentrations (turquoise encapsulated area). The black background area delineates night. (Newell-Price *et al.,* 2008).

Continuous subcutaneous hydrocortisone infusion (CSHI) therapy in Addison's disease, using infusion pumps usually utilized for infusing insulin in type 1 diabetes mellitus is technically feasible and safe (Løvås and Husebye, 2007). In a proof-of-concept study, CSHI in Addison's disease restored the circadian variation in cortisol (Figure 1.23) (Løvås and Husebye, 2007). CSHI was well-tolerated and was associated with improved self-reported health status (Løvås and Husebye, 2007). However, this study was not designed to determine the improvement in

health status with CSHI, because it was not placebo-controlled. Furthermore, an attempt was made only to mimic the circadian rhythm of cortisol secretion. However, the infusion technology allows cortisol pulses to be administered, e.g., in response to a protein-containing mid-day meal or a psychic stress.

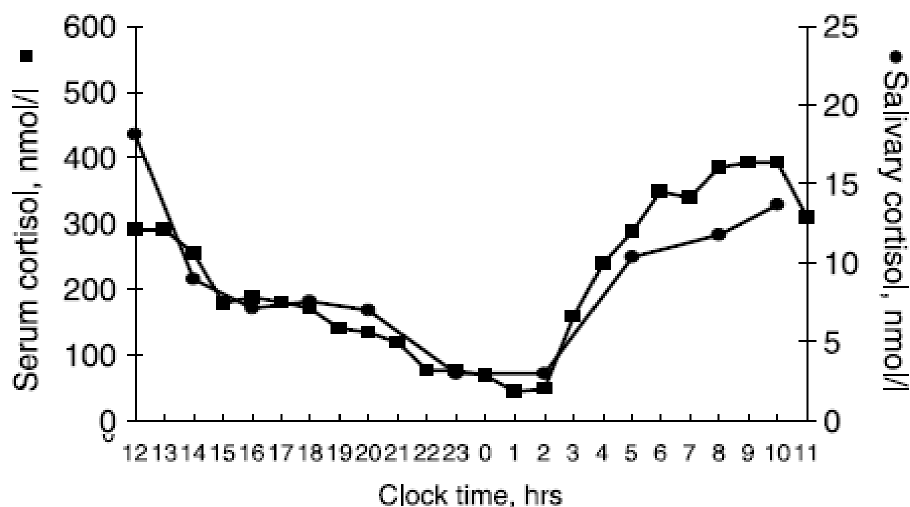


Figure 1.23. Serum and salivary cortisol profiles during continuous subcutaneous hydrocortisone infusion therapy. The data for one patient are shown. (Løvås and Husebye, 2007, European Journal of Endocrinology, 157, 109-12. © Society of the European Journal of Endocrinology (2007). Reproduced by permission).

Thus, by restoring the circadian and ultradian rhythms of cortisol secretion, CSHI therapy has the potential to improve subjective health status in Addison's disease. We designed a randomized, double-blind, placebo-controlled clinical trial to compare the effect of CSHI therapy with conventional, thrice-daily, oral hydrocortisone, on subjective health status in Addison's disease. This study remains in progress - the data available at the time of writing this thesis are presented in Chapter 9.

1.7 Summary

The studies in this thesis encompass an area of HPA axis regulation, specifically as it relates to the effects of VP, a less well studied influence at both central and peripheral sites in the axis. Our studies involve delineating the phenotype and molecular mechanisms of a rare

familial disease, AIMAH, in which cortisol secretion is regulated by heightened adrenal sensitivity to VP, examining the frequency of VP sensitivity in established adrenal incidentalomas and a search for new incidentalomas in cases thought to have an increased incidence of adrenal tumours (type 2 diabetes mellitus and metabolic syndrome), and finally, a study of the relevance of HPA axis regulation to overall well-being utilising patients with Addison's disease as a model.

1.8 Aims and Hypotheses

The aims and hypotheses tested in each thesis chapter are summarized below:

Chapter 2:

Aim:

1. To describe the phenotype of inherited VP-sensitive AIMAH in two families and in the children of an apparently sporadic case.

Hypothesis:

1. Preclinical forms of AIMAH exist and can be detected using conventional endocrine testing and adrenal imaging modalities.

Chapter 3:

Aim:

1. To determine the *in vivo* steroidogenic enzyme deficiencies that could account for inefficient steroidogenesis in two siblings with familial VP-sensitive AIMAH.

Chapter 4:

Aim:

1. To determine the molecular mechanisms involved in: (1) the pathogenesis of adrenal tumours in familial AIMAH and (2) the progression from early to advanced AIMAH.

Chapters 5 and 6:

Aim:

1. To determine the presumed monogenic basis of familial AIMAH.

Chapter 7:

Aims:

1. To determine the prevalence of VP-sensitivity in sporadic non-AIMAH adrenocortical tumours, utilizing PD-VP.

2. To determine if absent responses to PD-VP correlated with other biochemical tests suggesting SCS.

Hypotheses:

1. The aberrant ACTH-cortisol response to PD-VP seen in AIMAH would not be seen more generally in adrenal tumours.

2. An absent response to VP is indicative of pituitary corticotroph suppression due to cortisol excess from an adenoma, and would be more likely in patients with other tests suggestive of SCS.

Chapter 8:

Aims:

1. To determine the prevalence of subclinical Cushing's syndrome in patients with T2DM and the metabolic syndrome.

2. To determine the clinical utility (sensitivity and specificity) of nocturnal salivary cortisol in ambulatory patients with T2DM, obesity and poor glycaemic control.

Hypothesis:

1. Nocturnal salivary cortisol has a better specificity (lower false positive rate) than that reported for the dexamethasone suppression test in patients with T2DM and the metabolic syndrome.

Chapter 9:

Aim:

1. To evaluate the effect of circadian and ultradian glucocorticoid replacement on physical and psychological well-being in patients with Addison's disease using continuous subcutaneous hydrocortisone infusion therapy.

Hypothesis:

1. Circadian and ultradian glucocorticoid replacement improves physical and psychological well-being in patients with Addison's disease.

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

2.1 Introduction

ACTH-independent macronodular adrenal hyperplasia (AIMAH) is a rare (< 1%) cause of Cushing's syndrome (CS), typically presenting in the 5th and 6th decades of life (Lacroix, 2009). Several early reports of familial AIMAH suggested it could be inherited (Findlay *et al.,* 1993; Minami *et al.,* 1996; Miyamura *et al.,* 2002; Nies *et al.,* 2002). Nevertheless, it has been regarded a predominantly sporadic disease, with familial forms considered to represent exceptional cases. Accordingly, little effort thus far has been made to elucidate the genetic basis of familial AIMAH. Recently, several other families with AIMAH have been reported (Lee *et al.,* 2005; Vezzosi *et al.,* 2007; Staermose *et al.,* 2008). The clinical details of these families have been presented in Chapter 1 and are summarised in Table 2.1. Our report of three kindreds with familial AIMAH is the result of the work that will be discussed in this chapter (Gagliardi *et al.,* 2009). Overall, the emerging literature increasingly suggests that familial AIMAH is not infrequent, although a bias towards reporting familial disease is possible.

Table 2.1. Summary of familial ACTH-Independent Macronodular Adrenal Hyperplasia.

Reference	Proband	Family members
Findlay *et al.,* 1993	Female – 38, CS and bilateral adrenal hyperplasia	Daughter – 38, CS and bilateral adrenal enlargement
Minami *et al.,* 1996	Female - 60, CS and bilateral adrenal enlargement	Brother – 59, CS due to AIMAH Sister – 64, SCS and bilateral adrenal enlargement Brother – 54, SCS and bilateral adrenal enlargement
Miyamura *et al.,* 2002	Female – 68, CS due to AIMAH	Son – 38, bilateral macronodular adrenal glands, hypertension, obesity
Nies *et al.,* 2002	Female – 50, CS and marked enlargement of both adrenals	Sister – 34, CS and bilateral adrenocortical "adenomas" Female cousin – 38, CS and bilateral adrenocortical hyperplasia Two aunts – died aged 54 (cause unknown) – CS clinically Paternal grandmother – had CS clinically Father – 79, SCS and bilateral nodular adrenal hyperplasia
Lee *et al.,* 2005		Sisters – 46 and 58, CS and bilateral adrenal nodules
Vezzosi *et al.,* 2007	Two females* – 54 and 56, mild CS and bilateral macronodular adrenals	Father – 81, SCS, "thick" adrenals, no nodule Brother - 57, SCS, "thick" adrenals, no nodule
Staermose *et al.,* 2008	<u>Family 1</u> Female – 46, primary aldosteronism, SCS and bilateral adrenal masses	Mother – primary aldosteronism and bilateral macronodular adrenals Aunt – bilateral macronodular adrenals and SCS Sister – adrenal macronodule
	<u>Family 2</u> Male – 46, autonomous secretion of cortisol and aldosterone and bilateral adrenal masses	Female cousin – primary aldosteronism and bilateral adrenal nodular hyperplasia

*proband not specified; Abbreviations: AIMAH – ACTH-Independent Macronodular Adrenal Hyperplasia; CS – Cushing's syndrome; SCS – subclinical Cushing's syndrome

These reports of familial AIMAH, occurring in multiple generations, suggest that AIMAH may be inherited as a Mendelian disorder. CS has an incidence of 3 per million per year; thus the incidence of AIMAH is 3 per 100 million per year (Newell-Price *et al.,* 2006). Since AIMAH is exceptionally rare, it is statistically unlikely that multiple family members would be affected by chance alone. In several familial cases, the onset of CS was at a younger age (early to mid 30s) than sporadic AIMAH usually presents (5th and 6th decades); the earlier age of onset is also compelling for the inherited basis of AIMAH in these families. In many of these familial reports, females were solely affected or had more pronounced phenotypic expression, suggesting that sex steroids may modulate the phenotype. Overall, segregation analysis within these families favours autosomal dominant inheritance.

There are two intriguing aspects of the physiology of AIMAH. The first is the aberrant, ACTH-independent increase in cortisol production and secretion, in response to the physiological modulation of hormones or their exogenous administration, as discussed in Chapter 1 (Lacroix *et al.,* 2001). This was first described concurrently by Lacroix *et al.,* and Reznik *et al.,* in regards to food (gastric inhibitory polypeptide – GIP)-sensitive AIMAH (Lacroix *et al.,* 1992; Reznik *et al.,* 1992). The aberrant cortisol response to non-ACTH secretagogues is correlated with the expression of illegitimate or aberrant G-protein coupled receptors (GPCR) on AIMAH cells, which have become coupled to steroidogenesis (Lacroix *et al.,* 2001; Figure 1.4). Various other hormones may modulate cortisol production and secretion in AIMAH: these include vasopressin (VP), serotonin, luteinising hormone (LH) / human chorionic gonadotropin, catecholamines and glucagon (Lacroix *et al.,* 2001). The role of aberrant receptor expression in the pathogenesis of AIMAH is unknown; although in xenotransplanted murine models, ectopic expression of the LH or GIP receptor was sufficient to induce adrenocortical hyperplasia and CS (Mazzuco *et al.,* 2006a; Mazzuco *et al.,* 2006b). These data suggest that aberrant receptor expression occurs early, if it is not primary, in the

pathogenesis of AIMAH. A clinical implication of these *in vitro* data is that an aberrant cortisol response might be detectable as a preclinical manifestation of AIMAH.

The other intriguing feature of AIMAH is the discordance between the bilateral and massive macronodular adrenal glands and the relatively mild hypercortisolism. This is attributed to inefficient steroidogenesis, discussed in Chapter 3, and which, undoubtedly, is a major factor in the late-onset of CS in AIMAH compared with other causes of CS (Lindholm *et al.,* 2001). It could be expected therefore, that preclinical manifestations (e.g., macronodular adrenal glands) might be present and detectable years before the onset of CS.

Between 2005 and 2006, three brothers presented with CS due to VP-sensitive AIMAH. They all had an aberrant, ACTH-independent cortisol increase after exogenous administration of VP. With three siblings affected with an extremely rare disease, we hypothesised that in this kindred (AIMAH-01), AIMAH was familial. We also hypothesised that because of the typically late age-of-onset of CS due to AIMAH and the inefficiency with which AIMAH tumours synthesise cortisol, preclinical disease in the form of subtle abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis (nonsuppression to dexamethasone, ACTH suppression, aberrant or absent cortisol response to VP) and nodularity of the adrenal glands could be detected many years before CS developed. Accordingly, we devised a protocol to screen kindred members for CS, aberrant VP sensitivity and adrenal tumours. Since the three siblings from AIMAH-01 had an aberrant *in vivo* cortisol response to VP, we also wanted to determine which VP receptors were overexpressed (mRNA; RT-qPCR) by the adrenal tumours and thus, could be mediating the aberrant cortisol response.

During the course of this study, we were referred two other kindreds: AIMAH-02 comprised the adult children of an apparently sporadic case and in AIMAH-03, three siblings had had CS due to AIMAH. In addition to the aforementioned hypotheses, we hypothesised that in even

apparently sporadic cases (proband, AIMAH-02) occult familial disease could exist, but without screening, would remain undetected. Broadly, our aim was to describe the AIMAH phenotype in these three kindreds, since this could assist in the discovery of the genetic basis of familial AIMAH; which was the aim of the linkage, whole exome capture and next-generation sequencing studies we have performed (Chapters 5 and 6).

As will be discussed in this chapter, we found that preclinical forms of AIMAH, in otherwise phenotypically normal individuals, could be detected using our screening protocol (Gagliardi *et al.,* 2009). Our data suggest that the familial basis of AIMAH could have been previously overlooked, with subclinical AIMAH in relatives of apparently sporadic cases remaining undetected without screening. At the time of writing this thesis, the genetic basis of familial AIMAH has not been elucidated; hence we and others propose periodic (e.g., every 5 years) screening of family members of affected individuals, since this offers individuals at risk of developing CS early diagnosis and treatment – both of which are paramount in reducing the morbidity and mortality of CS (Lacroix, 2009). Eventual discovery of the genetic basis of familial AIMAH would allow screening of only those with the disease allele.

2.2 Research Methods

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee. Those with CS informed other kindred members of the study and distributed the study information sheet. All kindred members were eligible for evaluation and initiated contact with the study investigators. All participants provided written, informed consent; in those younger than 16 years, parental consent was obtained. Participants consented separately to the provision of a blood sample for DNA extraction, and in those undergoing adrenalectomy for CS, separate consent was obtained for retention of adrenal tissue for research.

Clinical Evaluation

A clinical assessment for characteristics of CS, with particular attention to early features such as weight gain, hypertension and mood change and concentration difficulties, was performed.

Endocrine Testing

We used standard screening tests for CS, in accordance with the Endocrine Society guidelines (Nieman *et al.,* 2008). These were: (1) nocturnal salivary cortisol (NSC) (reference range [RR]: < 13nmol/L); (2) 24 hour urine free cortisol (24h UFC) (adults) (RR: 50-350nmol/day); and (3) 1mg overnight dexamethasone suppression test (DST) (RR: 0900h cortisol < 50nmol/L after 1mg dexamethasone at 2300h the night prior). The protocol for collection of NSC is described in Chapter 8. Although measurement of ACTH is not a recommended screening test for CS, in these individuals we were seeking evidence of HPA axis suppression due to subtle adrenal overactivity; since this might have been revealed by a low or suppressed ACTH, we included morning plasma ACTH (RR: 10-60ng/L) in our screening protocol.

All those with CS in the three kindreds (except two siblings from kindred AIMAH-03, in whom evaluation was not performed) had VP sensitive-AIMAH (VPs-AIMAH). We therefore postulated that an aberrant cortisol response to VP, defined as an ACTH-independent cortisol increase following exogenous VP administration, might be one of the earliest biochemical abnormalities manifested by kindred members destined to develop AIMAH. VP stimulation testing was performed in adults using intravenous arginine VP, 1 I.U. per 70kg body weight (Pitressin, Link Pharmaceuticals, United Kingdom). The rationale for our use of this dose, rather than the conventional dose of 10 I.U. IM is discussed in Chapters 1 and 7. Patients fasted from midnight prior to the test, and smokers were asked to refrain from smoking on the day of the test. An intravenous cannula was inserted for blood sampling and VP administration. Patients lay supine for one hour prior to, and for the

duration of, the test. Serial blood samples were collected for ACTH and cortisol at -30, -15, 0, +5, +15, +30, +45, +60, +90, +120 minutes after VP administration. Blood pressure, heart rate and symptoms (pallor, chest or abdominal discomfort) were recorded. Based on our experience with VP testing of the HPA axis, we classified the VP response as normal if ACTH increased from baseline with a subsequent rise in cortisol (Torpy *et al.,* 1994). The VP response was abnormal if: (1) cortisol increased and ACTH decreased or was unchanged (aberrant response); or (2) neither ACTH nor cortisol increased (absent response).

Those with confirmed CS also underwent a corticotrophin-releasing hormone (CRH) stimulation test (1µg/kg CRH-Ferring, Ferring Pharmaceuticals, Germany) with serial measurement of ACTH and cortisol (from -30 mins to +60 mins at the intervals described for VP stimulation testing above); where the absence of an ACTH and cortisol response to CRH confirmed ACTH-independent CS.

Adrenal Imaging

Adrenal morphology was assessed by a non-contrast computed tomography (CT) scan (adults) or ultrasound (in children presenting for such evaluation). The reporting radiologists were unaware of the patients' clinical status. The adrenal glands were "normal" if size and morphology were within normal and age-appropriate reference limits.

In confirmed CS, an iodocholesterol scan was used to ascertain unilateral (unilateral tracer uptake) or bilateral (bilateral uptake) adrenal hyperfunction.

Histopathology of AIMAH Tumours

We reviewed the histology of all the familial AIMAH tumours with Dr Constantine Stratakis, Section on Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child

Health and Human Development, Division of Intramural Research, National Institutes of Health, Bethesda, Maryland, United States.

Assays

These were performed by the appropriate clinical testing laboratory in the Institute of Medical and Veterinary Science.

Plasma ACTH

ACTH was measured using the Immulite® 2000 assay (Siemens Medical Solutions Diagnostics Limited, United Kingdom). The coefficient of variation (CV) for the assay was 9.4% at 32ng/L.

Urinary Free Cortisol, Serum and Nocturnal Salivary Cortisol

These were measured using the Roche Elecsys Cortisol Electrochemiluminescence Immunoassay (Roche Diagnostics, United States). The CV for the urine cortisol assay was 12% at 91nmol/L, and for the serum cortisol assay, 7% at 70nmol/L. The functional sensitivity of the salivary cortisol assay was ≤ 8nmol/L. According to the product information, assay cross-reactivity was: cortisone (0.3%), dexamethasone (0.08%) and prednisolone (171%). For NSC we have established a normal range of < 13nmol/L as distinguishing normal individuals from those with CS (Chapter 8).

Vasopressin receptor RT-qPCR and data analysis

Adrenal tissue from patients with AIMAH undergoing resection for CS was obtained at surgery. Normal adrenal glands were obtained from patients undergoing nephrectomy for renal cancer (supplied by Professor Michael Stowasser and Associate Professor David Nicol, Princess Alexandra Hospital, Brisbane). All patients gave written, informed consent for the retention of adrenocortical tissue fragments for research. Tissue fragments were rapidly

frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted using the Qiagen RNeasy Lipid Tissue Midi Kit (Qiagen, Germany), according to the manufacturer's instructions.

RT-qPCR for the analysis of VP receptor expression on adrenal cells

Messenger RNA (mRNA) was reverse transcribed to cDNA using the Superscript III Reverse Transcriptase kit (Invitrogen, Australia). Reverse transcription of 400ng of total RNA was performed using the random hexamer priming method, according to the manufacturer's instructions. A reaction without reverse transcriptase (RT-) was performed using 400ng of pooled total RNA (equal amount of total RNA from all cases and controls) in order to detect genomic DNA contamination.

Semi- and quantitative RT-PCR of the VP receptors, *AVPR1A*, *AVPR1B* and *AVPR2* were each performed in a 20µl (10µl for RT-qPCR) reaction volume comprising 1X LC480 ProbeMaster (Roche Diagnostics, Australia), 200nM of each forward and reverse primer (GeneWorks, Australia), 100nM of Universal ProbeLibrary® (UPL) probe (Roche Diagnostics) (for RT-qPCR only) and an equal amount of cDNA from each of the cases and controls. The housekeeping genes proteasome subunit, beta type, 2 (*PSMB2*), 1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*) and hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*) were also amplified and served as loading controls.

All PCR reactions were performed using the LightCycler® 480 System (Roche Diagnostics) with a pre-denaturing step at 95°C for 10 minutes, and 40 cycles (45 cycles for RT-qPCR) at 95°C (10 seconds), 60°C (30 seconds) and 72°C (30 seconds) followed by a cooling step at 40°C for 1 minute. In both semi- and RT-qPCR, primers were designed using the ProbeFinder 2.35 at the Assay Design Centre for UPL Assay provided by Roche Applied Science (https://www.roche-applied-science.com). The primers are listed in Appendix 1, Table A.1.1.

Statistical analysis

Relative quantification using a standard curve method

The crossing point (C_p) from each signal was calculated based on the Second Derivative Maximum method. A set of serially diluted cDNAs was used to construct a standard curve (with at least 3-data points, $R^2 > 0.985$, PCR efficiency 95-105%) for every PCR system in each run (Applied Biosystems, 2004). Based on a successful standard curve, the amounts of starting RNA of both target and housekeeping genes were calculated as a linear function of logarithmic concentration and C_p. All estimated starting amounts were in arbitrary values. The starting amounts of each of the VP receptor genes were normalised against all starting amounts of reference genes generated from the same sample. Generated ratios from each target gene were transformed into log-ratios and differential analysis was performed using empirical Bayesian moderated *t*-statistics (Smyth, 2004). For each comparison, *p*-values were adjusted to control the false-discovery rate; differential analysis with adjusted p-value < 0.05 was considered significant (Benjamini and Hochberg, 1995).

2.3 Results and Discussion

Clinical Presentation of Cushing's syndrome in Kindred AIMAH-01

III-1

The proband (III-1, Figure 2.1) presented aged 69, with advanced CS (Table 2.2). Over the preceding four years he had experienced abdominal weight gain, hyperglycaemia, hypertension, proximal myopathy, multiple spontaneous fractures, bruising, cognitive change and depression. The plasma ACTH was suppressed and an adrenal CT scan showed bilateral macronodular adrenal glands, consistent with AIMAH. He had a 45% ACTH-independent increase in plasma cortisol after VP (Figure 2.2). Limited testing for other aberrant receptors **-** AVPR2 (1μg subcutaneous desmopressin, DDAVP, Minirin, Ferring Pharmaceuticals, Australia), GIP (mixed-meal) and serotonin (10mg oral metoclopramide, Maxolon, Valeant Pharmaceuticals, Australia) **-** was negative (performed supine, fasting and serum ACTH and

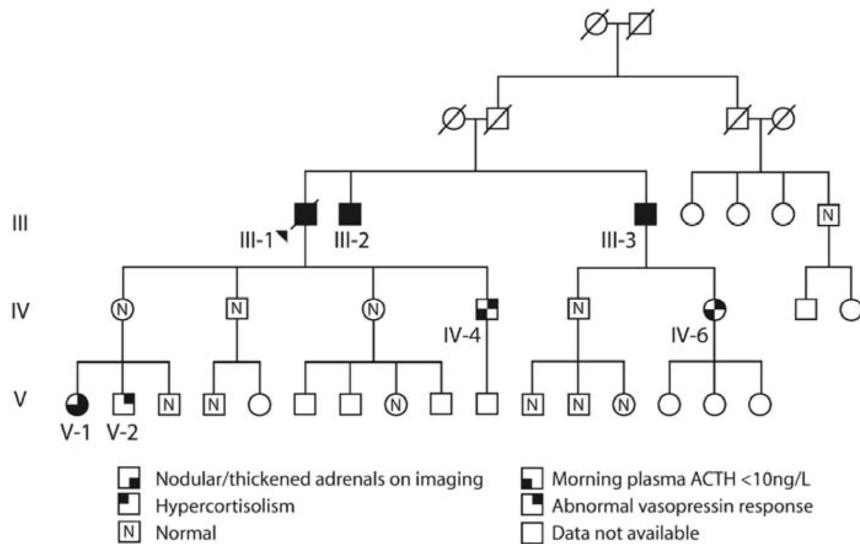


Figure 2.1. Pedigree of kindred AIMAH-01.

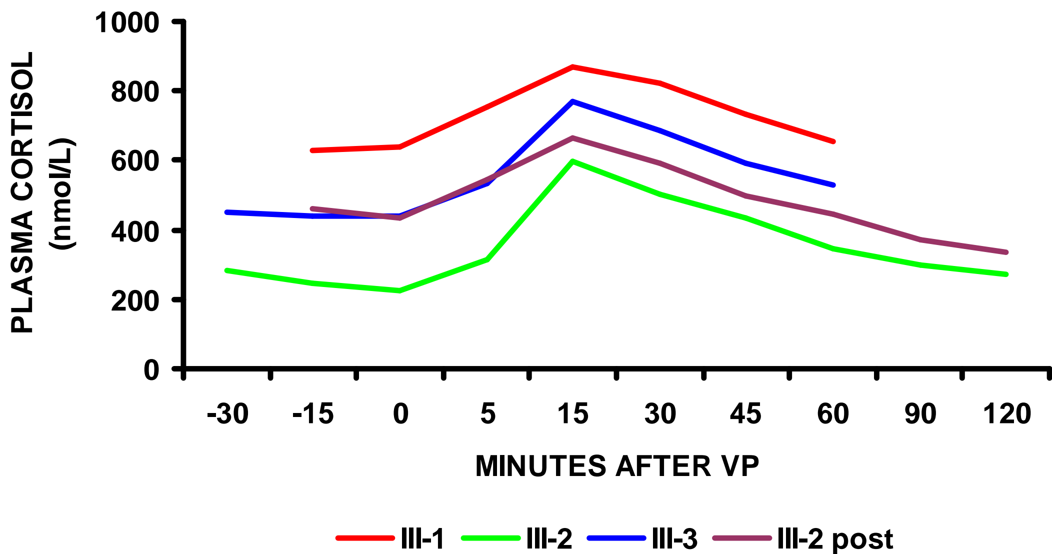


Figure 2.2. The aberrant cortisol response to vasopressin in AIMAH-01.
The data for III-1, III-2, III-3 (Figure 2.1) are shown.
III-2 post – vasopressin testing was also performed postoperatively.
Vasopressin was given at time=0. ACTH remained suppressed (< 10ng/L) throughout.

cortisol sampled half-hourly for two hours after each stimulus) (data not shown). A trial of ketoconazole was unsuccessful in achieving eucortisolism.

His clinical course was complicated by pulmonary embolism despite thromboprophylaxis, cardiac failure and nosocomial pneumonia – to all of which he was predisposed by the hypercortisolaemic state. Hypercortisolism produces a hypercoagulable state due to increased prothrombotic factors; the risk of thromboembolism is further enhanced by central obesity and proximal myopathy-associated immobility, which promote venous stasis (Van Zaane *et al.,* 2009). Hypercortisolaemia also produces a susceptibility to infection. Preoperative insertion of an inferior vena caval filter had been planned, but was expedited when he developed a spontaneous lower limb haematoma whilst therapeutically anticoagulated with heparin (platelet count normal). Whilst this seems paradoxical to the prothrombotic milieu of the hypercortisolaemic state, thinning of the skin and capillary fragility culminate in a bleeding tendency in CS.

After multidisciplinary discussions with him and his family, the decision was made to proceed with adrenalectomy. Laparoscopic bilateral adrenalectomy of macronodular adrenal glands (combined adrenal weight, 130gm; Figure 2.3) was curative. The histopathology was characteristic of AIMAH: clear and compact cells (Figure 2.4; Chapter 1). Despite cure, he died several months later from CS-associated complications; highlighting the morbidity and mortality of advanced CS.

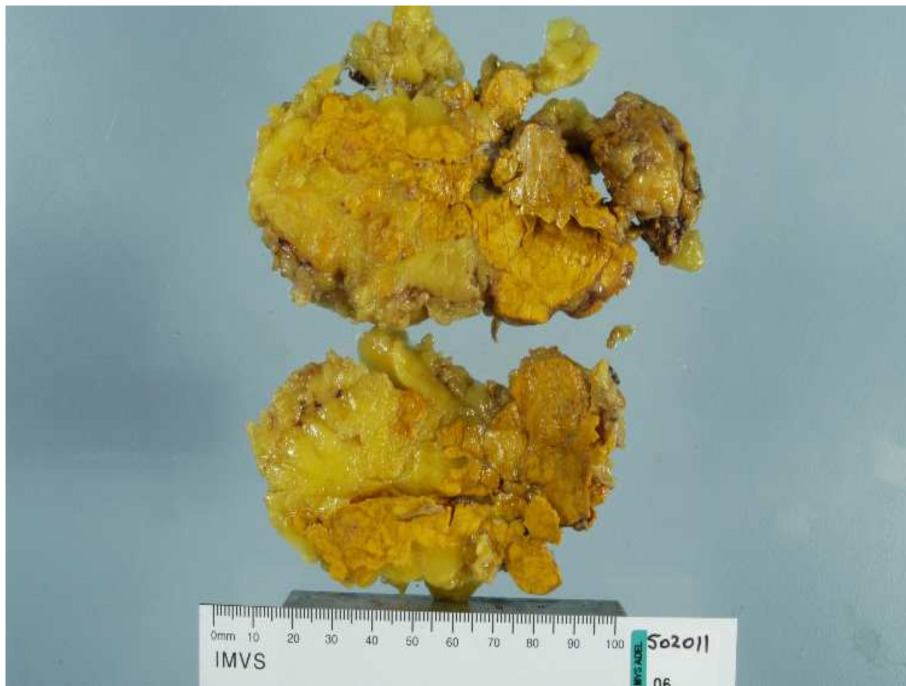


Figure 2.3. Resected macronodular adrenal glands from III-1 (AIMAH-01).

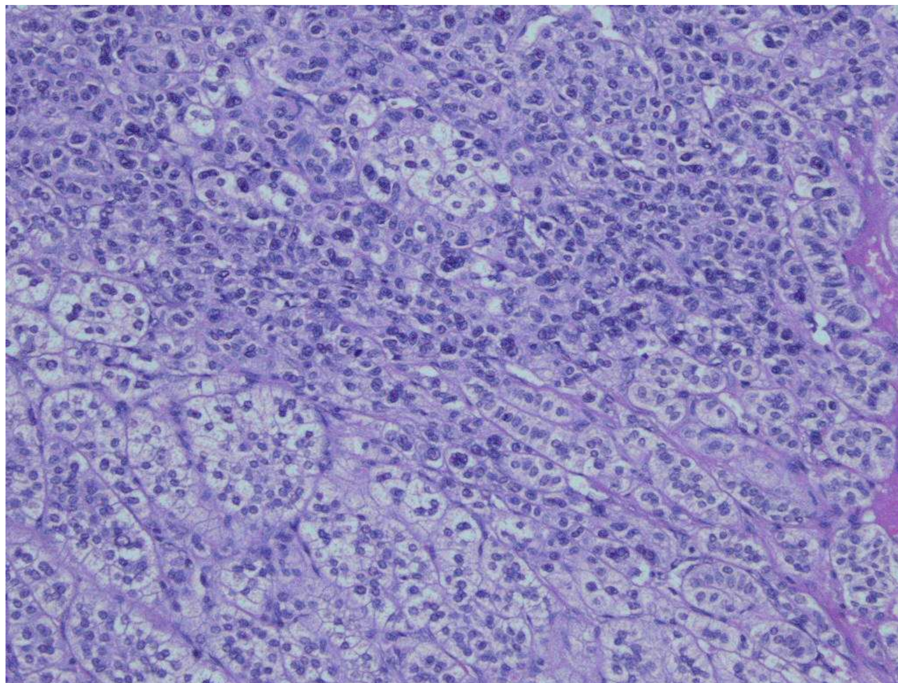


Figure 2.4. Histopathology of AIMAH tumour.
This specimen is from the adrenal tumour of III-1, AIMAH-01. In the lower left quadrant are *clear cells* (lipid-laden, cytoplasm rich) which form cordon nest-like structures, whilst in the upper right quadrant are *compact cells* (devoid of cytoplasm); the two characteristic cell types of AIMAH.

III-2

The proband's brother, aged 61 (III-2, Figure 2.1) self-presented for evaluation of possible CS. He had an 18 year history of hypertension, a 3 year history of depression and a recent 20kg weight gain. He was using nocturnal continuous positive airway pressure for obstructive sleep apnoea and had had bilateral carpal tunnel release. At presentation, he was acromegalic and review of personal photographs indicated this was longstanding (≈ 20 years). The serum insulin-like growth factor 1 was 159nmol/L (RR: 20-65nmol/L). Growth hormone levels did not suppress after a glucose load. Imaging of the pituitary gland revealed a 13mm X 16mm pituitary macroadenoma, which was resected transsphenoidally. He was cured briefly, but subsequently relapsed biochemically. Octreotide was commenced, and he has since achieved biochemical remission.

He had CS due to VPs-AIMAH, with a 105% ACTH-independent increase in plasma cortisol after low-dose VP (Figure 2.2). Bilateral adrenal hypertrophy was evident on adrenal CT scan. CRH stimulation testing confirmed ACTH-independent CS. He underwent a modified three-day aberrant receptor testing protocol (Lacroix *et al.,* 1999b). He had a 45% ACTH-independent increase in cortisol after 10mg oral metoclopramide (data not shown). This replaced the serotonin agonist cisapride, which is no longer available in Australia. There was no cortisol response to upright posture, 1mg intravenous glucagon (Glucagen, Novo Nordisk Pharmaceuticals, Australia), 200µg intravenous thyrotropin releasing hormone (Protirelin, Cambridge Laboratories Limited, United Kingdom), 1µg subcutaneous DDAVP or a mixed meal (all performed supine, fasting and serum ACTH and cortisol sampled half-hourly for two hours after each stimulus) (data not shown). This evaluation was performed whilst he was in biochemical remission of acromegaly post-operatively; octreotide had not been commenced. As a somatostatin analog, this could have abrogated a cortisol response to the mixed-meal. A computed tomography scan revealed bilateral adrenal hypertrophy. Adrenal scintigraphy using ^{131}I-19-iodocholesterol showed bilateral, though asymmetrical, uptake (Figure 2.5).

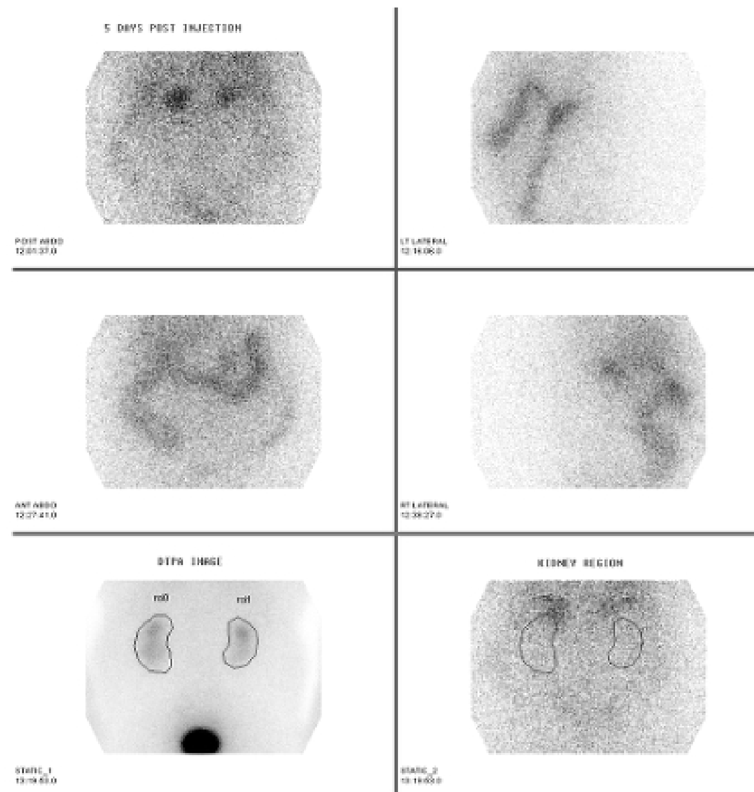


Figure 2.5. Adrenal scintigraphy in III-2 (AIMAH-01).
This was performed using ^{131}I-19-iodocholesterol. Bilateral, although slightly predominant in the right adrenal, uptake is seen in the top left panel.

Anaesthetic and surgical considerations directed he undergo a right adrenalectomy initially. He became eucortisolaemic after laparoscopic resection of a 55gm macronodular right adrenal gland (Figure 2.6). There was an improvement in hypertension and a reduction in weight. After low-dose VP, an aberrant, ACTH-independent 53% increase in plasma cortisol persists (Figure 2.2). Three years postoperatively plasma ACTH remains undetectable. He will have ongoing assessment for early detection of recurrent hypercortisolism.

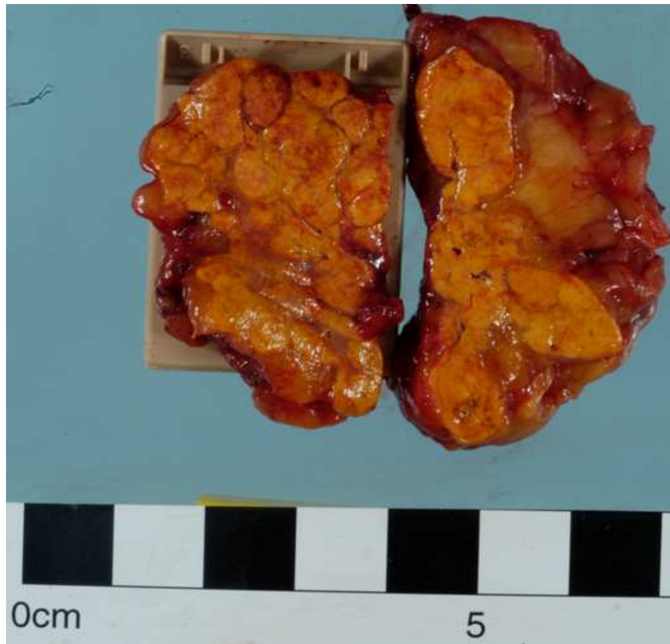


Figure 2.6. Resected macronodular right adrenal gland from III-2 (AIMAH-01).

III-3

The third brother, aged 65 (III-3, Figure 2.1), also self-presented for evaluation of possible CS. He had had central weight gain, poorly controlled hypertension, and memory loss and concentration difficulties. He had a history of osteoporosis and recurrent renal calculi. Incidental bilateral adrenal enlargement had been noted on a CT scan several years before presentation. He had VPs-AIMAH, with a 75% ACTH-independent cortisol increase after low-dose VP (Figure 2.2). CRH stimulation testing confirmed ACTH-independent CS. Adrenal scintigraphy showed right adrenal gland uptake (Figure 2.7), guiding its selection for resection. He underwent a laparoscopic right adrenalectomy of a hyperplastic gland (weight, 50gm) (Figure 2.8). He required hydrocortisone replacement for eight months postoperatively, consistent with relative suppression of the contralateral adrenal gland. Clinical history and early morning cortisol levels guided the duration of replacement. He is now eucortisolaemic, and there has been a sustained improvement in hypertension. Three years postoperatively, plasma ACTH remains undetectable. He will have ongoing surveillance for recurrent hypercortisolism.

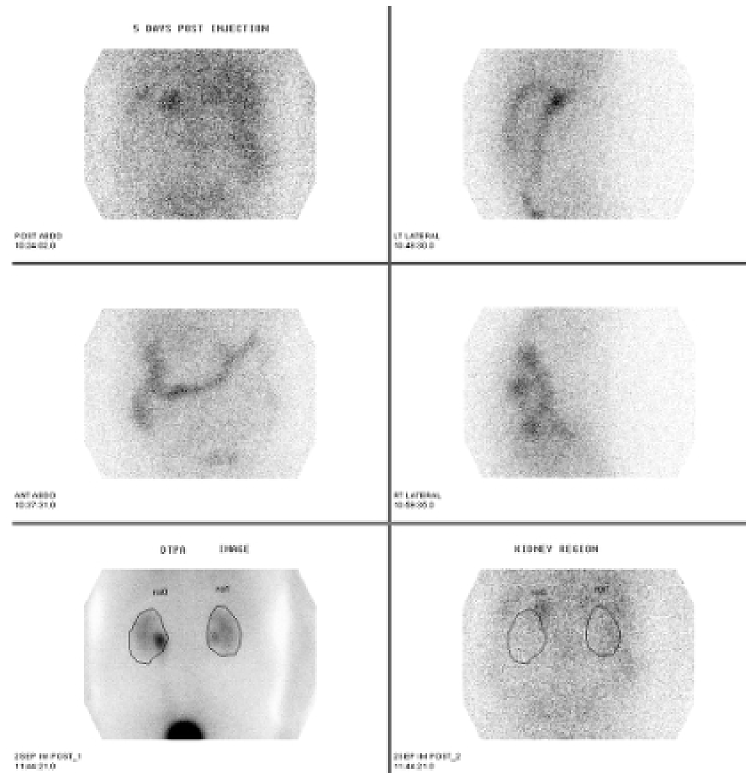


Figure 2.7. Adrenal scintigraphy in III-3 (AIMAH-01).
This was performed using ^{131}I-19-iodocholesterol. Unilateral (right) adrenal uptake is seen in the top left panel.

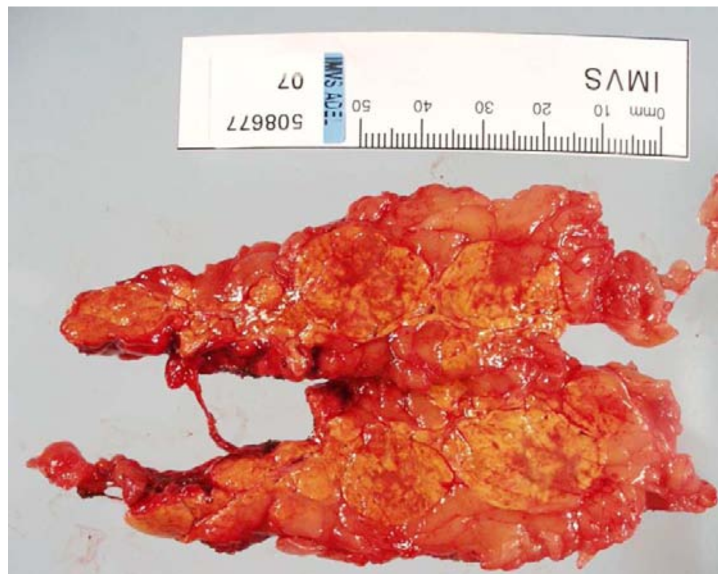


Figure 2.8. Resected macronodular right adrenal gland from III-3 (AIMAH-01).

During post-operative surveillance, he was found to have mild hypercalcaemia due to primary hyperparathyroidism. The biochemistry was: serum total calcium 2.63mmol/L (RR: 2.10-2.55mmol/L), ionised calcium 1.35mmol/L (RR: 1.10-1.25mmol/L), parathyroid hormone (PTH) 4.7pmol/L (RR: 0.8-5.5pmol/L) and vitamin D 125nmol/L (RR: 60-160nmol/L). A parathyroid sestamibi study was normal. A full parathyroid exploration was performed; and he underwent a subtotal (three and a half) parathyroidectomy for parathyroid hyperplasia. In the early postoperative period, PTH was undetectable (< 0.3pmol/L) and he was hypocalcaemic (ionised calcium 1.06mmol/L, total calcium 1.98mmol/L). He received supplemental calcium briefly, and four months later, calcium and PTH were normal.

These latter two cases illustrate the heterogeneity of AIMAH, even amongst related individuals. The aberrant cortisol response to exogenous ligands varied (III-2 also responded to metoclopramide, although neither of his siblings did). At the time of writing this thesis, we do not know whether or not common mechanisms are responsible for the development of pituitary or parathyroid adenomas and AIMAH in these patients. The presentation is reminiscent of, though atypical for, the MEN1 syndrome, since these patients generally present with primary hyperparathyroidism as the first manifestation, and although adrenal tumours (including AIMAH) may occur in 20-40% of patients, they are predominantly nonfunctional (Skogseid *et al.,* 1992; Burgess *et al.,* 1996). Others have also reported thyroid, parathyroid, parotid, pituitary and uterine leiomyomatous tumours in patients with AIMAH and their relatives (Nagai *et al.,* 1999; Hsiao *et al.,* 2009; Sato *et al.,* 2006). Two siblings with familial AIMAH also had meningiomas (Lee *et al.,* 2005). In our studies, we have only pursued investigation of other clinically apparent endocrine tumours, i.e., apart from screening for AIMAH, we have examined each person for clinical signs of other endocrine tumours (e.g., acromegaly, goitre) and investigated further if clinically indicated. All had fasting serum calcium measured.

Adrenocortical expression of VP receptors by AIMAH-01 tumours

Semi-quantitative RT-PCR (Figure 2.9) showed no obvious differences in *AVPR1A* expression between AIMAH-01 and normal adrenal. However, by RT-qPCR analysis there was a 1.5-fold upregulation of *AVPR1A* ($p < 0.002$) in the AIMAH-01 tumours, compared with normal adrenal gland. In both semi- and RT-qPCR, relatively low level (cycle threshold > 30) expression of *AVPR1B* was observed in AIMAH-01 tumours, but not in normal adrenal (Figure 2.9). *AVPR2* was not expressed in the AIMAH-01 tumours or in normal adrenal (Figure 2.9).

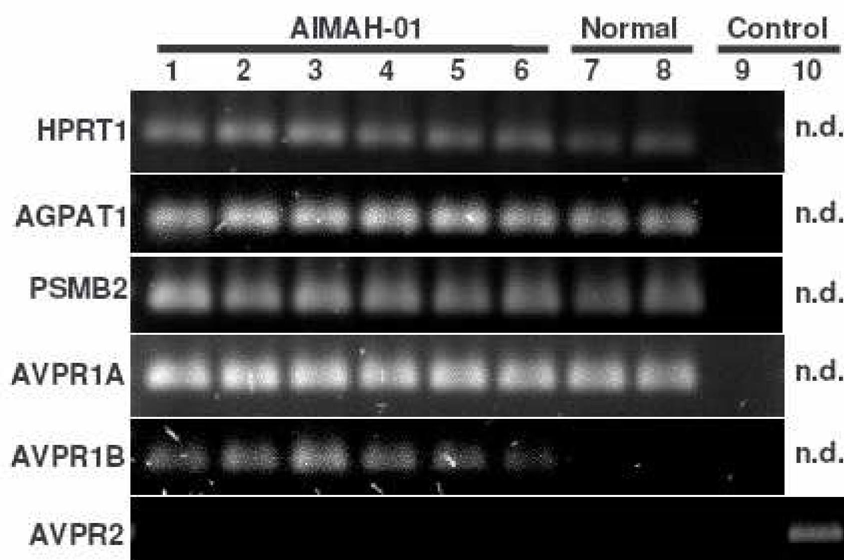


Figure 2.9. Expression of vasopressin receptors by AIMAH-01 adrenal tumours.
VP receptor genes: *AVPR1A, AVPR1B, AVPR2*; Reference genes: *HPRT1*, *AGPAT1*, *PSMB2*.
Lane 1 and 2 – patient III-2; Lane 3 and 4 – patient III-1; Lane 5 and 6 – patient III-3; Lane 7 and 8 – normal adrenal; Lane 9 – RT minus; Lane 10 – human kidney (positive control); n.d. – not determined. Patient ID corresponds to Figure 2.1.
There are no obvious differences in the adrenal (AIMAH-01 and normal samples) expression of housekeeping genes and *AVPR1A*. No bands are observed in the normal samples for *AVPR1B*. None of the adrenal samples expressed *AVPR2*. The expression of *AVPR2* in kidney excludes the possibility of false negative results in *AVPR2* adrenal sample analysis. No bands are observed in RT-minus reactions for all genes, confirming an absence of genomic DNA.

These data suggest that the *in vivo* aberrant cortisol response to VP in the three siblings from AIMAH-01 was mediated by adrenal ectopic expression of AVPR1B and/or overexpression of eutopic AVPR1A. This finding is consistent with the many reports of the aberrant expression of various GPCR, including the VP receptor, detected in AIMAH (Lacroix *et al.,* 2001). Others have shown that the aberrant receptors are coupled to steroidogenesis, such that *in vivo* modulation of circulating levels of the receptor ligand (either by its exogenous administration or physiological stimulation; discussed in Chapter 1) or *in vitro* incubation of AIMAH cells with the receptor ligand, stimulate cortisol production (Lacroix *et al.,* 1992; Reznik *et al.,* 1992; Lacroix *et al.,* 1999a; Goodarzi *et al.,* 2003; Lacroix *et al.,* 1997a; Cartier *et al.,* 2003; Bertherat *et al.,* 2005; de Miguel *et al.,* 2010; Figure 1.4). Furthermore, pretreatment with a receptor antagonist, when available, abrogates the *in vivo* and *in vitro* cortisol increment (Lacroix *et al.,* 1992; Daidoh *et al.,* 1998). Similarly, we postulate that in the AIMAH-01 tumours, the VP receptors have become coupled to steroidogenesis, enabling the adrenal tumours to secrete cortisol in direct response to VP, rather than *via* the usual ACTH-dependent mechanism, discussed in Chapter 1.

Our data do not allow us to determine which of these receptor subtypes is predominant in mediating the VP-sensitive aberrant cortisol response in AIMAH-01. This would require assessment of the *in vitro* cortisol response of cultured AIMAH-01 adrenal cells (not available) to VP administration in the presence and absence of the AVPR1A antagonist, OPC-21268 (Daidoh *et al.,* 1998). Alternatively, pretreatment with the orally administered OPC-21268 prior to VP administration in our patients with VP-sensitive AIMAH, could determine the relative importance of AVPR1A *vs* AVPR1B in mediating the *in vivo* cortisol response.

Phenotyping of Kindred AIMAH-01

The results of phenotyping are shown in Table 2.2 and are summarised in Figure 2.1.

Table 2.2. Phenotyping data of kindred AIMAH-01.

Patient, Age	UFC (50-350nmol/d)	NSC (<13nmol/L)	ACTH (10-60ng/L)	DST (<50nmol/L)	VP stimulation (1 I.U./70kg)			Adrenal imaging
					Result	ACTH	F	
III-1, 69	1386	-	<10	-	Abnormal	↔	↑	Bilateral macronodular adrenals
III-2, 61	263	22	<10	339	Abnormal	↔	↑	Bilateral adrenal hypertrophy
III-3, 65	346	22	<10	501	Abnormal	↔	↑	Bilateral macronodular adrenals
III-4, 60	103	<8	38	10	-			Normal
IV-1, 46	136	8	15	13	Normal	↑	↑	Normal
IV-2, 44	121	9	36	20	-			Normal
IV-3, 42	172	<8	10	21	Normal	↑	↑	Normal
IV-4, 40	-	<8	<10	-	Abnormal	↔	↔	Normal
IV-5, 44	278	<8	16	14	Normal	↑	↑	Normal
IV-6, 42	190	9	<10	60	Abnormal	↔	↔	Normal
V-1, 17	121	<8	<10	15	Abnormal	↓	↔	L adrenal thickening
V-2, 24	-	<8	47	-	Abnormal	↔	↔	Normal
V-3, 22	-	<8	35	-	Normal	↑	↑	Normal
V-4, 15	23	-	22	11	-			Normal
V-5, 18	-	<8	-	-	-			Normal
V-6, 19	-	-	-	-	-			Normal
V-7, 9	-	<8	13	13	-			-
V-8, 13	-	<8	11	8	-			Normal
V-10, 8	-	<8	-	-	-			Normal
V-11, 17	151	<8	44	11	Normal	↑	↑	Normal
V-12, 19	132	-	20	18	Normal	↑	↔	Normal
V-13, 11	-	<8	11	<8	-			Normal

ACTH – morning plasma ACTH; DST – dexamethasone suppression test – result listed is 0900h cortisol after 1mg dexamethasone at 2300h night prior; F – cortisol; NSC – nocturnal salivary cortisol (for children, sample collected at bedtime); UFC – urinary free cortisol; VP (vasopressin) stimulation – increase↑, decrease↓, no change ↔ in measured hormone; "-" result not available

VP testing was well-tolerated, with only transient symptoms, including nausea and pallor, experienced. No-one had clinical features of CS or, apart from III-4 (Figure 2.1; discussed later), any other endocrine disorder at the time of evaluation. There were four other family members who had biochemical abnormalities (suppressed ACTH and absent or abnormal VP response). We believe that subtle biochemical abnormalities such as these precede the onset of CS by many years. In addition, one person (V-1, Figure 2.1) also had a left adrenal nodule. Adrenal incidentalomas are uncommon in her age group (late teenage years) and this nodule could represent the beginning of generalised adrenal nodularity (Kloos *et al.,* 1995). Although AIMAH affects the adrenals bilaterally, nodularity may progress asynchronously (N'Diaye *et al.,* 1999). Whether the subtle biochemical abnormalities truly are markers of preclinical disease will only be determined by discovery of the causative gene and/or longitudinal surveillance of these individuals.

III-4 (Figure 2.1) was phenotypically normal on screening for AIMAH. However, he was known to have had a large retrosternal multinodular goitre for many years, but had recently developed clinical and radiological evidence of airway obstruction, and is awaiting thyroidectomy. His thyroid function tests have been normal (TSH 0.89mIU/L; Free T4 13pmol/L). He has a sister, who has not presented for screening for AIMAH, but who also had a thyroidectomy for goitre. Whilst goitre is not uncommon in the general population, we can not exclude that, in these individuals, thyroid tumorigenesis is a non-adrenal manifestation of the AIMAH phenotype. It is currently not known whether there are common underlying mechanisms predisposing individuals in this family to various endocrine tumours. Other factors, such as secondary mutations, or environmental or epigenetic factors, could determine which tumours develop or become clinically apparent.

The phenotyping data suggest familial AIMAH in AIMAH-01 is autosomal dominantly inherited with reduced penetrance. Reduced penetrance is suggested because IV-4 (Figure

2.1) has two children (V-1, V-2; Figure 2.1) who have preclinical manifestations of AIMAH, and is therefore an obligate carrier of the AIMAH disease allele, but she herself is phenotypically normal. The data suggest that preclinical forms of AIMAH can be detected with biochemical and adrenal imaging investigations. Careful surveillance will afford these individuals the opportunity for timely treatment of CS.

If AIMAH is autosomal dominantly inherited in AIMAH-01, then we would expect one parent of the three affected siblings to have been affected. Apparently, neither parent had clinical features of CS. Their mother died aged 83. Her certified cause of death was a cerebrovascular accident, on a background of hypertension. Photographs were not available for review. Their father did not have clinical features of CS, despite living well into his 8th decade (photographs reviewed). Alternatively, if we consider that the only "definitely affected" individuals are the three siblings from whom we have diagnostic histopathology, then a recessive disorder is not excluded, although there is no known history of consanguinity. In such a case, the phenotype of individuals IV-4, IV-6, V-1 and V-2 (Figure 2.1) may be a "carrier phenotype". An example of this is autosomal recessive hypophosphataemia, where homozygotes have skeletal dysplasia and hypophosphataemia, whilst heterozygotes have osteomalacia and milder hypophosphataemia (Mäikitie *et al.,* 2010). Another example is reported in a family with Charcot-Marie Tooth (CMT) neuropathy in which compound heterozygotes of two mutations in the SH3 domain and tetratricopeptide repeats 2 gene (*SH3TC2*) manifest CMT, whereas individuals harbouring only one disease allele have mild mononeuropathy of the median nerve or other axonal neuropathy (Lupski *et al.,* 2010).

Clinical presentation of the proband of kindred AIMAH-02

The proband (II-1, Figure 2.10) presented, aged 75, with weight gain, hypertension and cognitive difficulties. He had advanced CS due to AIMAH. He had an ACTH-independent 47% cortisol increase after VP (Figure 2.11).

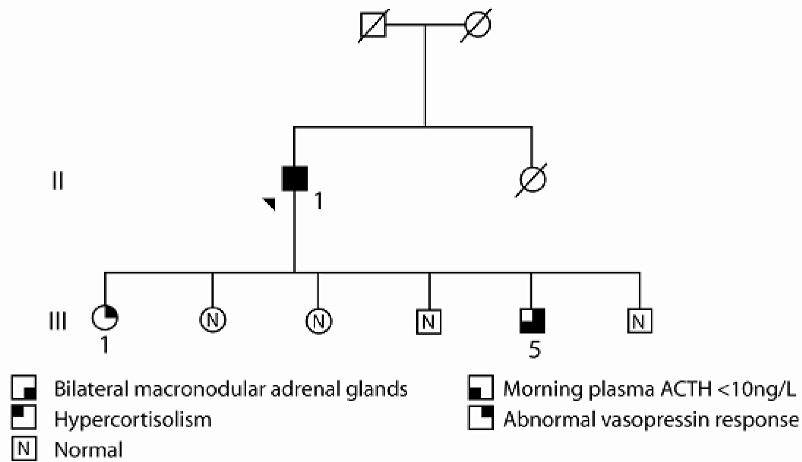


Figure 2.10. Pedigree of kindred AIMAH-02.

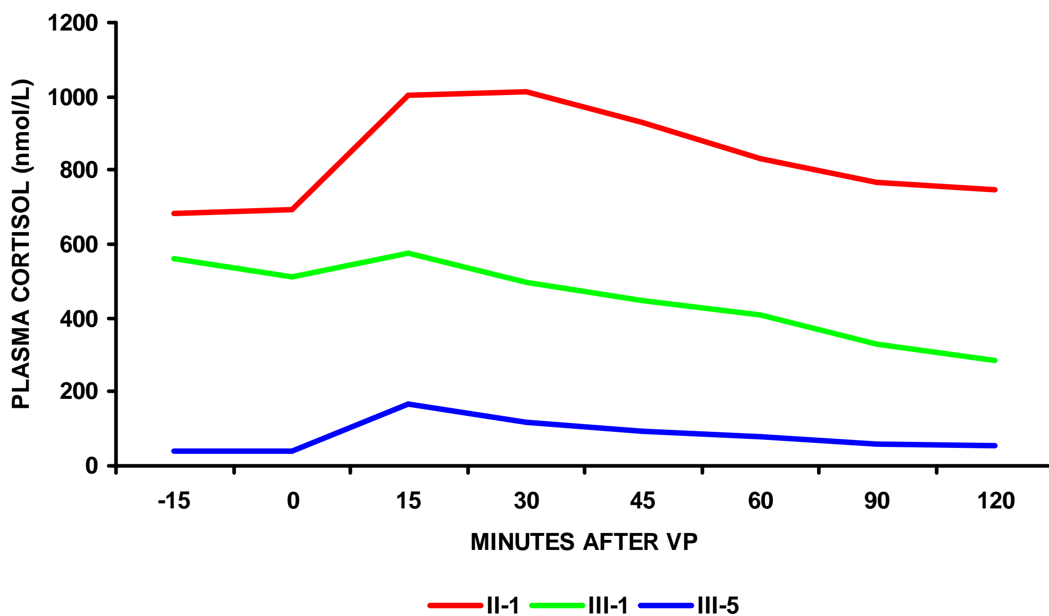


Figure 2.11. The aberrant cortisol response to vasopressin in AIMAH-02.
The data for II-1, III-1, III-5 (Figure 2.10) are shown.
Vasopressin was given at time=0. ACTH remained suppressed (< 10ng/L) throughout in II-1, and in III-1, ACTH and cortisol remained unchanged. III-5 was given 2mg of dexamethasone at midnight prior to the test and 1mg immediately prior to vasopressin administration. ACTH remained suppressed throughout.

Too frail for surgery, ketoconazole 200mg daily was commenced, he became eucortisolaemic, and to my knowledge, has remained so, three years later. With knowledge of the AIMAH-01 kindred, the treating clinician referred the proband's six adult children for evaluation. At the time of preliminary discussions with the family, we informed them that their father may have had sporadic AIMAH, in which case familial screening would not be useful. They remained keen to proceed with evaluation.

Phenotyping of kindred AIMAH-02

We phenotyped the proband's six adult children. Clinical history and examination excluded CS and other endocrinopathy in all. The results of phenotyping are shown in Figure 2.10 and Table 2.3. VP stimulation testing was well tolerated.

Individual III-1 (Figure 2.10), aged 44, had an absent VP response, but was otherwise clinically and biochemically eucortisolaemic. Although her initial thyroid examination was normal, she subsequently had a right hemithyroidectomy for a large (29 X 24 X 17mm) thyroid nodule which was diagnosed after she became acutely aware of a painful neck lump. Macrofollicular nodular hyperplasia was observed on histological examination of the surgical specimen. This is fascinating since, histologically, this is not dissimilar to the hyperplasia of AIMAH, and may be a nonadrenal manifestation of the AIMAH phenotype.

Individual III-5 (Figure 2.10), aged 49, also had no clinical features of CS. However, he had a suppressed plasma ACTH, bilateral macronodular adrenal glands and plasma cortisol did not suppress to dexamethasone. Under HPA axis suppression (dexamethasone 2mg at midnight, and 1mg immediately prior to VP), he had a 340% ACTH-independent cortisol increase (Figure 2.11). Thus, he also had VPs-AIMAH, with subtle biochemical abnormalities of the HPA axis, but did not have CS. Segregation analysis in this family is consistent with autosomal dominant inheritance. Furthermore the data suggest that familial AIMAH may

masquerade as sporadic disease – since in the absence of screening, III-5 would not have been recognised as having early AIMAH. The clinical implication of these data is that in apparently sporadic cases, familial disease may have been undiagnosed.

Personal photographs of the proband's parents were reviewed - neither appeared to have CS. His sister had died aged in her 40s, from metastatic breast cancer.

Table 2.3. Phenotyping data of kindred AIMAH-02.

Patient, Age	UFC (50-350nmol/d)	NSC (<13nmol/L)	ACTH (10-60ng/L)	DST (<50nmol/L)	VP stimulation (1 I.U./70kg)			Adrenal imaging
					Result	ACTH	F	
II-1, 75	835	-	<10	-	Abnormal	↔	↑	Bilateral macronodular adrenals
III-1, 44	132	<8	21	33	Abnormal	↔	↔	Normal
III-2, 51	75	<8	12	28	Normal	↑	↑	Normal
III-3, 38	91	<8	10	40	-			Normal
III-4, 42	145	<8	26	14	Normal	↑	↑	Normal
III-5, 49	143	9	<10	88	Abnormal	↔	↑	Bilateral macronodular adrenals
III-6, 47	150	<8	20	18	Normal	↑	↑	Normal

ACTH – morning plasma ACTH; DST – dexamethasone suppression test – result listed is 0900h cortisol after 1mg dexamethasone at 2300h night prior; F – cortisol; NSC – nocturnal salivary cortisol (for children, sample collected at bedtime); UFC – urinary free cortisol; VP (vasopressin) stimulation – increase↑, decrease↓, no change ↔ in measured hormone; "-" result not available

Clinical presentation of Cushing's syndrome in kindred AIMAH-03

III-2

The proband (III-2, Figure 2.12), aged 46, was referred for assessment of incidentally discovered bilateral macronodular adrenal glands. He had a history of poorly controlled hypertension, ischaemic heart disease, alcohol-induced chronic pancreatitis and previous distal pancreatectomy and an ischaemic and alcohol-related cardiomyopathy. In the years prior to presentation, he had had weight gain and depression. On examination, he was overweight, hypertensive, had facial fullness and plethora, and prominent dorsocervical fat pads and purple abdominal striae. He had ACTH-independent CS due to AIMAH (Table 2.4). Due to his comorbidities, metyrapone was commenced and surgery deferred. He ultimately underwent a right adrenalectomy; surgical considerations guided selection of the adrenal. The resected macronodular adrenal gland weighed 119gm. The postoperative course was complicated by sepsis. Urinary free cortisol remained elevated and metyrapone was continued. He died one year later from cardiac failure.

III-3

The proband's brother (III-3, Figure 2.12), aged 45, was referred for assessment of bilateral adrenal incidentalomas. He had longstanding treatment-resistant hypertension. When reviewed, he was Cushingoid and had truncal adiposity, facial fullness and dorsocervical fat pads. On preoperative adrenal imaging, the right adrenal gland was larger; guiding selection of that gland for resection (weight, 95gm). Postoperatively, he became eucortisolaemic; hypertension improved, and over several months, there was amelioration of his Cushingoid body habitus. Plasma ACTH remained suppressed, suggesting subtle, autonomous hyperfunction of the remaining adrenal. At his most recent review, five years after adrenalectomy, he was again Cushingoid, and UFC and NSC were elevated, 436nmol/day, and 14.2nmol/L, respectively, consistent with recurrent CS. He has been offered a second adrenalectomy.

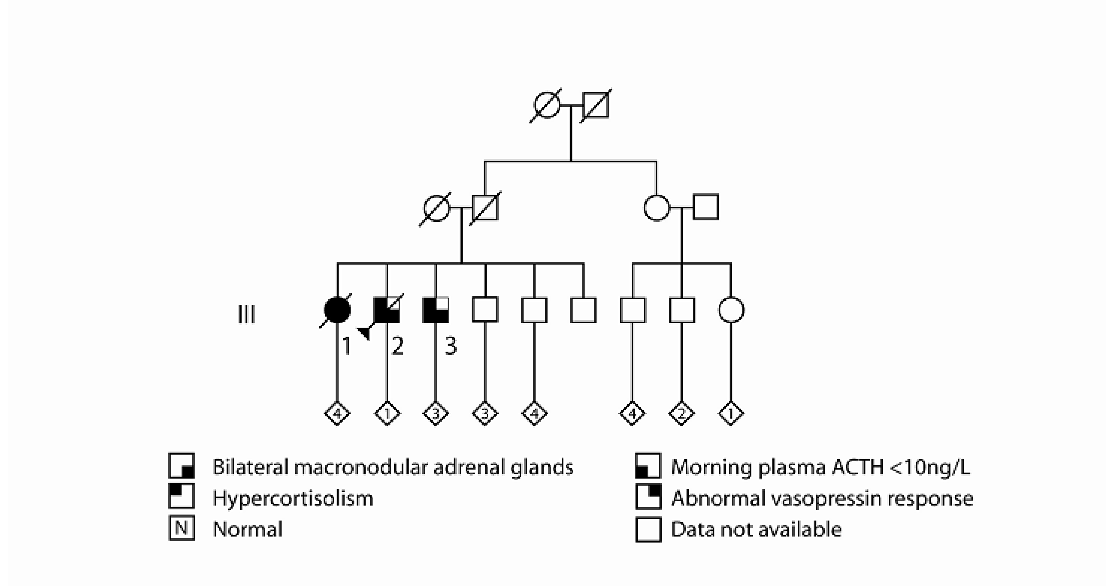


Figure 2.12. Pedigree of kindred AIMAH-03.

III-1

This sibling (III-1, Figure 2.12) was referred with an 18 month history of hypertension, a 6 month history of increasing fatigue and generalised weakness and central weight gain. She also had a bruising tendency, thinning of her skin, cutaneous infections and difficulty standing from the seated position. She was Cushingoid and had facial plethora, hypertension, dorsocervical fat pads, centripetal obesity, purple abdominal striae and mild proximal lower limb weakness. She had ACTH-independent CS due to AIMAH (Table 2.4). She underwent a modified three-day aberrant receptor screening protocol (Lacroix *et al.,* 1999b). There was no cortisol response to posture, a mixed-meal, GnRH, protirelin, glucagon or metoclopramide. After 10 I.U. VP IM, there was a 97% increase in serum cortisol (Figure 2.13). She was commenced on metyrapone whilst awaiting adrenalectomy. She was selected for a unilateral adrenalectomy, given its success in her sibling, III-3. Since the adrenal glands were symmetrically enlarged, surgical considerations guided selection of the left adrenal for resection (weight 50gm). Postoperatively, she became eucortisolaemic and both her weight and hypertension improved. At her last review, three years postoperatively, ACTH remained suppressed, consistent with subtle autonomous hyperfunction of the remaining adrenal,

although there were no features to suggest recurrent CS. She has since died unexpectedly from a large cerebrovascular accident.

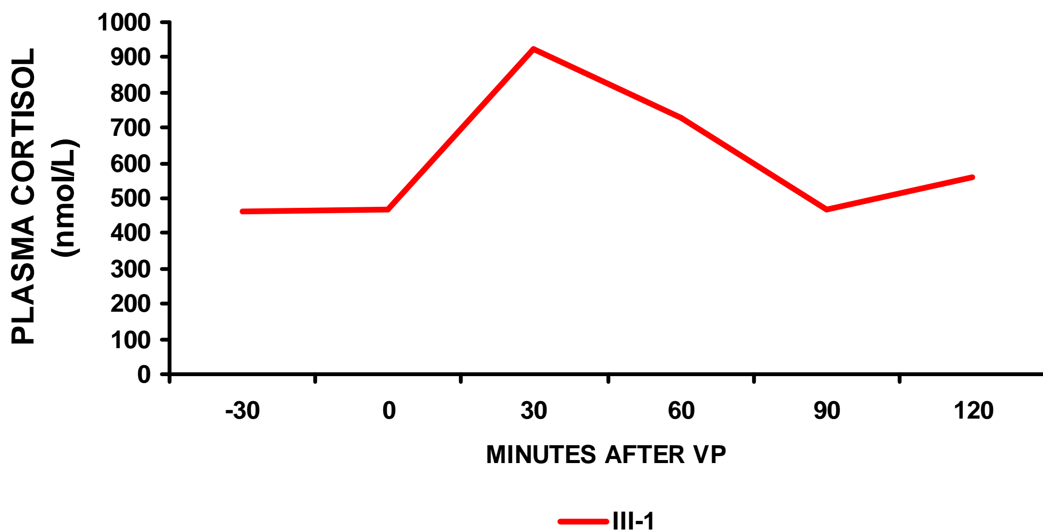


Figure 2.13. The aberrant cortisol response to vasopressin in AIMAH-03.
The data for III-1 (Figure 2.12) are shown. Vasopressin was given at time=0. ACTH remained undetectable throughout.

Phenotyping of kindred AIMAH-03

This was performed by Dr Cheri Hotu, Auckland General Hospital. The data are summarised in Table 2.4. There were no other affected individuals. Although their paternal aunt has an adrenal adenoma, this is likely to be an incidentaloma, since these are common in her age group, having a prevalence of at least 3% in those aged over 50 (NIH Consensus Statement, 2002; Kloos *et al.,* 1995). Furthermore, the clinical presentation of AIMAH in this kindred has been in the 5th decade; and since she was aged 76 at the time of evaluation, we would have expected her to have had other disease manifestations. Clinical details regarding the parents of the siblings were not available.

Table 2.4. Phenotyping data of kindred AIMAH-03

Patient, Age	UFC (100-300nmol/d)	ACTH (2-11pmol/L)	DST (<50nmol/L)	VP stimulation 10 I.U. IM			Adrenal imaging
				Result	ACTH	F	
III-1, 46	435	<1.1	650	Abnormal	↔	↑	Bilateral macronodular adrenals
III-2, 46	1045	<1.1	673	-			Bilateral macronodular adrenals
III-3, 45	360	<1.5	650	-			Bilateral macronodular adrenals
III-4, 51	176	3.97	-	-			Normal
III-5, 49	171	5.06	-	-			Normal
III-6, 38	125	4.4	-	-			Normal
II-2, 76	71	2.5	-	-			1.5cm left adrenal adenoma

ACTH – morning plasma ACTH; DST – dexamethasone suppression test – result listed is 0900h cortisol after 1mg dexamethasone at 2300h night prior; F – cortisol; UFC – urinary free cortisol; VP (vasopressin) stimulation – increase↑, decrease↓, no change ↔ in measured hormone; "-" result not available

The onset of CS in AIMAH-03 occurs in the 5th decade which is much earlier than in AIMAH-01 and -02, which to date has occurred in the 7th and 8th decades, respectively, further exemplifying the clinical heterogeneity of AIMAH. Why this might be remains speculative, but since AIMAH cells produce cortisol inefficiently (discussed in Chapter 3), the earlier clinical onset of CS in AIMAH-03 could suggest more rapid adrenocortical cell proliferation, or less inefficient steroidogenesis, in AIMAH-03.

Histopathology of AIMAH Tumours

The AIMAH-01 (III-1, III-2, III-3) and AIMAH-03 (III-1, III-3) adrenal nodules were comprised of clear cells and compact cells (Figure 2.4). The histology of AIMAH tumours can be broadly classified based on the characteristics of the internodular cortical tissue: (1) Type 1 - internodular cortical atrophy and (2) Type 2 - internodular hyperplasia (Stratakis and Boikos, 2007). The basis for the two histological subtypes is uncertain since they do not seem to be associated with any particular aberrant receptor response or clinical features; however it has been proposed that they might represent different genetic or paracrine influences on the adrenal (Stratakis and Boikos, 2007).

All the familial AIMAH tumours were of type 2 histology. This histology could be the hallmark of familial disease – with diffuse hyperplasia of nodular and internodular cortex reflecting the presence of the genetic (germline) mutation from the commencement of adrenal organogenesis. We propose that the type 2 histology is further evidence of the familial nature of this disease in these kindreds, since it suggests that the causative genetic mutation is present from the commencement of adrenal development, such that the adrenal glands are diffusely hyperplastic, with areas where, perhaps due to secondary mutations, discrete nodularity has developed. In contrast, however, in another study, three familial AIMAH tumours and two sporadic AIMAH tumours with germline mutations (menin, adenomatous polyposis coli) were all of type 1 histology (Hsiao *et al.,* 2009).

Alternatively, the two histologies could represent different stages of disease – e.g., type 2 histology could occur in early disease and type 1 in advanced disease – where the formerly hyperplastic internodular tissue has become compressed by expanding adrenal nodules. The histology of our AIMAH cases disputes this theory, however, since several individuals (III-1 – AIMAH-01 and III-1, III-3 – AIMAH-03) had advanced AIMAH and type 2 histology.

2.4 Conclusion

In summary, these data suggest that AIMAH is inherited as a Mendelian disorder in AIMAH-01, -02 and -03, and that preclinical AIMAH exists and can be detected with biochemical and adrenal imaging studies. Screening the adult children of an apparently sporadic case detected one case of preclinical AIMAH. Although AIMAH has been largely regarded a sporadic disease, we suspect that in the absence of familial screening, subtle familial forms have remained undiagnosed. Subtle manifestations of CS such as obesity, hypertension and T2DM may incorrectly be attributed to the metabolic syndrome. Each proband from the three families reported here was diagnosed with advanced CS, illustrating the difficulties associated with the diagnosis of CS in general, but particularly in AIMAH, due to the insidious onset of signs and symptoms. In these and other families with AIMAH, screening of kindred members affords them the opportunity to have preclinical disease detected, allowing careful surveillance for, and the opportunity for early treatment of, CS. Tentative recommendations for screening have been made, although a consensus opinion has not been reached (Lacroix, 2009). The optimal frequency of screening and age at which to commence screening is not known. We propose that screening should have commenced in kindred members by at least 10 years before the presentation of CS in their family member. Screening could be repeated every five years, providing the investigations are normal; more frequent (perhaps annual) reassessment may benefit those with biochemical or adrenal imaging abnormalities. Discovery of the genetic basis of familial AIMAH would assist in the clinical care of these families because those who do not have the disease allele could be appropriately excluded

from surveillance; enabling care to be directed to those who carry the disease allele. Discovery of the genetic basis of familial AIMAH would also enrich our understanding of adrenal tumorigenesis in AIMAH, with the potential to inform our knowledge of the pathogenesis of adrenocortical tumours more generally.

Having phenotyped these three families as far as was practically possible, we performed a linkage study, with the intent of identifying chromosomal regions (loci) potentially harbouring the disease allele. The data will be presented in Chapter 5. As already discussed, an intriguing aspect of the physiology of AIMAH tumours is the inefficiency with which cortisol is produced due to deficiencies and differential cellular localisation of steroidogenic enzymes; we tried to better characterise these deficiencies by profiling basal and ACTH-stimulated steroid hormones and their intermediates in two patients from AIMAH-01. These data are presented in the next chapter.

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

3.1 Introduction

Inefficient steroidogenesis is a unique feature of ACTH-independent macronodular adrenal hyperplasia (AIMAH) tumours, not observed in other adrenocortical tumours, and accounting for the discordance between the massive macronodular adrenal glands and the relatively mild hypercortisolism of AIMAH. This is in stark contrast to the hypercortisolism associated with the micronodular adrenal glands of primary pigmented nodular adrenocortical disease (PPNAD) or to the florid Cushing's syndrome that develops due to a relatively small adrenocortical adenoma. Immunohistochemical and enzyme immunoreactivity studies in AIMAH have identified a distinctive pattern of enzyme expression: exclusive expression of 3β-hydroxysteroid dehydrogenase (3βHSD) in clear cells and predominant expression of 17α-hydroxylase (CYP17A1) in compact cells, rendering these cells co-dependent for steroidogenesis, since progesterone produced by clear cells must reach compact cells for cortisol production (Figure 3.1) (Sasano *et al.,* 1994; Wada *et al.,* 1996; Aiba *et al.,* 1991). The differential cellular localization of steroidogenic enzymes may account for the discordance between the massive adrenal glands and the relatively mild adrenocortical hormone hypersecretion. Thus, hormone hypersecretion is the result of a massively increased total adrenocortical cell mass, rather than augmented steroidogenesis per cell. Expression patterns of other steroidogenic enzymes have been inconsistent, but include reduced 21-hydroxylase and CYP11A1 (Sasano *et al.,* 1994; Koizumi *et al.,* 1994; Wada *et al.,* 1996; Morioka *et al.,* 1997).

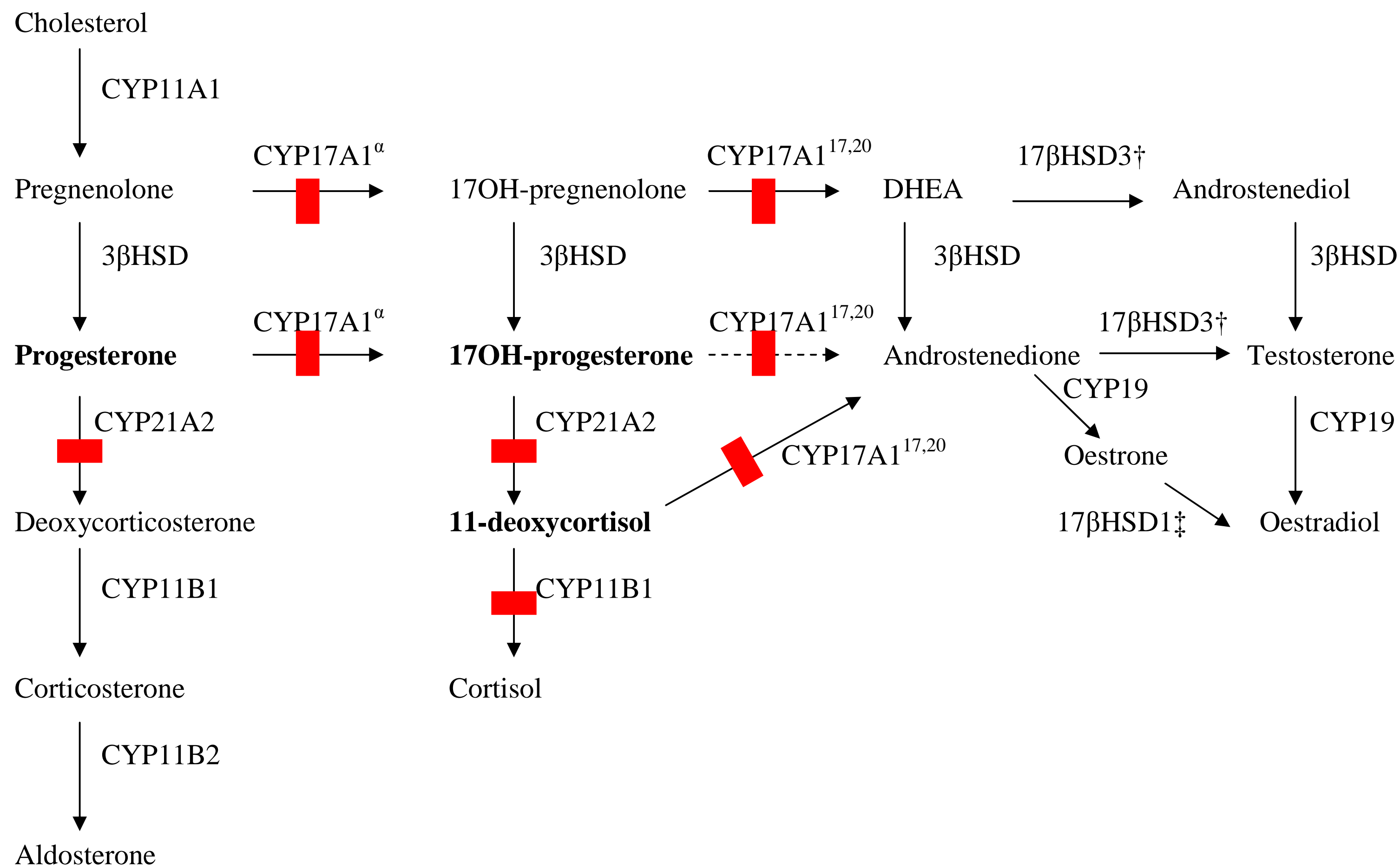


Figure 3.1. The steroidogenic pathway showing the enzyme defects ( ) in AIMAH-01, and elevated steroid intermediates (in bold) as suggested by measurement of basal and ACTH-stimulated adrenocortical hormones and steroid intermediates. CYP11A1 – cholesterol side chain cleavage enzyme; CYP11B1 - 11β-hydroxylase; CYP11B2 – aldosterone synthase; CYP17A1$^{\alpha}$ - 17α-hydroxylase of CYP17; CYP17A1^{17,20} – 17,20 lyase activity of CYP17; CYP19 – aromatase; CYP21A2 – 21-hydroxylase; DHEA - dehydroepiandrosterone; 3βHSD - 3β-hydroxysteroid dehydrogenase; 17βHSD1,3 - 17β-hydroxysteroid dehydrogenase 1,3; *insignificant amounts of 17OH-progesterone are converted to androstenedione in humans; †testis; ‡placenta, ovary

In patients with AIMAH, exogenous administration of various hormones, or physiological modulation of their endogenous levels have been shown, both *in vivo* and *in vitro*, to stimulate cortisol production (Lacroix *et al.,* 1992; Horiba *et al.,* 1995; Lacroix *et al.,* 1997a; Lacroix *et al.,* 1997b; Cartier *et al.,* 2003; Tatsuno *et al.,* 2004). This is due to the cellular expression of illegitimate or aberrant G-protein coupled receptors that have become coupled to steroidogenesis (Lacroix *et al.,* 1997a; Lacroix *et al.,* 1997b; Mune *et al.,* 2002; Cartier *et al.,* 2003; Tatsuno *et al.,* 2004; de Miguel *et al.,* 2010). However, the cortisol response to synthetic ACTH (Synacthen®) is generally more robust (as defined by the percentage increase in cortisol from baseline) than any aberrant response (Lacroix *et al.,* 1999; Lacroix *et al.,* 1997b; Bourdeau *et al.,* 2001; Cartier *et al.,* 2003; Lee *et al.,* 2005). This is despite reduced expression of the ACTH receptor on AIMAH cells compared with normal adrenals (Antonini *et al.,* 2006; Assie *et al.,* 2010). Although the expression of the ACTH and aberrant receptors has not been directly compared, the cortisol responses may reflect the relative abundance of the receptors; alternatively, the aberrant, illegitimate receptors which do not normally regulate steroidogenesis, may be less efficiently coupled to the steroidogenic pathway than the ACTH receptor, which is involved in the physiological regulation of cortisol secretion.

Macronodular adrenal hyperplasia develops in longstanding (ACTH-dependent) Cushing's disease, and it has been proposed that AIMAH may be a form of tertiary hyperadrenalism, where autonomous adrenal hyperfunction and pituitary suppression have developed after longstanding ACTH stimulation (Hermus *et al.,* 1988). However, as discussed in Chapter 1, there are no data in AIMAH to substantiate this postulate. The mechanisms underlying differential steroidogenic enzyme expression in AIMAH are poorly understood. Moreover, that the differential expression is unique to AIMAH suggests that these steroidogenic alterations might be a primary defect, rather than a by-product of a dedifferentiated tumour cell. Early in the pathogenesis of AIMAH, a non-hyperplastic adrenal gland with cellular steroidogenic enzyme defects might produce sufficiently low cortisol to diminish

glucocorticoid negative feedback, resulting in stimulation of pituitary ACTH secretion, and hence adrenocortical cell proliferation. Ultimately, the acquisition of a sufficiently increased cell mass could result in hypercortisolism and ACTH suppression.

The aim of this study was to identify the enzyme deficiencies that could account for inefficient steroidogenesis in two affected individuals (III-2, III-3; Figure 2.1) from the first AIMAH kindred, AIMAH-01, by measurement of adrenal steroid hormones and their intermediates before and after Synacthen® administration. There are few published data of steroid intermediate concentrations in this patient group (Wada *et al.,* 1996). We hypothesised that measurement of plasma steroid intermediates might have clinical utility in the diagnosis of AIMAH, for which specific diagnostic tests are lacking. Congenital adrenal hyperplasia (CAH) is an inherited syndrome caused by various steroidogenic enzyme defects and characterised by elevated ACTH, adrenal hyperplasia, elevated steroid intermediates and androgens, and a corresponding phenotype. The strategy of measuring ACTH-stimulated steroid intermediates in CAH is diagnostically reliable, even relative to genetic testing for 21-hydroxylase, 11β-hydroxylase and 17α-hydroxylase deficiencies (Stewart, 2003).

We chose to perform an *in vivo* study, since this would provide data integrating all steroidogenic alterations in AIMAH, allowing us to detect those which were evident at a glandular level and thus might be clinically relevant defects. In contrast, an *in vitro* study, (which we also performed; Chapter 4) would have allowed us to study partitioned elements of cellular function (e.g., gene or protein expression).

3.2 Research Methods

Individuals III-2 and III-3 (AIMAH-01; Figure 2.1) underwent a standard (250μg) short synacthen test (Synacthen®, tetracosactrin, ACTH(1-24), Novartis Pharmaceuticals Pty Ltd, Australia) with measurement of cortisol, aldosterone, dehydroepiandrosterone (DHEA), and

steroid intermediates (androstenedione, 17-hydroxyprogesterone, progesterone, pregnenolone, 17-hydroxypregnenolone, 11-deoxycorticosterone and corticosterone) at baseline, and 30 and 60 minutes after administration of Synacthen®. This study was performed 18 months after single adrenalectomy in each patient. Both were eucortisolaemic, based on normal 24 hour urinary free cortisol, although ACTH suppression persisted.

For all steroid hormones (except aldosterone and corticosterone) and the intermediates measured, comparison was made with historical control data (*n*=17; 6 males; age 18-36 years) (Munabi *et al.,* 1986). The control data for aldosterone and corticosterone were derived from evaluation of six (males; age not stated) and 10 normal adults (gender and age not stated), respectively (Raff and Findling, 1990; Nishida *et al.,* 1977). We also compared the enzyme defects suggested by measurement of steroid intermediates with the enzyme deficiencies suggested by microarray study of the AIMAH tumours from these patients. These data will be presented in Chapter 4.

Assays

Cortisol

The serum cortisol assay has been described in Chapter 2.

Aldosterone

Serum aldosterone was measured using the Coat-A–Count® solid-phase ^{125}I radioimmunoassay (Siemens Medical Solutions Diagnostics, United States). The CV for the assay was 3.4% at a mean of 243pg/mL.

DHEA Sulphate

DHEAS was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay (IMMULIT®2000 DHEA-SO_4, Siemens Medical Solutions Diagnostics, United

States). The CV for the assay was 9.8% at 52µg/dL. The product information reported less than 0.2% cross-reactivity with other steroids.

Progesterone

Progesterone was measured using the ARCHITECT Progesterone assay (chemiluminescent microparticle immunoassay, Abbott Laboratories, United States). The product information reported the following cross-reactivities: corticosterone (4.6%), 17-hydroxyprogesterone (2.9%) and 11-deoxycorticosterone (1.8%).

17-hydroxyprogesterone

17-hydroxyprogesterone was measured using the Coat-A-Count® solid-phase ^{125}I radioimmunoassay (Siemens Medical Solutions Diagnostics). The CV for the assay was 8.9% at 3.7nmol/L and 7% at 14.7nmol/L. The product information reported the following cross-reactivities: 11-deoxycortisol (2.1%), 17-hydroxypregnenolone (3.2%) and 17-hydroxypregnenolone sulphate (3.8%).

Androstenedione

Androstenedione was measured using the Immulite®2000 Androstenedione, a solid-phase, competitive chemiluminescent enzyme immunoassay (Siemens Medical Solutions Diagnostics). The CV for the assay was 4.1% at 3.63ng/ml. The product information reported cross-reactivity with testosterone (1.4%).

Other steroid precursors

Pregnenolone, 17-hydroxypregnenolone, 11-deoxycortisol and corticosterone were performed by Quest Diagnostics (San Juan Capistrano, California, United States) using a commercially available liquid chromatography tandem mass spectrometry method. There was no cross-reactivity with other human steroids (personal communication, Quest Diagnostics).

3.3 Results and Discussion

The steroid hormone and intermediate concentrations before and after Synacthen® are shown in Table 3.1. The historical control data are presented as mean ± 2 standard deviations. Basal and stimulated cortisol production was normal, suggesting that any steroidogenic enzyme deficiencies are relatively compensated for by the increased adrenocortical cell mass so that, overall, cortisol production is maintained.

Aldosterone levels were lower than in published controls, suggesting a defect in aldosterone synthesis due to reduced aldosterone synthase (CYP11B2) expression (Raff and Findling, 1990). The low aldosterone levels were unexpected because there had been no prior clinical or biochemical evidence of aldosterone deficiency. Although clinically manifest aldosterone deficiency might have been circumvented by steroid precursors with mineralocorticoid activity (e.g., deoxycorticosterone; not measured in our patients); both patients had had normal aldosterone levels at initial evaluation (III-2 – 180pmol/L; III-3 – 200pmol/L), i.e., there had never been suggestion, biochemically, of aldosterone deficiency.

Medication-induced changes in aldosterone levels may be another explanation for the low aldosterone levels observed in the participants during this study. III-2 was taking lisinopril and III-3 was taking irbesartan – both of which may decrease aldosterone levels (Funder *et al.,* 2008). Also, preceding the study, neither individual had been prescribed a specific daily sodium intake; a relatively high sodium intake prior to evaluation may have reduced aldosterone levels (Funder *et al.,* 2008). In retrospect, measurement of renin would have been useful, since this is an important regulator of plasma aldosterone.

The aldosterone control data used were validated by another group who independently derived similar aldosterone levels after Synacthen® (*n*=14; males; age 21-27 years) (Kigoshi *et al.,* 1980). ACTH and aldosterone levels after Synacthen® are poorly correlated, suggesting that

Table 3.1. Basal and ACTH-stimulated (Synacthen®) steroids and steroid intermediate concentrations in III-2 and III-3 (AIMAH-01).

250µg Synacthen® was administered intravenously at time = 0.

Steroid/Intermediate	III-2	III-3	Controls
Cortisol (nmol/L)			
0 minutes	336	353	(82.77, 413.85)
30 minutes	**809**	**895**	(358.67, 800.11)
60 minutes	885	**1007**	(441.44, 993.24)
Aldosterone (pmol/L)			
0 minutes	**60**	**30**	(221.6, 365.64)
30 minutes	**310**	**270**	(761.75, 1071.99)
60 minutes	310	280	no data
Androstenedione (nmol/L)			
0 minutes	2.3	2.4	(2.16, 4.68)
30 minutes	4	5.5	(1.85, 8.97)
60 minutes	3.9	7.3	(2.36, 7.96)
DHEA-S (nmol/L)			
0 minutes	**0.8**	**1**	(2.35, 14.45)
30 minutes	**0.8**	**1**	(2.16, 15.13)
60 minutes	0.8	1.1	(0, 17.02)
Progesterone (nmol/L)			
0 minutes	<1	<1	(0.16, 0.8)
30 minutes	**5.8**	**11**	(0.13, 2.67)
60 minutes	**6.3**	**12**	(0, 2.93)
17-hydroxyprogesterone (nmol/L)			
0 minutes	2.6	**3.6**	(0.48, 2.91)
30 minutes	**17.2**	**29.4**	(0.73, 7.50)
60 minutes	**18.1**	**32**	(1.42, 7.72)
Pregnenolone (nmol/L)			
0 minutes	0.25	1.61	(0, 11.12)
30 minutes	2.21	5.34	(0.38, 11.5)
60 minutes	7.87	6.26	(2.4, 16.94)
17-hydroxypregnenolone (nmol/L)			
0 minutes	0.69	0.39	(0, 7.74)
30 minutes	6.68	5.75	(0, 31.94)
60 minutes	6.05	8.07	(0, 38.41)
11-deoxycortisol (nmol/L)			
0 minutes	3.29	3.06	(0, 5.14)
30 minutes	**22.14**	**30.35**	(1.3, 5.58)
60 minutes	**23.41**	**29.22**	(1.97, 6.47)
Corticosterone (nmol/L)			
0 minutes	16.36	14.85	(3.81, 42.31)
30 minutes	100.05	**173.26**	(48.5, 135.31)
60 minutes	114.96	141.73	(49.68, 145.86)

The control data are presented as (mean – 2 standard deviations, mean + 2 standard deviations)

even after administration of supraphysiological doses of ACTH, other factors are important in determining aldosterone concentrations (Schiffrin *et al.,* 1984). If there had been a defect in aldosterone synthesis, corticosterone levels (the precursor to aldosterone) would be expected to be elevated. However, corticosterone levels were normal in III-2; in III-3, basal corticosterone was normal, whilst stimulated levels were high-normal (Table 3.1).

Compared with historical controls, stimulated levels of 11-deoxycortisol, the immediate precursor of cortisol, were higher in III-2 and III-3, suggesting CYP11B1 deficiency (Table 3.1; Figure 3.1) (Munabi *et al.,* 1986). There was a trend to higher progesterone and 17-hydroxyprogesterone concentrations suggesting deficiency of 21-hydroxylase (CYP21A2), and perhaps also CYP17A1 (Table 3.1; Figure 3.1). Elevated progesterone levels were previously reported in two patients with AIMAH (Wada *et al.,* 1996). Relative cellular deficiencies of these enzymes in AIMAH have been shown previously (Koizumi *et al.,* 1994; Morioka *et al.,* 1997). Measurement of steroid intermediates did not suggest 3βHSD deficiency, which is expressed solely in the clear cells of AIMAH (Koizumi *et al.,* 1994; Aiba *et al.,* 1991).

DHEAS levels were lower than in controls (Table 3.1). This could be due to cellular deficiency of CYP17A1 (Figure 3.1). An alternative explanation however is that the control group was younger (18-36 years) than the study participants (63 and 67 years) when evaluated. The age difference between the patient and control groups is relevant because DHEA and DHEAS levels decline markedly with age, such that serum concentrations in 70-80 year olds are approximately 20% of those in 20-30 year olds (Orentreich *et al.,* 1992).

Where in the pathogenesis of AIMAH inefficient steroidogenesis develops is not known. Is inefficient steroidogenesis a consequence of poorly differentiated tumour cells, or is it the primary defect in AIMAH, and hyperplasia therefore a compensatory response, analogous to

CAH? In contrast to CAH hyperplasia in AIMAH maintains normal and may eventually result in elevated cortisol levels, perhaps due to a more subtle steroidogenic synthetic defect.

The limitations of this study are the small number of patients evaluated, and comparison with historical controls, rather than an age- and gender-matched control group. As a logical extension of this study, we have performed genome-wide gene expression profiling of AIMAH-01 tumours, in order to determine the mechanisms involved in the pathogenesis of AIMAH and to profile the cellular mechanisms of inefficient steroidogenesis. These data are presented in Chapter 4.

3.4 Conclusion

In the AIMAH-01 patients who were evaluated whilst eucortisolaemic after single adrenalectomy, the cortisol response to Synacthen® was normal. However, measurement of steroid intermediates suggested reduced activity of CYP11B1 and CYP17A1, consistent with the immunohistochemical findings reported by others. The lower aldosterone levels in our patients are likely to be multifactorial – including the effect of medications and possibly variable salt intake, which was not regulated preceding the evaluation; rather than due to a true synthetic defect, since there had not been prior or subsequent clinical or biochemical evidence of aldosterone deficiency. Our data suggest that individual cellular synthetic defects in AIMAH are masked by adrenocortical hyperplasia. Measurement of plasma steroid intermediates therefore is unlikely to be of any clinical utility in the diagnosis of AIMAH. Ancillary studies (immunohistochemistry, Western blotting) are needed to confirm whether CYP11B1, CYP17A1 and CYP21A2 are reduced in AIMAH-01.

Ultimately, the coalescence of clinical and biochemical data, together with gene expression, and eventually, when identified, the genetic basis of familial AIMAH, may provide a detailed aetiopathogenic model for inefficient steroidogenesis in, and the development of, AIMAH.

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

4.1 Introduction

By identifying differentially expressed genes (DEG; upregulated or downregulated) between tumour and normal (from the same individual or another) tissue, gene expression profiling may provide insights into the molecular mechanisms of tumorigenesis (Golub *et al.,* 1999). As discussed in Chapter 1, microarray platforms are a powerful tool because they allow the systematic analysis of the expression of thousands of genes in a single experiment (Brown and Botstein, 1999). Furthermore, the data obtained are without bias from existing biological knowledge; thus the methodology has the potential to identify novel molecular mechanisms.

Notwithstanding the power of microarray analysis, it is necessary to be mindful of the limitations of the technology, the data analysis and thus the gene expression data obtained. These have been discussed in Chapter 1. Determining the DEG typically involves identifying those genes which fulfil arbitrarily determined criteria, e.g., fold-change (FC) (e.g., a minimum absolute FC of two) and a *p*-value threshold (e.g., $p < 0.05$, after multiple test correction; described in Methods, Statistical analysis, this chapter). However, statistically significant DEG may not be biologically significant; and biologically significant differences may not achieve statistical significance. For example, minor FC of many genes in the same pathway, although individually appearing insignificant, in concert may have important consequences for the function of that pathway (Subramanian *et al.,* 2005).

When we conducted our studies there were two published gene expression studies of sporadic ACTH-independent macronodular adrenal hyperplasia (AIMAH) tumours (Bourdeau *et al.,*

2004; Lampron *et al.,* 2006). These studies are described here and are summarized in Table 4.1. A third study was published recently (Table 4.1; Almeida *et al.,* 2011).

Bourdeau *et al.,* compared gene expression from eight AIMAH tumours (three GIP-dependent; i.e., demonstrating *in vivo* ACTH-independent cortisol secretion in response to stimulation of endogenous GIP secretion by the oral ingestion of food) from unrelated individuals with commercially available pooled (*n*=62) total adrenal RNA, using a National Cancer Institute custom array containing ≈ 10,000 human PCR-amplified cDNA clones (Bourdeau *et al.,* 2004). The criteria for DEG were a minimum two-fold difference in expression values in at least six of eight AIMAH samples. There were 113 DEG (82 upregulated) (Bourdeau *et al.,* 2004).

Gene function was initially annotated using Gene Ontology; the function of each gene was then annotated manually using the data available in the literature (PubMed: http://www.ncbi.nlm.nih.gov/pubmed and GeneCards®: http://www.genecards.org/) (personal communication, Dr Isabelle Bourdeau). Upregulated genes encoded proteins involved in the cell cycle, transcriptional regulation, chromatin remodelling and cell adhesion (Bourdeau *et al.,* 2004). Several immunoglobulin genes and genes involved in insulin signalling and lipid metabolism were downregulated (Bourdeau *et al.,* 2004). Several DEG were involved in Wnt/β-catenin signalling (Bourdeau *et al.,* 2004).

Table 4.1. Summary of published gene expression data in AIMAH (part 1 of 2)

Study; Design; Platform; Criteria for differential gene expression	DEG	DEG – Functions/Pathways
Bourdeau *et al.*, 2004 • *n*=8 (3 GIP dependent) AIMAH tumours from unrelated individuals *vs* pooled total adrenal RNA (62 individuals) • NCI custom array • Minimum two-fold difference in expression values and in at least six of eight AIMAH samples	Overall 113 (82 upregulated) Cluster analysis (GIP-dependent AIMAH) 778	Overexpressed Cell cycle; Transcriptional regulation; Chromatin remodelling; Cell adhesion Underexpressed Immunoglobulin genes; Insulin signalling; Lipid metabolism Overexpressed Adrenal tumorigenesis Underexpressed Steroidogenesis Wnt/β-catenin signaling
Lampron *et al.*, 2006 • *n*=5 GIP-dependent AIMAH *vs* adrenal tumours from patients with Cushing's disease (*n*=5) *vs* pooled total adrenal RNA (62 individuals) • Affymetrix HG-U133 plus 2.0 gene chip array • alpha of 0.05, present "flag" in at least two of the five AIMAH and minimum two-fold increase in intensity. Downregulated probe sets, required present "flag" in pooled total adrenal RNA tissue	Overall 723 probe sets of which 461 corresponded to a gene or a sequence with a known functional implication	• Cell adhesion • DNA, RNA structural organization • Intracellular signal transduction • Cell-surface linked signalling • Immunological processes • Cell growth and development, oncogenesis, apoptosis • Transcriptional control • Metabolic, anabolic and protein modification processes

Abbreviations: AIMAH – ACTH-Independent Macronodular Adrenal Hyperplasia; DEG – differentially expressed genes; GIP – gastric inhibitory polypeptide; NCI – National Cancer Institute

Table 4.1. Summary of published gene expression data in AIMAH (part 2 of 2)

Study; Design; Platform; Criteria for differential gene expression	DEG	DEG – Functions/Pathways
Almeida *et al.*, 2011 • 7 nodules, from 1 patient; each nodule *vs* 3 commercially available pools of human adrenal total RNA • Nodules numbered 1-7 in order of increasing diameter • Illumina Sentrix HumanRef-8 Expression BeadChips • Minimum two-fold change	Nodule 1: 1315 Nodule 2: 1287 Nodule 3: 1243 Nodule 4: 1220 Nodule 5: 1124 Nodule 6: 1254 Nodule 7: 1132	Pathways Nodule 1 • Circadian rhythm • Starch and sucrose metabolism • Aminosugars metabolism • MAPK signalling pathway • ECM-receptor interaction • Nicotinate and nicotinamide metabolism Nodule 7 • Ribosome • p53 signalling pathway • Circadian rhythm • Pathways in cancer • ECM-receptor interaction • Colorectal cancer • MAPK signalling pathway

Abbreviations: AIMAH – ACTH-Independent Macronodular Adrenal Hyperplasia; DEG – differentially expressed genes; ECM – extracellular matrix; GIP – gastric inhibitory polypeptide; MAPK – mitogen-activated protein kinase

Cluster analysis of microarray data is used to assign the global data set into smaller subsets. By definition, the members of a subset are more similar to each other than to members of other subsets. In this study, cluster analysis identified 778 DEG in GIP-dependent AIMAH (Bourdeau *et al.,* 2004). Amongst the upregulated genes were some which were known to be involved in tumorigenesis: cyclin-dependent kinase inhibitor 1C (*CDKN1C*), the gap junction protein, alpha 1, 43kDa (*GJA1*) and the regulatory subunit type-2B of protein kinase A (*PRKAR2B*) (Bourdeau *et al.,* 2004). Underexpressed genes included those encoding steroidogenic enzymes (*CYP11A1* – cholesterol side-chain cleavage enzyme; *CYP17A1* – 17α-hydroxylase; *CYP21A2* – 21-hydroxylase) (Bourdeau *et al.,* 2004).

In microarray analysis, hierarchical clustering identifies clusters from previously defined clusters (Figure 4.1). Hierarchical clustering showed a distinct clustering of GIP-dependent AIMAH relative to non-GIP-dependent AIMAH, reflecting similarities between the GIP-dependent tumours (Figure 4.1) (Bourdeau *et al.,* 2004). Data from patients with GIP-dependent AIMAH placed them under a unique subdivision of the branches reflecting gene similarities, when compared with the other AIMAH samples (Figure 4.1). Upregulated genes in GIP-dependent AIMAH included a member of the RAS oncogene family (*RAB31*), WNT1 inducible signalling pathway protein 2 (*WISP2*), and other genes related to Wnt/β-catenin signalling; this latter pathway was also upregulated in AIMAH overall (Bourdeau *et al.,* 2004). These data suggest heterogeneity of gene expression changes and thus, molecular mechanisms, operating between different subtypes of AIMAH.

In another study, Lampron *et al.,* compared gene expression in GIP-dependent AIMAH nodules (*n*=5) from unrelated individuals with adrenal tumours from patients with ACTH-dependent Cushing's disease (*n*=5), using the Affymetrix HG-U133 plus 2.0 gene chip array (Lampron *et al.,* 2006). The aims of the study were to identify the genes responsible for the ectopic expression of the GIP receptor and the molecular mechanisms and signalling pathways

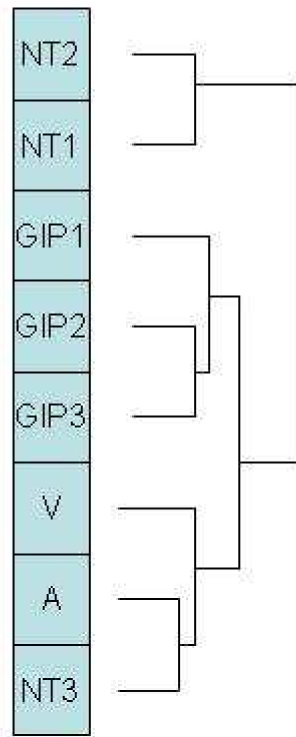


Figure 4.1. Dendrogram showing hierarchical clustering of gene expression in sporadic AIMAH. There were similarities in the gene expression profiles between the patients with GIP-dependent AIMAH (GIP1-3) compared to all other patients with AIMAH (patients with β-adrenergic (A), vasopressin (V) or with no aberrant receptors (NT1-3) revealed *in vivo*. (Bourdeau *et al.,* 2004).

involved in the formation of AIMAH. The comparison between two types of adrenal tumours was performed in order to maintain to a minimum the number of non-specific proliferation-associated genes identified as differentially expressed.

A feature of the array used in that study is that each probe set can be assigned a "flag" or detection call (present, marginal or absent) based on intensity (Affymetrix Gene Chip Operating System) (www.affymetrix.com). The criteria for DEG were an alpha of 0.05 (Student's two-tailed heteroscedastic *t* test), a present "flag" in at least two of the five GIP-dependent AIMAH and a minimum two-fold increase in intensity. For downregulated probe sets, the present "flag" needed to be scored in pooled total adrenal RNA (62 individuals), which served as a control. Using these criteria, 723 probe sets were differentially expressed;

of these, 461 corresponded to a gene or a sequence with a known functional implication (Lampron *et al.,* 2006). Gene function analysis was performed using data from the Affymetrix NetAffx Analysis centre and the National Centre for Biotechnology Information's (NCBI) various databases. The GFINDer (Genome function integrated discoverer) tool (http://www.medinfopoli.polimi.it/GFINDer) was used to identify predominant gene ontology terms in the data set. Selected genes were validated by RT-PCR. DEG functions are listed in Table 4.1 (Lampron *et al.,* 2006).

In the most recent AIMAH gene expression paper, transcriptome profiling and oligonucleotide array comparative genomic hybridization (oligo-aCGH) (for chromosomal gains or losses) were performed in seven differently-sized nodules from one patient (Almeida *et al.,* 2011). A principal aim of this study was to identify whether genetic changes (chromosomal gains or losses; gene expression) in hyperplastic nodules from the same patient correlated with nodule size. The expression profile of each nodule was compared with three commercially available pools of total adrenal RNA. After normalisation, the data for each sample was $\log_{10}$-transformed and a Z score calculated (by subtracting the overall average gene intensity from the raw intensity data for each gene, then dividing that result by the standard deviation of all of the measured intensities). Changes in gene expression (ratio) between different Z-transformed datasets (nodules compared with the average of the normal adrenal pools) were calculated as differences between the corresponding Z scores and then divided by the standard deviation of each Z difference dataset. A two-fold change was the threshold for detection of DEG in adrenal nodules compared with the pooled normal adrenal samples.

Chromosomal gains were more frequent in larger nodules, and overall were more frequent than chromosomal losses (Almeida *et al.,* 2011). Genes involved in Wnt signalling (*WNT4*, *WISP1*) were overexpressed in the AIMAH nodules (Almeida *et al.,* 2011). Some overexpressed genes occurred in regions of chromosomal gains (e.g., tissue factor pathway

inhibitor 2, *TFPI2*), whilst others did not (e.g., tetraspanin 8, *TSPAN8*). Amongst the 50 most overexpressed genes, half were amplified in the CGH data, whilst only nine of the 50 most underexpressed genes were in regions of chromosomal loss (Almeida *et al.,* 2011). Functional analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) revealed enrichment in the smallest and largest nodules, for metabolic and oncogenic pathways, respectively (Table 4.1) (Almeida *et al.,* 2011). Gene set enrichment analysis (GSEA) was used to correlate the microarray expression data with chromosome gene sets (Almeida *et al.,* 2011). DEG between the largest and smallest nodule revealed enrichment (by GSEA, described later) for genes on chromosome 20q13 and 14q23 (Almeida *et al.,* 2011).

These studies have enhanced our understanding of the molecular mechanisms involved in sporadic AIMAH. However, much remains to be understood. To date, there are no published gene expression data from familial AIMAH. Whether common mechanisms of tumorigenesis exist between familial AIMAH and sporadic AIMAH or other adrenocortical tumours (ACT; carcinoma, adenoma) has also not been investigated; we explored this using GSEA, described later in this chapter. We also analysed the familial AIMAH expression data using Motif Activity Response Analysis (MARA), an algorithm which models gene expression in terms of transcription factors which are predicted to be changing their activity (The FANTOM Consortium and the Riken Omics Science Centre, 2009). The theoretical basis of MARA is described briefly later in this chapter.

We chose to use microarrays because they provide genome-wide gene expression data. We anticipated that this might generate new hypotheses as to the pathways involved in the pathogenesis of AIMAH, which could provide directions for further study. Also, genome-wide expression data would allow us to compare our familial AIMAH expression data with the existing gene expression data of sporadic AIMAH.

The principal aim of this study was to gain insights into the molecular mechanisms and pathways involved in the development of the familial adrenal tumours in AIMAH-01 (Chapter 2). In so doing, we anticipated that we might confirm existing knowledge, but also identify novel mechanisms of tumorigenesis. Since inefficient steroidogenesis is a cardinal and unique feature of AIMAH, we wanted to determine the mechanisms, known or novel, operating within the steroidogenic pathway which could contribute to inefficient steroidogenesis in AIMAH-01 and compare these data with the *in vivo* steroid intermediate data (Chapter 3). When we conducted this study, the bioinformatics analyses we utilised – Ingenuity Pathway Analysis (IPA), GSEA and MARA – had not, to our knowledge, previously been applied to AIMAH expression data.

The second aim of this study was to identify the mechanisms involved in the progression from early to advanced AIMAH. Tumour nodules were classified based on the manifest biochemical and clinical features of Cushing's syndrome. Hence, early tumours were from III-2 and III-3 (AIMAH-01; Figure 2.1), both of whom had subtle clinical and biochemical features of Cushing's syndrome, whilst the advanced tumour nodules were from the proband, III-1 (AIMAH-01; Figure 2.1), who was floridly hypercortisolaemic.

4.2 Research Methods

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee.

Tissue Collection and RNA extraction

Adrenal tissue from patients with AIMAH undergoing resection for Cushing's syndrome was obtained at surgery. Normal adrenal glands were obtained from patients undergoing nephrectomy for renal cancer (supplied by Professor Michael Stowasser and Associate Professor David Nicol, Princess Alexandra Hospital, Brisbane). All patients gave written, informed consent for the retention of adrenocortical tissue fragments for research.

Adrenocortical fragments were rapidly frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted using the Qiagen RNeasy Lipid Tissue Midi Kit (Qiagen, Germany), according to the manufacturer's instructions.

Gene expression studies

Genome-wide gene expression profiling was performed using the Affymetrix GeneChip® Human Gene 1.0 ST array (Adelaide Microarray Centre, SA Pathology). We studied RNA from two adrenal nodules from each of III-1, III-2 and III-3 (Figure 2.1, AIMAH-01) and normal adrenal cortex (*n*=2).

The Affymetrix GeneChip® Human Gene 1.0 ST array is comprised of oligonucleotide probes which hybridise specific transcripts (cDNA) (Affymetrix, 2007b). The array has over 760,000 distinct probes for over 28,000 genes (Affymetrix, 2007a). Compensation for non-specific hybridisation to a probe is achieved by multiple probes (which together comprise a probe set) for each gene; each probe hybridises to a specific region of the transcript. Hybridisation is measured as a fluorescence intensity signal – more intense fluorescence indicates more abundant transcript. All RNA had a minimum RNA integrity number (quality score) of 7.5.

Labelling and array hybridisation

This was performed according to the whole transcript sense target labeling assay manual from Affymetrix (Affymetrix, 2007c). Total RNA was labelled using two-cycle cDNA synthesis; this was followed by cDNA hybridisation to, washing, staining and scanning of, the arrays, as per the manufacturer's protocol (Affymetrix, 2007c).

Statistical analysis

The raw expression data were normalised with the robust multi-chip average (RMA) function using the aroma.affymetrix package (Irizzary *et al.,* 2003; Bolstad *et al.,* 2003; Bengtsson *et al.,* 2008). Array quality was examined using normalised unscaled standard error (NUSE) boxplots (not shown). All arrays were of good quality; however in the statistical analysis higher weighting was given to better quality arrays (Ritchie *et al.,* 2006). Tumour samples from the same individual were considered biological replicates.

The analysis was performed in the "R" statistical environment (http://www.r-project.org/). The moderated *t*-test, using empirical Bayes linear modelling (LIMMA) to borrow information across genes, was performed (Smyth, 2004). Two comparisons were made: (1) all AIMAH *vs* normal adrenal; and (2) advanced *vs* early AIMAH. A multiple test correction was performed using the method of Benjamini and Hochberg to adjust the *p*-value in order to control the false discovery rate (FDR) (Benjamini and Hochberg, 1995). The many thousands of gene-wise comparisons render a multiple test correction essential in the analysis of microarray data to minimise the number of false-positive DEG. The criteria for DEG were: an adjusted *p*-value (or FDR) < 0.05 and an absolute fold-change (FC) ≥ 2.

Data visualisation

A principal components analysis (PCA) plot of the expression data was constructed using Partek® Genomics Suite™, Version 6.4 (Partek Inc, St Louis, Missouri, United States). This is a method of visualising the global similarities and differences between the samples studied, without specifying what the similarities and differences are. The first principal component (PC) is PC1, which, by definition, explains the maximum amount of variation in the data that can be attributed to any single PC. Each subsequent PC, to a maximum of 10 PC, explains a decreasing percentage of the remaining variation in the data. The closer two samples are on a

PCA plot, the more “similar” they are with regards to the PC (axis) being considered; likewise, the farther apart two samples are, the more “different” they are.

A heat map of the 100 most statistically significantly upregulated and downregulated (by FC) genes was generated in Multiexperiment Viewer (MeV) program using unsupervised clustering (Saeed *et al.,* 2006).

Microarray validation

Microarray validation was performed using RT-qPCR. The primers and protocol are described in Appendix 1. For RT-qPCR of every selected gene, each sample was studied in triplicate. Gene expression was normalized to the expression of the housekeeping gene, proteasome subunit beta type-2 (*PSMB2*). Concentrations were derived in reference to standard curves constructed for each gene, from known dilutions of pooled cDNA from the AIMAH-01 tumours and the normal adrenals. A minimum of three data points was used to construct the standard curve. The average expression of the triplicates for each sample was taken as the final value. The mean concentration (± standard error) of the genes in the AIMAH-01 tumour and normal adrenal groups were compared using the Student’s *t*-test. A *p*-value < 0.05 was taken as statistically significant for DEG.

Bioinformatics analyses of gene expression data from AIMAH-01 tumours

We used Ingenuity Pathway Analysis (IPA), Motif Activity Response Analysis (MARA) and Gene Set Enrichment Analysis (GSEA) of the following two comparisons: (1) all AIMAH *vs* normal adrenal; and (2) advanced *vs* early AIMAH.

Ingenuity Pathway Analysis (IPA)

Genes fulfilling DEG criteria (absolute FC ≥ 2 and FDR < 0.05) were submitted to IPA to determine which gene functions and/or molecular pathways were over-represented in the data

set and hence could be potentially involved in the adrenal tumorigenesis of AIMAH-01 (Ingenuity Systems®; http://www.ingenuity.com/). The Ingenuity® Knowledge Base contains Ingenuity® Supported Third Party Information - manually reviewed information from third party databases including Gene Ontology (http://www.geneontology.org/); Kyoto Encyclopaedia of Genes and Genomes (KEGG) metabolic pathway information (http://www.genome.jp/kegg/); NCBI Databases – Entrez Gene, RefSeq, Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/) and others. The full list of sources of content is at http://www.ingenuity.com/library/index.html.

Motif activity response analysis (MARA)

This is available as an automated online tool (http://test.swissregulon.unibas.ch/cgi-bin/mara). MARA is an algorithm which attempts to explain DEG in terms of altered transcription factor (motif) activity. This method is based on the principle that gene transcription is driven by regulatory sites in DNA (promoter regions, motifs) that are recognized by transcription factors (The FANTOM Consortium and the Riken Omics Science Centre, 2009). The focus is on regulatory regions near the transcription start sites (TSS) of genes, since these regions contain many functional regulatory sites (The ENCODE Project Consortium, 2007). A discussion of the theoretical basis of MARA development is beyond the scope of this thesis; however a summary of the key principles is presented below.

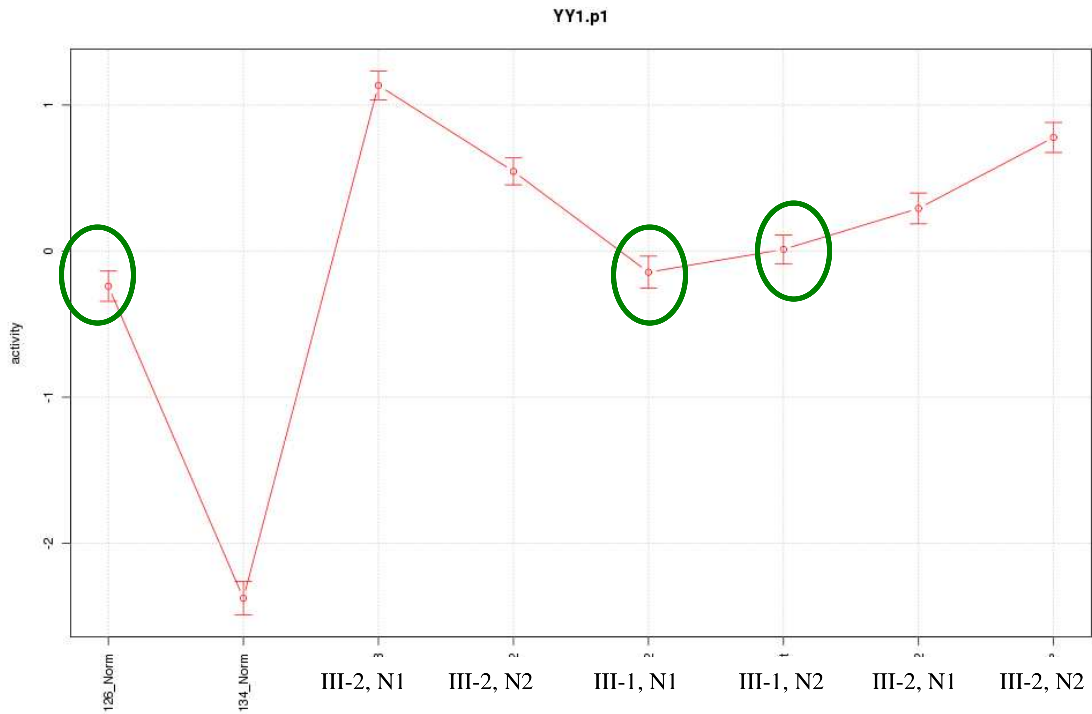
Transcription factor binding sites (TFBS) for proximal promoters were predicted using the MotEvo algorithm and knowledge of (1) mammalian regulatory motifs (databases: JASPAR - http://jaspar.cgb.ki.se, TRANSFAC® - http://www.gene-regulation.com/cgi-bin/pub/databases/transfac); (2) proximal promoter regions (-300, +100 base pairs) for each TSS; and (3) alignments with orthologous regions from other mammals (The FANTOM Consortium and the Riken Omics Science Centre, 2009; Pachkov *et al.,* 2007; van Nimwegen, 2007). Probe set data are associated with promoters and MARA models gene expression

patterns in terms of predicted TFBS, and reports the statistical significance (z-value) for that motif changing its activity across samples. A list of the top 20 genes predicted to be regulated by the motif is also provided. We considered motifs to be significant only if there was no overlap in the activity profile when all tumour samples were compared with both normal samples (Figure 4.2).

Gene set enrichment analysis (GSEA)

GSEA analyzes expression data at the level of gene sets and is primarily intended to generate hypotheses for further study. The aim of using GSEA to analyse the AIMAH expression data was to determine if there was enrichment of published DEG sets of other ACT, or pathways or other mechanisms implicated in adrenal or endocrine tumorigenesis in the preranked (in descending order of t-statistic) AIMAH-01 gene lists. We used the preranked GSEA function, with default settings. Gene duplicates and probe sets for which a gene or transcript had not been identified by the time of the analysis, were removed from all gene sets prior to analysis. If the members of the gene set were at the top (Figure 4.3, panel A), bottom (Figure 4.3, panel B) or were clustered at the top and bottom (Figure 4.3, panel C) of the ranked gene lists, then the gene set would be correlated with the AIMAH-01 phenotype. Conversely, if the members of the gene set were randomly distributed (Figure 4.3, panel D) throughout the ranked gene lists, then there would be no correlation with the AIMAH-01 phenotype. Gene sets were considered enriched based on FDR (q-value for enrichment) < 10%, a slightly more stringent threshold than usually recommended (< 25%) (Subramanian *et al.,* 2005; Mootha *et al.,* 2003; http://www.broadinstitute.org/gsea/doc/GSEAUserGuide.pdf).

A.



B.

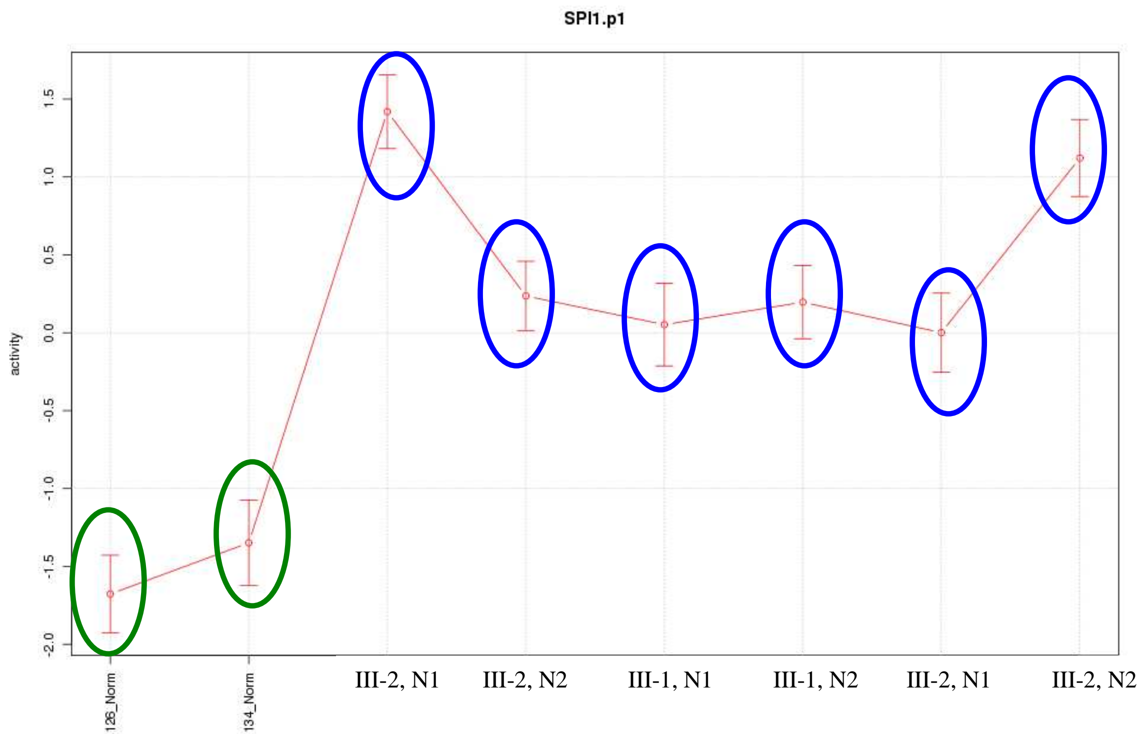


Figure 4.2. Motif activity response analysis activity profiles.

A. Motif activity not considered significant since N_126 activity profile is comparable to the AIMAH samples.

B. Motif activity considered significant if the activity profile was consistent for all samples within each group and differed between the normal and AIMAH groups.

Normal group: 126_Norm, 134_Norm. AIMAH-01: III-1, III-2, III-3 (Figure 2.1, AIMAH-01 pedigree)

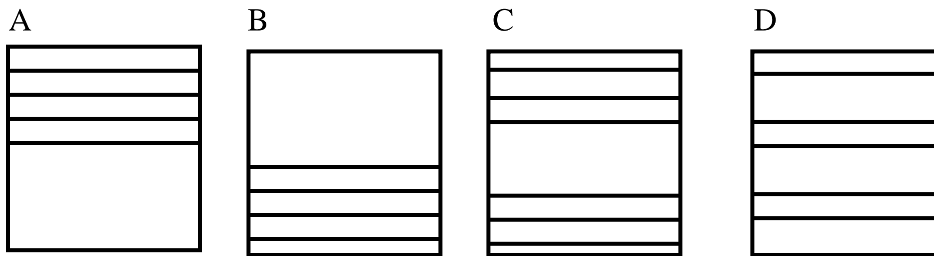


Figure 4.3. Gene set enrichment analysis.
Gene set enrichment analysis determines where the members of a predefined gene set are positioned in a ranked gene list from another experiment. In the figure, the gene list is represented by the rectangle; the members of the gene set are represented by lines in the box. In panel A, the members of the gene set are located at the top of the gene list (non-random) – the gene list is positively enriched for the members of the gene set. In panel B, the members of the gene set are located at the bottom of the gene list (non-random) – the gene list is negatively enriched for the members of the gene set. Panel C is another example of the non-random distribution of members of a gene set in a ranked gene list. In panel D, the members of the gene set are distributed randomly throughout the gene list – so there is no enrichment for the members of the gene set.

The gene sets we selected included DEG between human ACT (adrenocortical carcinoma or adenoma, AIMAH, primary pigmented nodular adrenocortical disease) or their comparison with normal (Table 4.2). An additional four studies were selected because the expression data represented genes modulated by: (1) steroidogenic factor-1 (SF-1), an orphan nuclear receptor important in adrenal development and function; (2) menin, a tumour suppressor gene associated with endocrine neoplasia; (3) ACTH; (4) cAMP-dependent protein kinase A pathway activation. Each may have a role in normal adrenal physiology and/or in adrenal tumorigenesis. These have been described in Chapter 1.

Table 4.2. Gene sets used for Gene Set Enrichment Analysis (part 1 of 3). Table legend is on page 152.

Human Adrenocortical Tumour studies				
Reference	**Study**	**Data format**	**Platform**	
Criteria for differential gene expression				**Nō of DEG**
Lampron *et al.,* 2006; GSE4060	GIPsAIMAH	GIPsAIMAH (*n*=5) *vs* Cushing's disease (*n*=5)	Affymetrix HG-U133 plus 2.0 array	461
Alpha of 0.05 (Student's two-tailed heteroscedastic *t* test), a present "flag" in at least two of the five GIP-dependent AIMAH and a minimum two-fold increase in intensity; for downregulated probe sets, the present "flag" needed to be scored in pooled normal adrenal RNA (*n*=62).				
Bourdeau *et al.,* 2004	AIMAH	AIMAH (*n*=8) *vs* normal adrenal gland (pooled, *n*=62)	National Cancer Institute custom array	102
Minimum two-fold difference in expression values in at least six of eight AIMAH samples				
Almeida *et al.,* 2011	AIMAH	AIMAH (*n*=1), 7 nodules; each nodule *vs* 3 commercially available adrenal gland pools	Illumina Sentrix HumanRef-8 Expression BeadChips	620-702
Two-fold change				
Ye *et al.,* 2007; GSE8514	APA	GPCR in APA (*n*=5) *vs* normal adrenal gland (*n*=10)	Affymetrix HG-U133 plus 2.0 array	95
$p < 0.2$				
West *et al.,* 2007	Paediatric ACT	ACT (*n*=24) *vs* normal adrenal cortex (*n*=7)	Affymetrix HG-U133 plus 2.0 array	838
$p < 0.001$				
Soon *et al.,* 2009 GSE12368	ACT	ACC (*n*=12) *vs* ACA (*n*=16)	Affymetrix HG-U133 plus 2.0 array	177
B-statistic ≥ 2; M value ≤ -2 and ≥ 2				

Human Adrenocortical Tumour studies				
Reference	**Study**	**Data format**	**Platform**	
Criteria for differential gene expression				**Nō of DEG**
Laurell *et al.,* 2009	ACT	ACC (n=11) *vs* (normal adrenal cortex (n=4) and ACA (n=17))	in-house manufactured cDNA array	76
Log_2fold change > 1 and $p < 0.001$				
Giordano *et al.,* 2003	ACT	ACC (n=11) *vs* normal adrenal cortex (n=4) & ACC *vs* ACA (n=3)	Affymetrix HG_U95Av2 array	82
$p < 0.01$ and fold-change > 3 or < 0.3333				
Giordano *et al.,* 2009 GSE10927	ACT	ACC (n=33) *vs* ACA (n=22) ACC *vs* normal adrenal cortex (n=10); ACA *vs* normal adrenal cortex	Affymetrix HG_U133 plus 2.0 array	2976 3270 273
|Fold-change| > 1.5 and $p < 0.001$				
de Reyniès *et al.,* 2009	ACT	ACA (n=58) *vs* ACC (n=34); aggressive ACC (n=21) *vs* good prognosis ACC (n=13)	Affymetrix HG_U133 plus 2.0 array	1058 1353
$p < 0.01$				
de Fraipont *et al.,* 2005	ACT	ACA (n=33) *vs* ACC (n=24)	custom array (230 genes only)	22
Cluster analysis to identify genes which distinguished carcinoma from adenoma				
Bassett *et al.,* 2005	APA, CPA - Steroidogenesis	APA and CPA (n=12) *vs* normal adult adrenal gland (n=9)	Affymetrix HG_U133A and B array	19
$p < 0.05$				

<table>
<tr><th colspan="5">Human Adrenocortical Tumour studies (continued)</th></tr>
<tr><th>Reference</th><th>Study</th><th>Data format</th><th colspan="2">Platform</th></tr>
<tr><th colspan="4">Criteria for differential gene expression</th><th>Nō of DEG</th></tr>
<tr><td>Fernandez-Ranvier et al., 2008a</td><td>ACT</td><td>ACA (n=78) vs ACC (n=11)</td><td>Affymetrix HG_U133 plus 2.0 array</td><td>37</td></tr>
<tr><td colspan="5">|Fold-change| > 8, False-discovery rate < 5% and adjusted $p < 0.01$</td></tr>
<tr><td>Fernandez-Ranvier et al., 2008b</td><td>ACT; genes on 11q13</td><td>ACA (n=43) vs ACC (n=11)</td><td>Affymetrix HG_U133 plus 2.0 array</td><td>25</td></tr>
<tr><td colspan="5">|Fold-change| > 2 and $p < 0.05$</td></tr>
<tr><td>Horvath et al., 2006c</td><td>PPNAD</td><td>PPNAD (n=1) vs normal adrenal (n=1)</td><td>SAGE Analysis</td><td>140</td></tr>
<tr><td colspan="5">Five-fold difference (or higher) in the normalized expression of all tags and $p < 0.05$</td></tr>
<tr><th colspan="5">Experimental studies</th></tr>
<tr><td>Doghman et al., 2007</td><td>SF-1 overexpression</td><td>Doxycycline treated vs untreated H295R TR/SF-1 wildtype cells</td><td>from RNG/MRC resource</td><td>97</td></tr>
<tr><td colspan="5">Absolute value of log-fold change > 0.7, mean log-expression level > 9 and log-odds of differential expression (statistical score) > 0</td></tr>
<tr><td>Scacheri et al., 2006</td><td>Men1 regulated genes</td><td>Men1 conditional null murine islets vs wildtype islet cells</td><td>MOE430A Affymetrix GeneChip</td><td>184</td></tr>
<tr><td colspan="5">$p < 0.01$</td></tr>
<tr><td>Schimmer et al., 2006</td><td>Genes regulated by: ACTH
PKA
PKC</td><td>Murine adrenal tumour cell (Y1) line</td><td>National Institute on Aging 15K mouse cDNA microarray</td><td>
1273
1554
418</td></tr>
<tr><td colspan="5">False-discovery rate < 0.05</td></tr>
<tr><td>Aghajanova et al., 2010</td><td>PKA-regulated genes</td><td>cAMP treated vs untreated endometriosis tissue</td><td>Affymetrix Human Gene 1.0 ST array</td><td>159</td></tr>
<tr><td colspan="4">|Fold-change| ≥ 1.5</td><td></td></tr>
</table>

Table 4.2. Gene sets used for Gene Set Enrichment Analysis (part 3 of 3). Table legend is on page 152.

Table 4.2. Gene sets used for Gene Set Enrichment Analysis (legend)
ACA – adrenocortical adenoma; ACC – adrenocortical carcinoma; ACT – adrenocortical tumour; ACTH – adrenocorticotropic hormone; AIMAH – ACTH-independent macronodular adrenal hyperplasia; APA – aldosterone producing adenoma; cAMP – cyclic AMP; DEG – differentially expressed gene GIP – gastric inhibitory polypeptide; GIPsAIMAH – GIP-sensitive AIMAH; GPCR – G protein coupled receptor; H295R TR/SF-1 wildtype – human adrenocortical cell line transfected to overexpress SF-1 wildtype; Men1 – menin gene; PKA – protein kinase A; PKC – protein kinase C; PPNAD – primary pigmented nodular adrenocortical disease; RNG/MRC - Réseau National des Génopoles/Medical Research Council; SAGE – serial analysis of gene expression; SF-1 - steroidogenic factor 1

4.3 Results and Discussion

Differentially expressed genes in AIMAH-01

Although the number of samples studied was small, the availability of tumours from three siblings was a powerful component of our study design – since background variation (or “noise”) due to genetic differences between unrelated individuals was kept to a minimum. Nevertheless, in the discussion to follow, it is necessary to be mindful that the small sample size of the tumour and normal adrenal groups is a limitation of this study and may have reduced our ability, statistically, to detect differential gene expression. Furthermore, we did not select specific cell types (e.g., clear or compact cells selected using laser capture) for study. Thus our studies reflect global tissue gene expression changes in AIMAH, and differential expression between heterogeneous tissue components may have been diluted.

A principal components analysis (PCA) plot of the expression data is depicted in Figure 4.4. The PCA plot of the AIMAH-01 and normal adrenal expression data illustrates two important aspects of the data: (1) at least in PC1, the tumour samples were more similar to each other, than to the unrelated normal samples and (2) the nodules from III-2 and III-3 (“early”) were more similar to each other than they were to III-1 (“advanced”), validating the tumour subclassification.

Applying the criterion of an adjusted *p*-value or FDR < 0.05, there were 1131 (all AIMAH *vs* normal) and 347 (advanced *vs* early AIMAH) statistically significantly DEG. Applying the *additional* criterion of an absolute FC ≥ 2, the numbers of DEG were as follows: all AIMAH *vs* normal 367 (162 genes upregulated); advanced *vs* early 101 (53 upregulated) (Appendix 2; Tables A.2.1 and A.2.2). Thus, most of the statistically significantly DEG showed low-level (< 2) FC differences. A heatmap of the 100 most statistically significantly upregulated and downregulated genes (by FC) was generated in MeV (microarray data visualisation tool) using unsupervised clustering (Figure 4.5) (Saeed *et al.,* 2006).

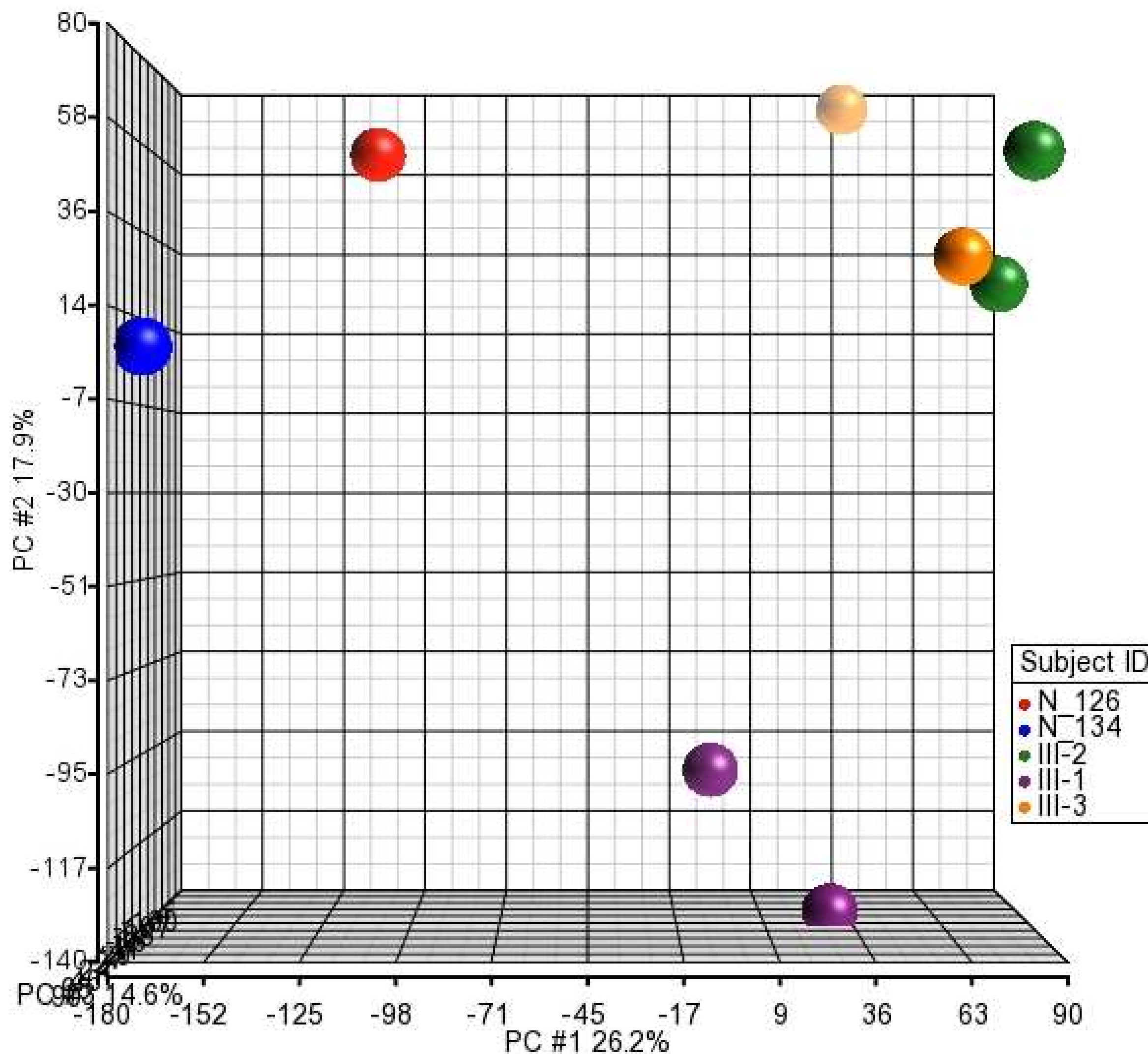


Figure 4.4. Principal components analysis plot of AIMAH-01 tumours and normal adrenal cortex expression data.
AIMAH-01: III-1, III-2, III-3 (Figure 2.1); Normal: N_126, N_134
The first principal component (PC #1) is shown on the *x*-axis and explains the maximum amount (26.2%) of variation in the gene expression data. The second principal component (PC #2) is shown on the *y*-axis and explains the second most amount of remaining variation in the gene expression data (17.9%). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

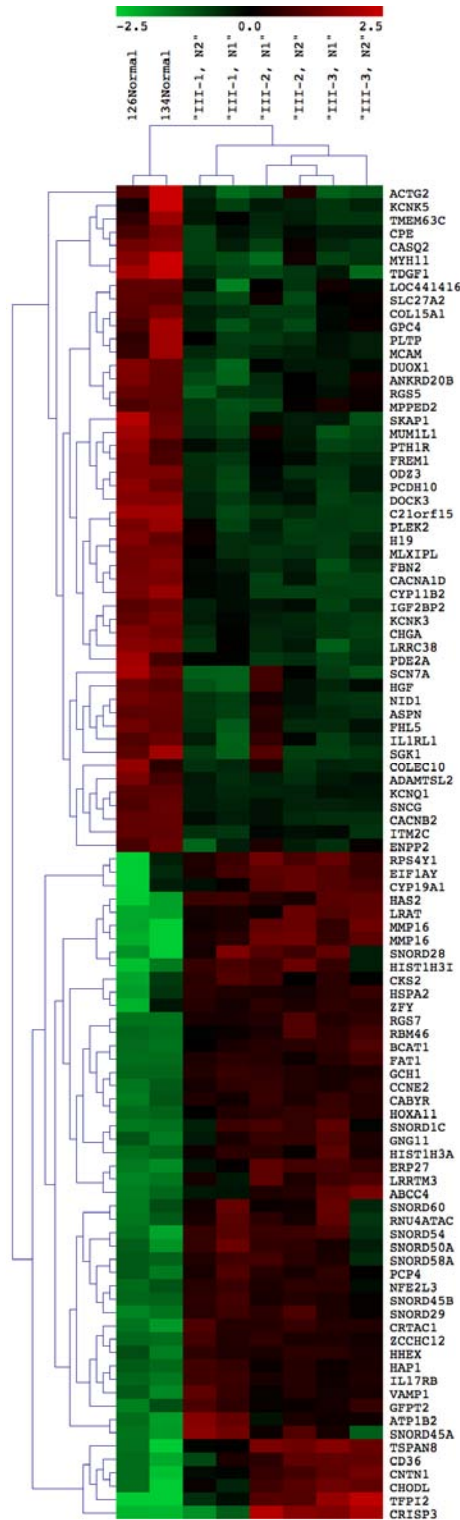


Figure 4.5. Heat map of 100 most differentially expressed (according to fold-change) genes in the AIMAH-01 tumours. The comparison is with normal adrenal. The 50 most upregulated and 50 most downregulated genes are shown. The expression values of the probe sets were adjusted so that each probe set had the same mean expression value. Red indicates expression above the mean expression value for all probe sets; green indicates expression below the mean expression value for all probe sets. Normal – N126, N134; AIMAH-01 (Figure 2.1) – III-1 – proband, advanced AIMAH; III-2 and III-3 – early AIMAH; N1, 2 – nodule 1, 2. This figure was generated in MeV.

The expression array results for (all AIMAH *vs* normal) are presented in an MA plot (Figure 4.6), where M represents the difference in expression observed for each probe and is given as $\log_2$ of the ratio between the intensities for each probe ($\log_2$fold ratio); and A represents the $\log_2$ of the average intensities (Mean (Original)) for a specific probe across all the arrays performed (Partek® Genomics Suite™).

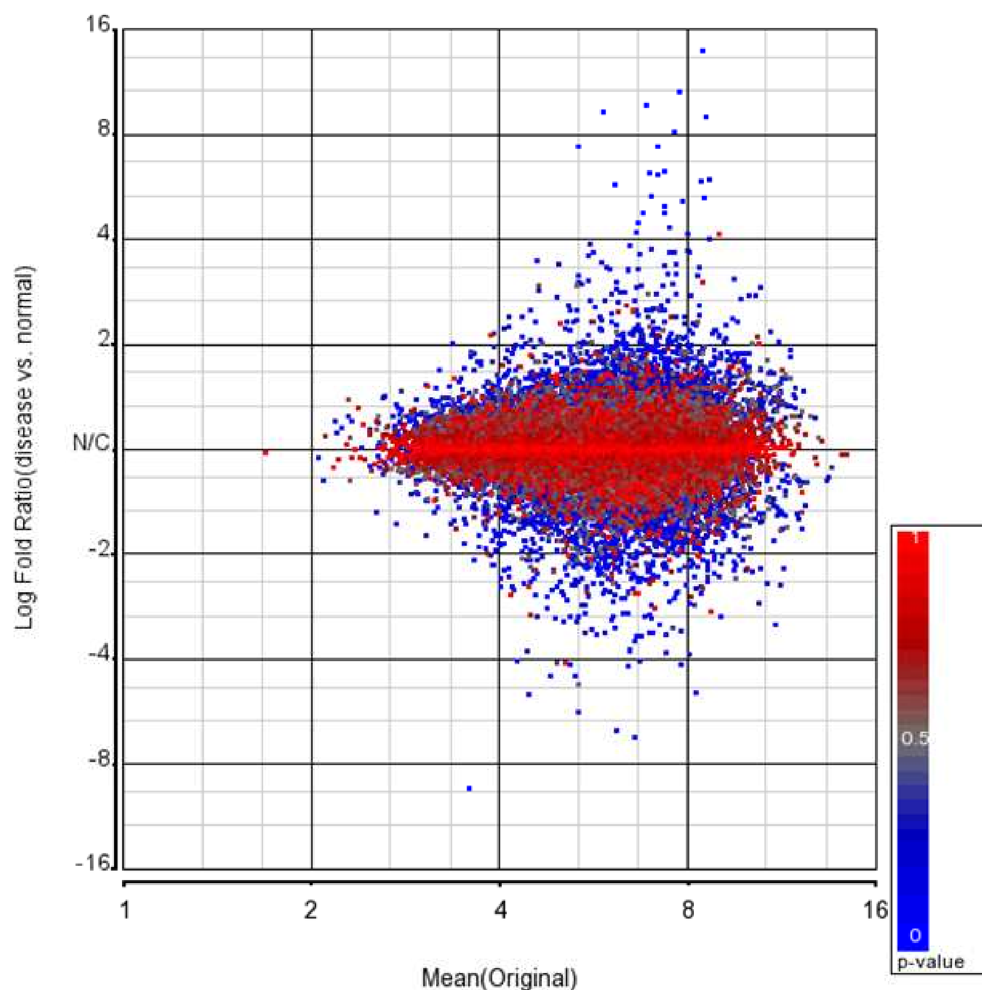


Figure 4.6. MA plot for all AIMAH *vs* normal. The probes with significant adjusted *p*-values are indicated in blue. This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

The number of DEG in the familial AIMAH-01 tumours was less than that reported in the non-familial GIP-dependent AIMAH tumours (Lampron *et al.,* 2006; Bourdeau *et al.,* 2004). This could be due to technical factors known to influence microarray data including: different arrays and laboratory conditions used in these studies or different statistical tests applied to the

data in the three studies or different criteria for DEG (Table 4.1). In GIP-dependent AIMAH there were 778 DEG compared with only 113 in all subtypes of AIMAH; the smaller number of DEG in our AIMAH-01 tumours may be due to their non-GIP-dependence (Bourdeau *et al.,* 2004). This suggests distinct molecular mechanisms operating in GIP-dependent and non-GIP-dependent AIMAH.

The cortisol-glucocorticoid receptor complex is an important transcriptional regulator of many genes; gene expression profiles may vary according to the nature (continuous *vs* pulsatile) of glucocorticoid exposure (McMaster *et al.,* 2011). Cortisol production in AIMAH may be intermittent or cyclical, contributing to the atypical and subtle clinical presentations of Cushing's syndrome, and we postulate that some of the DEG in the AIMAH tumours may reflect the nature and severity of hypercortisolism.

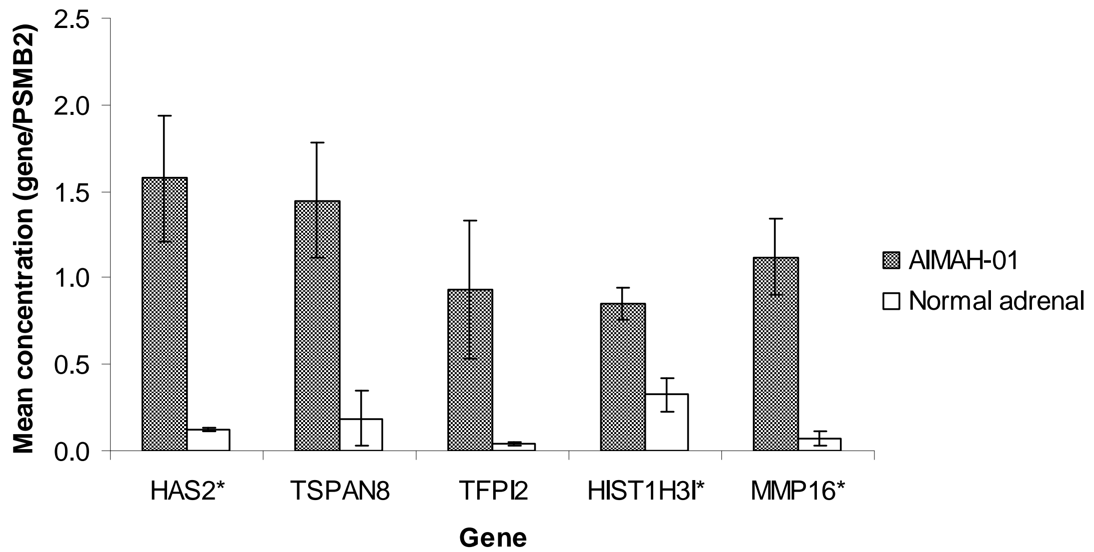
We confirmed differential expression of seven of the most highly upregulated and downregulated (absolute FC ≥ 2) and statistically significantly (FDR < 0.05) DEG (Table 4.3). In addition, three genes (actin, gamma 2, smooth muscle, enteric - *ACTG2*, tissue factor pathway inhibitor 2 - *TFPI2*, tetraspanin 8 - *TSPAN8*) showed a trend towards differential expression as suggested by the microarray data (Table 4.3; Figure 4.7). Interestingly, some of these genes were also differentially expressed in sporadic AIMAH (Almeida *et al.,* 2011; Lampron *et al.,* 2006).

Table 4.3. Ten highly differentially expressed genes in AIMAH-01.

Gene name	Log_2FC	FDR	FC	*p*-value	MARA motifs*
Upregulated genes					
Tetraspanin 8 (TSPAN8)	3.56	0.008	+ 7.7	0.065	FOX{I1,J2}; IKZF2; IRF7; SpI1
Matrix metallopeptidase 16 (membrane-inserted) (MMP16)	3.42	0.001	+15.7	0.035	NHLH1_2
Tissue factor pathway inhibitor 2 (TFPI2)	3.27	0.002	+21.3	0.217	
Hyaluronan synthase 2 (HAS2)	2.95	0.001	+12.5	0.05	LHX3_4; ZBTB16
Histone cluster 1, H3i (HIST1H3I)	2.69	0.006	+2.6	0.034	
Downregulated genes					
Actin, gamma 2, smooth muscle, enteric (ACTG2)	-3.12	0.035	-137	0.235	POU6F1; SOX5; MEF2{A,B,C,D}
Teratocarcinoma-derived growth factor 1 (TDGF1)	-2.95	0.001	-57.5	0.016	
Myosin, heavy chain 11, smooth muscle (MYH11)	-2.81	0.008	-13	0.013	MEF2{A,B,C,D}; TAL1_TCF{3,4,12}
Sodium channel, voltage-gated, type VII, alpha (SCN7A)	-2.37	0.017	-5.5	0.0002	
Src kinase associated phosphoprotein 1 (SKAP1)	-2.34	0.003	-7.7	0.01	

Log_2FC – according to microarray; FDR – false-discovery rate (*p*-value obtained after multiple test correction); FC (fold-change) obtained by reverse transcription-quantitative PCR; MARA – motif activity response analysis; *p*-value (RT-qPCR) – for differential mean gene expression (normalized to proteasome subunit beta type-2 - *PSMB2*) between AIMAH and normal adrenal

*motifs predicted by MARA to have altered activity. The gene listed is one of the top 20 genes regulated by the transcription factor.
Motif abbreviations: FOXI1 – forkhead box I1; FOXJ2 – forkhead box J2; IKZF2 – interferon regulatory factor 7; IRF7 – interferon regulatory factor 7; LHX3 - LIM homeobox 3; LHX4 – LIM homeobox 4; MEF2A/B/C/D - myocyte enhancer factor 2A/B/C/D; NHLH1 - nescient helix loop helix 1; NHLH2 - nescient helix loop helix 2; POU6F1 - POU class 6 homeobox 1; SOX5 - SRY (sex determining region Y)-box 5; SpI1 – spleen focus forming virus (SFFV) proviral integration; TAL1 - T-cell acute lymphocytic leukemia 1; TCF3- transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47); TCF4/12 – transcription factor 4/12; ZBTB16 - zinc finger and BTB domain containing 16



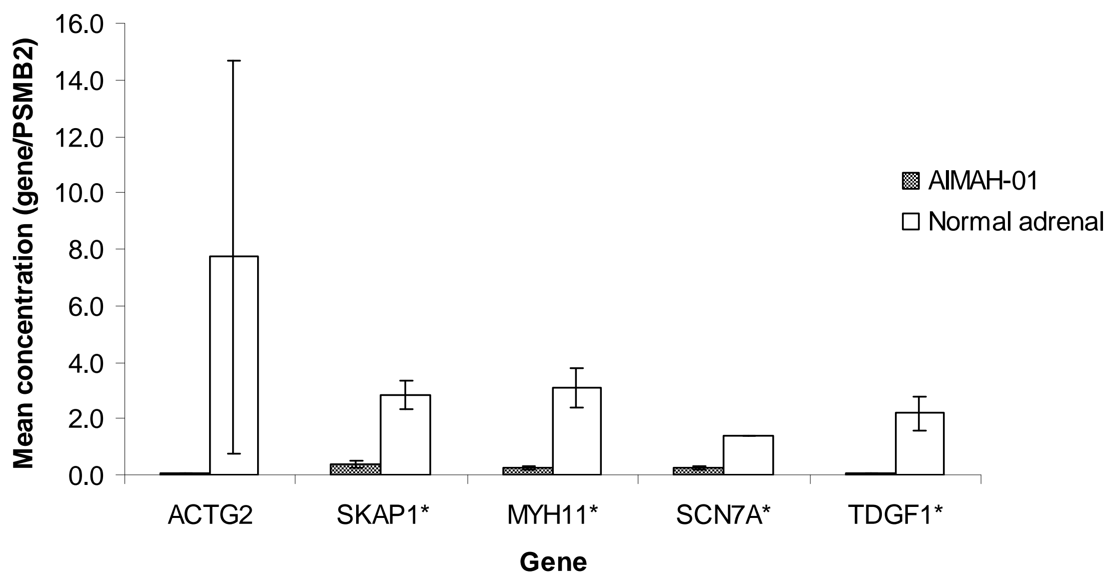


Figure 4.7. Selected significantly differentially expressed genes in AIMAH-01 tumours. The expression in normal adrenal is also shown. Microarray analysis identified these genes as having the largest fold-change. Differential expression was confirmed by RT-qPCR. Gene expression was normalized to the housekeeping gene, proteasome subunit beta type-2 (*PSMB2*). Error bars denote the standard error. The standard error for the normal adrenal group for *SCN7A* was 0.002. $*p < 0.05$, for a between group difference in gene expression. The full gene names are listed in Table 4.3.

TFPI2 and *TSPAN8* were also amongst the most overexpressed genes in all seven AIMAH nodules from one patient in whom genome-wide gene expression profiling was reported recently (Almeida *et al.,* 2011). TSPAN8 is a cell surface glycoprotein that is known to complex with integrins and mediates signal transduction events that are involved in the regulation of cell development, activation, growth and motility (Berditchevski, 2001; Hemler, 2005). It also induces angiogenesis in tumour and tumour-free tissues (Gesierich *et al.,* 2006).

TFPI2 is a serine proteinase inhibitor that has broad and potent inhibitory actions on proteases; thus protecting the extracellular matrix from degradation (Rao *et al.,* 1998; Izumi *et al.,* 2000; Konduri *et al.,* 2001; Jin *et al.,* 2001). Underexpression of *TFPI2* in many human solid tumours (melanoma, lung, liver, pancreas) is one of the mechanisms by which such tumours acquire their metastatic potential (Nobeyama *et al.,* 2007; Rollin *et al.,* 2005; Wong *et al.,* 2007; Sato *et al.,* 2005). The overexpression of *TFPI2* in AIMAH tumours may be one of the molecular mechanisms by which these tumours have a benign course, with no known propensity to invade adjacent tissues or metastasise. There was relatively lower (≈ 13-fold) expression of *TFPI2* in the advanced compared with early AIMAH nodules, a molecular marker perhaps of more aggressive growth potential in the advanced tumour.

We confirmed downregulation of teratocarcinoma-derived growth factor 1 (*TDGF1*); a gene which is upregulated in aldosterone-producing adenomas, and is implicated in adrenal tumorigenesis since it inhibits apoptosis of adrenocortical tumour (H295R) cells *in vitro* (Table 4.3; Figure 4.7; Williams *et al.,* 2010). It is expressed only in the zona glomerulosa (ZG); the presence and absence of the ZG in normal adrenal and AIMAH samples, respectively, is the most likely reason for *TDGF1* downregulation (Williams *et al.,* 2010).

We also confirmed downregulation of myosin, heavy chain 11, smooth muscle (*MYH11*) (Table 4.3; Figure 4.7), a gene which is downregulated in solid tumours (meningioma, colonic

adenomas) (Watson *et al.,* 2002; Sabates-Bellver *et al.,* 2007). The gene product is a subunit of a hexameric protein that consists of two heavy chain subunits and two pairs of non-identical light chain subunits. It functions as a major contractile protein, converting chemical energy into mechanical energy via the hydrolysis of ATP. Downregulation of *MYH11* was also found in GIP-dependent AIMAH (Lampron *et al.,* 2006).

We confirmed overexpression of matrix metallopeptidase 16 (membrane-inserted) (*MMP16*) (Table 4.3; Figure 4.7). MMP proteins are involved in the breakdown of extracellular matrix in normal physiological processes (e.g., embryonic development) and in disease processes (e.g., tumour metastasis). MMP16 is a direct-acting protease, dissolving the basement membrane (the supportive scaffolding that underlies epithelial cells and ensheathes blood vessels) and induces cellular transmigration (Hotary *et al.,* 2006). The finding of *MMP16* upregulation in AIMAH is surprising since this is a disease that is not known to have metastatic potential. Metastasis is unlikely to be regulated by one gene however, and it may be that other genetic changes counteract the effects of these genes and thus, are overall not conducive to metastasis in AIMAH.

Overexpression of hyaluronan synthase 2 (*HAS2*) in the AIMAH tumours was also confirmed (Table 4.3; Figure 4.7). *HAS2* is one of three *HAS* genes which synthesizes the protein hyaluronan (hyaluronic acid, HA), a constituent of the extracellular matrix. HA appears to be involved in tumour progression; it is present in greater amounts in tumour than in normal tissues, and high HA production correlates with invasive and metastatic tumour behaviour (Knudson, 1996). *In vitro*, increased production of HA specifically directed by *HAS2*, promotes anchorage-independent growth (correlates with tumorigenicity *in vivo*) and tumorigenicity of a human fibrosarcoma cell line (Kosaki *et al.,* 1999). Thus, amongst the most upregulated DEG were several genes involved in tumorigenesis and metastasis. This is intriguing for a disease which exhibits benign behaviour.

AIMAH has been regarded a benign disease because of the cytological appearance of the cells, and because there has never been clinical evidence of metastasis. However, the histological distinction between benign and malignant adrenal tumour cells can be difficult (Pohlink *et al.,* 2004). Furthermore, if AIMAH cells did have metastatic potential, then due to inefficient steroidogenesis, which in a metastatic cell might be even more pronounced, several decades may be required before metastases manifest clinically with Cushing's syndrome. This may never eventuate, due to the late age at which patients with AIMAH typically present (5th or 6th decades) (Lieberman *et al.,* 1994). Also, as discussed in Chapter 1, prospective surveillance of AIMAH tumours suggests that the cells do not have a massively increased rate of proliferation; this may be another mechanism by which metastases in AIMAH could remain clinically occult, although proliferation might be accelerated in metastatic cells compared with their benign counterparts (Lacroix *et al.,* 1997a; N'Diaye *et al.,* 1999).

As discussed in Chapter 2, the three siblings from AIMAH-01 from whom we had tumours, demonstrated an *in vivo* aberrant cortisol response to vasopressin (VP). After administration of VP, there was an ACTH-independent increase in cortisol; such responses to VP or other secretagogues have been previously demonstrated in AIMAH and, in general, are correlated with overexpression of a specific G-protein coupled receptor for a given secretagogue (Lacroix *et al.,* 2001). In our microarray analysis, the VP receptor genes (*AVPR1A*, *AVPR1B* and *AVPR2*) were not differentially expressed (Table 4.4). This was a surprising finding, because the patients had demonstrated definite aberrant cortisol responses to VP administration (Chapter 2; Gagliardi *et al.,* 2009). We further investigated VP receptor expression in AIMAH-01 using RT-qPCR and, as discussed in Chapter 2, found overexpression of *AVPR1A* and ectopic expression of *AVPR1B*, in the AIMAH tumours (Gagliardi *et al.,* 2009). The most likely explanation for the discrepancy between the microarray and RT-qPCR data is that the differential expression (*AVPR1A* – 1.5-fold upregulation; *AVPR1B* – ectopic expression, but at a low-level, since cycle threshold > 30)

was too subtle to be detected by the array. Other possible reasons for not detecting, by microarray analysis, the differential expression of the VP receptors include tissue heterogeneity (thought less likely since the mRNA used in both studies was from the same nodule) or other technical factors associated with the arrays or the small sample size in our study.

Insulin-like growth factor 2 (*IGF2*) is consistently overexpressed in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA) and with normal adrenal cortex; FC increases vary from three- to over 100-fold (Giordano *et al.,* 2003; Slater *et al.,* 2006). *IGF2* was upregulated in 80-90% of carcinoma samples studied by microarray (Giordano *et al.,* 2003; Soon *et al.,* 2009). Overexpression of *IGF2* mRNA and protein has been confirmed by RT-qPCR and immunohistochemistry, respectively (Soon *et al.,* 2009). IGF2 is mitogenic and is directly involved in the proliferation of the human ACC cell line (H295R), operating via the IGF1 receptor (Logie *et al.,* 1999). *IGF2* was upregulated in advanced *vs* early AIMAH (Table 4.4). Thus, IGF2 may be involved in disease progression in AIMAH-01.

Other genes involved in the IGF signalling pathway may be differentially expressed in ACC; although the results are less consistent than for IGF2 – since in some studies these genes were upregulated, whilst in others they were downregulated (Velázquez-Fernández *et al.,* 2005; Slater *et al.,* 2006; Laurell *et al.,* 2009). These include the receptors for IGF1 and IGF2, IGF1R and IGF2R; and binding proteins for IGF, IGFBP3 and IGFBP6 (Velázquez-Fernández *et al.,* 2005; Slater *et al.,* 2006; Laurell *et al.,* 2009). None of these were differentially expressed in AIMAH-01 (Table 4.4).

Table 4.4. AIMAH-01 expression data for selected genes, comparison with normal adrenal cortex.

Gene symbol	Gene name	Locus	Log_2FC	*t* statistic	FDR	Probe number
AVPR2	arginine vasopressin receptor 2	Xq28	-0.0106	-0.0761	0.9784	25
AVPR1B	arginine vasopressin receptor 1B	1q32	0.2522	1.6799	0.3792	25
AVPR1A	arginine vasopressin receptor 1A	12q14-q15	0.2408	1.1586	0.5642	32
FGFR1	fibroblast growth factor receptor 1	8p11.2-p11.1	-0.6823	-4.5199	0.0361	63
FGFR4	fibroblast growth factor receptor 4	5q35.1-qter	-1.1384	-7.1120	0.0066	48
IGF2	insulin-like growth factor 2 (somatomedin A)	11p15.5	-0.4728	-1.3041	0.5099	25
IGF2*			1.584	4.8635	0.0481	25
IGF2R	insulin-like growth factor 2 receptor	6q26	-0.6144	-3.5239	0.0781	58
IGFBP3	insulin-like growth factor binding protein 3	7p13-p12	-0.0484	-0.1689	0.9512	29
IGFBP6	insulin-like growth factor binding protein 6	12q13	-0.5012	-2.4413	0.2000	26
MEN1	multiple endocrine neoplasia I	11q13	-0.0589	-0.4362	0.8565	42
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	5q31.3	-0.3884	-3.2249	0.0999	41

Abbreviations: FC – fold-change; FDR – false discovery rate

*data given are for comparison between advanced *vs* early AIMAH

The fibroblast growth factors FGF-1 and FGF-2 are expressed in the adrenal cortex and are powerful mitogens for adult steroidogenic adrenocortical cells and H295R cells (Feige and Baird, 1991). FGF-1 and FGF-2 both bind to the growth factor receptors, FGFR1 and FGFR4; these receptors are also expressed in the adrenal cortex (Partanen *et al.,* 1991; Hughes, 1997). Both receptors have been found to be overexpressed in some ACC, but not others (Giordano *et al.,* 2003; de Fraipont *et al.,* 2005; Slater *et al.,* 2006; Laurell *et al.,* 2009; Soon *et al.,* 2009; de Reyniès *et al.,* 2009). Their overexpression may contribute to the proliferative potential of ACC. In contrast, in the AIMAH-01 expression data, there was a two-fold downregulation of FGFR4 (Table 4.4).

Overexpression of the glucocorticoid receptor (*NR3C1*) by 1.6- to 4-fold in ACC compared with ACA and with normal adrenal cortex, has now been reported by several groups (de Reyniès *et al.,* 2009; Giordano *et al.,* 2009; Soon *et al.,* 2009; Tacon *et al.,* 2009). This is a recent discovery, and its significance in the pathogenesis and treatment of ACC requires clarification. *NR3C1* was not differentially expressed in AIMAH-01 (Table 4.4).

The ACTH receptor (*MC2R*) was present, although downregulated (RT-qPCR) in a study of GIP-dependent AIMAH compared with normal adrenal cortex, although it was virtually absent in nonhyperplastic or nontumoral adjacent atrophic tissues (Antonini *et al.,* 2006). It was postulated that in the absence of circulating ACTH, there may be common mechanisms regulating GIP and ACTH receptor expression (Antonini *et al.,* 2006). Most patients with AIMAH have an exaggerated cortisol response to ACTH (Synacthen®; ACTH(1-24)) administration (Mircescu *et al.,* 2000). The exaggerated response may be due to hyperplasia – a massively increased number of cells expressing MC2R (albeit perhaps lower individual cellular expression). The expression of *MC2R* in the absence of circulating ACTH was an intriguing finding since *in vitro*, ACTH upregulates its own receptor, possibly by regulating one of the cAMP response elements in its promoter (Lebrethon *et al.,* 1994b; Mountjoy *et al.,*

1994; Morita *et al.,* 1995). Relative to normal adrenal cortex, MC2R was upregulated both in ACC (relative mean expression ± standard error of mean; 1.37 ± 0.67) and in ACA (3.04 ± 0.38); within each group relative expression was higher in functioning tumours (Tacon *et al.,* 2009). This suggests *MC2R* expression is more closely associated with tumour function, than malignancy (Tacon *et al.,* 2009). In our data set, *MC2R* was not differentially expressed (Table 4.4).

As discussed in Chapter 1, the menin gene, *MEN1*, is a classical tumour suppressor gene; homozygous inactivation of *MEN1* results in tumours in the multiple endocrine neoplasia type 1 (MEN1) syndrome. Menin mRNA and protein were highly expressed (RT-PCR; Western blot) in sporadic ACA and ACC causing Cushing's syndrome compared with corresponding adjacent tumour tissues and compared with ACT causing primary aldosteronism (Bhuiyan *et al.,* 2001). Menin was not differentially expressed in AIMAH-01 (Table 4.4).

In solid tumours, somatic copy number (CN) variations (CNV) frequently concur with gene expression analyses – i.e., genes affected by CNV frequently have expression patterns that correlate with CN (Cancer Genome Atlas Research Network, 2008). We performed CNV analysis of the AIMAH-01 tumours – the methods and results are presented in Chapter 5. In our comparisons of DEG and CNVs in AIMAH-01 tumours, there was no gene overlap. These data suggest that other factors (e.g., transcription factors) operate to maintain gene expression normal even when there has been an alteration (amplification or deletion) in gene dosage. Conversely, DEG in AIMAH-01 appear unrelated to variations in gene dosage, but could be modulated by changes in transcription factor activity or mRNA degradation. Alternatively, it is possible that there was no correlation because the tumour DNA (CNV studies) and RNA (gene expression studies) were extracted from different nodules and AIMAH is a heterogeneous disease process. As discussed earlier, Almeida *et al.,* studied AIMAH nodules for gene expression using microarrays and chromosomal amplifications or

deletions using CGH (Almeida *et al.,* 2011). In contrast to our data, they found half of the 50 most overexpressed genes to be amplified in CGH, although only nine of the 50 most underexpressed genes were located in regions of chromosomal losses (Almeida *et al.,* 2011).

Bioinformatics analyses of AIMAH-01 gene expression data

Gene function

IPA was used to assign gene function to the DEG (absolute FC ≥ 2 and FDR < 0.05) lists. The gene function lists for (all AIMAH *vs* normal) and (advanced *vs* early AIMAH) are found in Appendix 3; Tables A.3.1 and A.3.2. DEG functions for (all AIMAH *vs* normal) included metabolism (lipid, carbohydrate, amino acid), cell signalling and interaction, immunological processes, cell development, cell growth and proliferation, cancer, molecular transport and cell assembly and organization. Many of these functions were reported previously (Bourdeau *et al.,* 2004; Lampron *et al.,* 2006). DEG functions (advanced *vs* early AIMAH) included small molecule biochemistry, lipid metabolism, protein synthesis, cell cycle, cell signalling and interaction, cell movement, cell assembly and organization, cell function and maintenance, cell growth and proliferation and cancer.

IPA was also used to determine pathways that were overrepresented by the DEG. Selected pathways will be discussed later; however the pathways are listed in Appendix 3; Tables A.3.3 and A.3.4.

Comparison of AIMAH-01 tumours with other adrenocortical tumours

These data were obtained using GSEA. We sought to determine whether there was enrichment in our preranked (by *t* statistic) gene lists with published DEG sets of other ACT. The data are presented in Appendix 4; Tables A.4.1 and A.4.2. The (all AIMAH *vs* normal) ranked gene list was concordantly enriched for DEG in sporadic AIMAH (Figure 4.8; Figure 4.9, Panels A-G) (Bourdeau *et al.,* 2004; Almeida *et al.,* 2011). The study by Bourdeau *et al.,*

included sporadic AIMAH tumours with sensitivity to various exogenous ligands (GIP, *n*=3; VP, *n*=1; partial VP, *n*=1; β-receptor stimulation, *n*=1; none, *n*=3) (Bourdeau *et al.,* 2004). The gene lists were negatively enriched for downregulated DEG in a study of sporadic GIP-dependent AIMAH (Figure 4.9, Panel H; Lampron *et al.,* 2006). There was no enrichment for upregulated DEG in GIP-dependent AIMAH (Lampron *et al.,* 2006). These data suggest that some molecular mechanisms are shared between sporadic and familial AIMAH tumours; whilst distinct mechanisms may operate in different AIMAH subtypes (e.g. GIP- *vs* VP-sensitive).

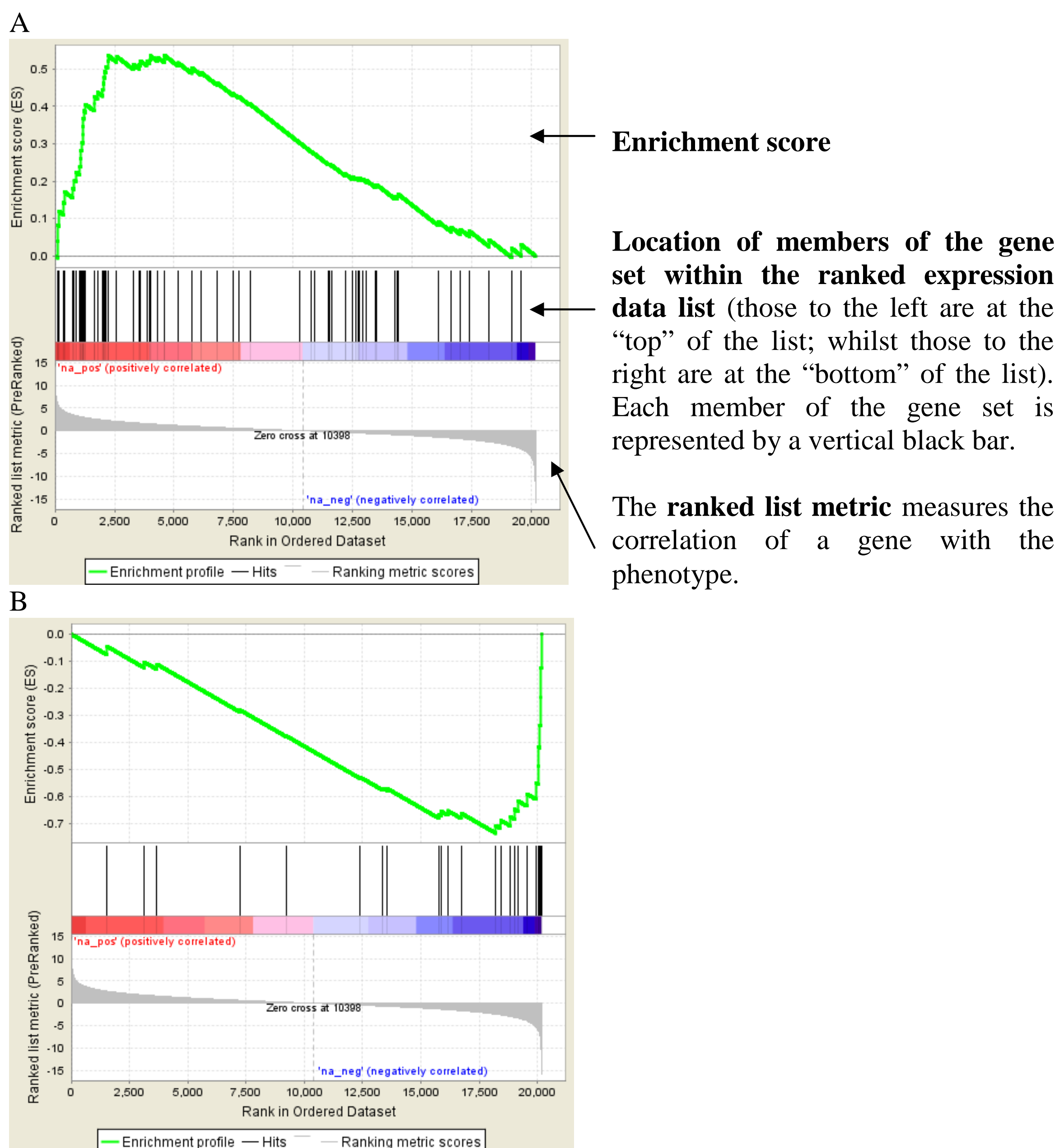


Figure 4.8. Familial AIMAH-01 enrichment plot for upregulated (Panel A) and downregulated (Panel B) genes in sporadic AIMAH. Upregulated genes in the sporadic AIMAH gene set were preferentially located at the top of our preranked gene list, and downregulated genes were preferentially located at the bottom of our preranked gene list; consistent with concordant enrichment of the sporadic AIMAH gene set in our ranked gene list. (Bourdeau *et al.,* 2004).

Enrichment plot: this provides a graphical view of the enrichment score (ES) for a gene set. The ES reflects the degree to which a gene set is over-represented at the top or bottom of a ranked list of genes. The *top portion of the plot* shows the running ES for the gene set as the analysis walks down the ranked list. The score at the peak of the plot is the ES for the gene set. The *middle portion of the plot* shows where the members of the gene set appear (each represented by a black line) in the ranked list of genes. The leading edge subset of a gene set is the subset of members that contribute most to the ES. For a positive ES (Panel A), the leading edge subset is the set of members that appear in the ranked list prior to the peak score. For a negative ES (Panel B), it is the set of members that appear subsequent to the peak score. The *bottom portion of the plot* shows the ranked list metric, which measures a gene's correlation with a phenotype. The value of the ranking metric goes from positive to negative as you move down the ranked list.

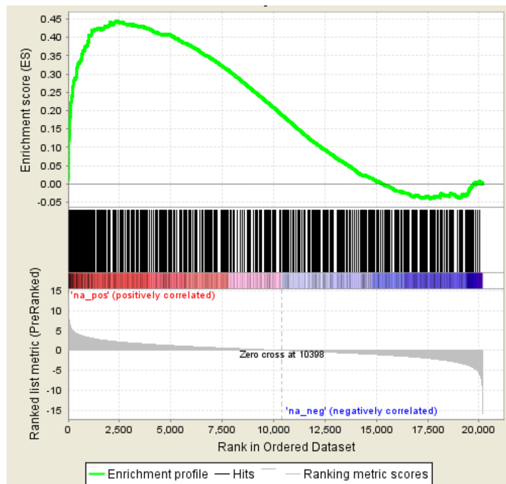
Figure 4.9. Gene Set Enrichment Analysis Plots.

Panel code	Top panel*	Bottom panel*
A	Almeida *et al.*, 2011: N1, Upregulated genes AIMAH	Almeida *et al.*, 2011: N1, Downregulated genes AIMAH
B	Almeida *et al.*, 2011: N2, Upregulated genes AIMAH	Almeida *et al.*, 2011: N2, Downregulated genes AIMAH
C	Almeida *et al.*, 2011: N3, Upregulated genes AIMAH	Almeida *et al.*, 2011: N3, Downregulated genes AIMAH
D	Almeida *et al.*, 2011: N4, Upregulated genes AIMAH	Almeida *et al.*, 2011: N4, Downregulated genes AIMAH
E	Almeida *et al.*, 2011: N5, Upregulated genes AIMAH	Almeida *et al.*, 2011: N5, Downregulated genes AIMAH
F	Almeida *et al.*, 2011: N6, Upregulated genes AIMAH	Almeida *et al.*, 2011: N6, Downregulated genes AIMAH
G	Almeida *et al.*, 2011: N7, Upregulated genes AIMAH	Almeida *et al.*, 2011: N7, Downregulated genes AIMAH
H	Lampron *et al.*, 2006: Downregulated genes AIMAH	
I	Giordano *et al.*, 2003: Upregulated genes	Giordano *et al.*, 2003: Downregulated genes
J	Soon *et al.*, 2009: Upregulated genes	Soon *et al.*, 2009: Downregulated genes
K	de Reyniès *et al.*, 2009: Upregulated genes in ACC	de Reyniès *et al.*, 2009: Downregulated genes in ACC
L	de Reyniès *et al.*, 2009: Upregulated genes in aggressive ACC	de Reyniès *et al.*, 2009: Downregulated genes in aggressive ACC
M	Horvath *et al.*, 2006: Upregulated genes in PPNAD	Horvath *et al.*, 2006; Downregulated genes in PPNAD
N	Schimmer et al, 2006: Upregulated genes by ACTH	
O	Giordano *et al.*, 2009: Upregulated genes in ACA	Giordano *et al.*, 2009: Downregulated genes in ACA
P	Doghman *et al.*, 2007: Upregulated genes by SF-1	
Q	West *et al.*, 2007: Upregulated genes in paediatric ACT	West *et al.*, 2007: Upregulated genes in paediatric ACT
R	Scacheri *et al.*, 2006: Upregulated genes by menin	

Abbreviations: ACA – adrenocortical adenoma; ACC – adrenocortical carcinoma; ACTH – adrenocorticotropic hormone; NX – nodule X; PPNAD – primary pigmented nodular adrenocortical disease; SF-1 steroidogenic factor-1

These plots were derived using Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea).
See Legend, Figure 4.8, for explanation of the enrichment plots.

A



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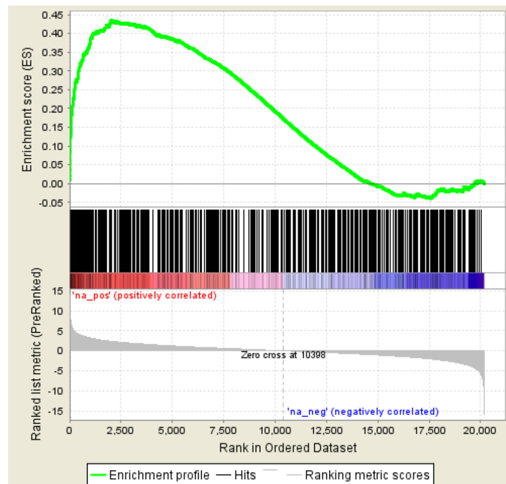

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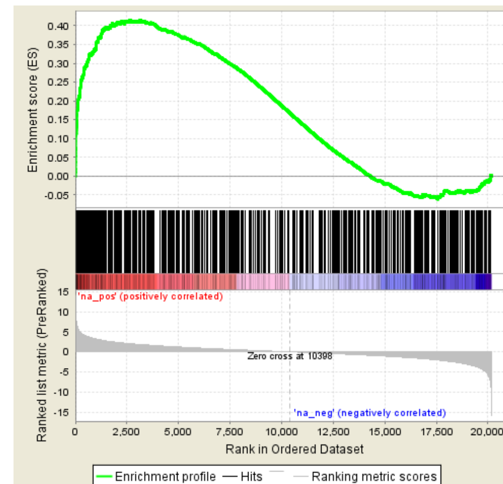

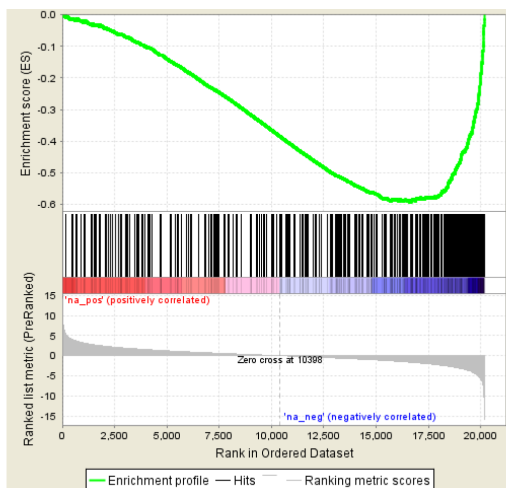

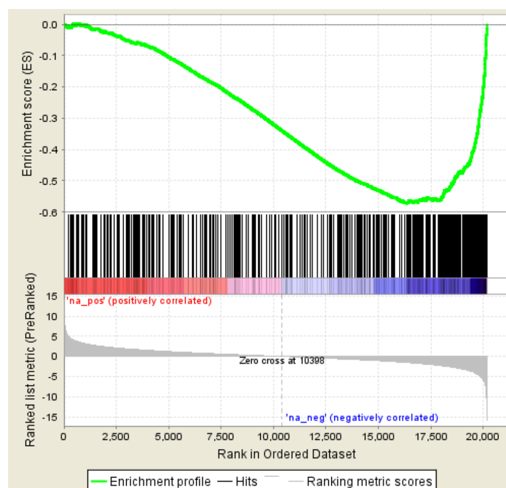

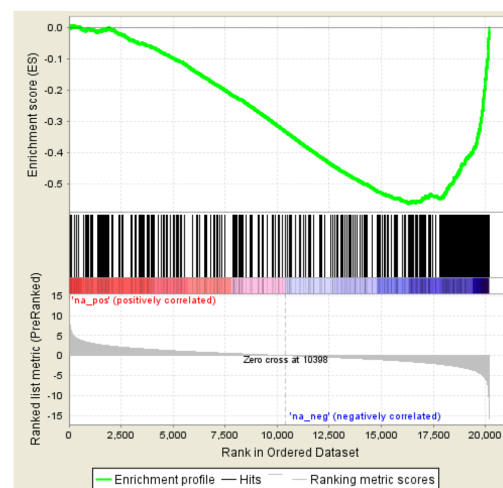

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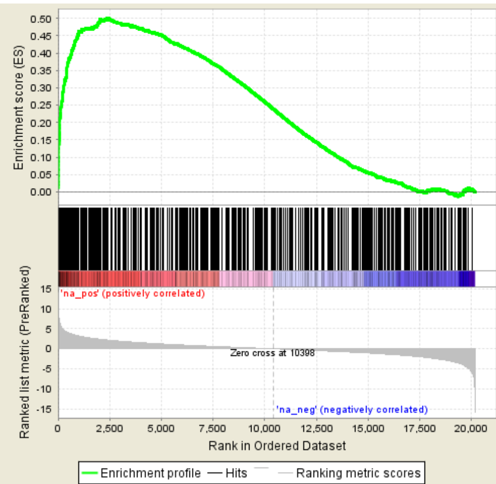

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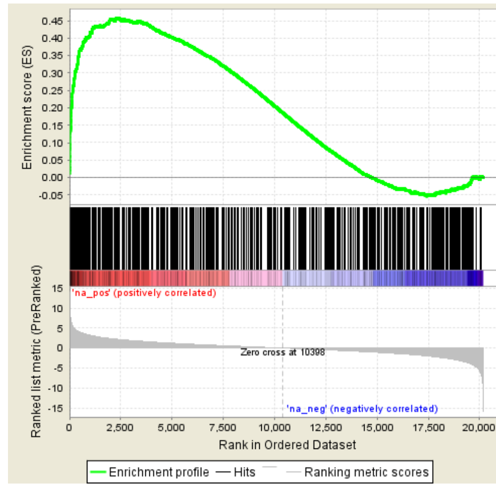

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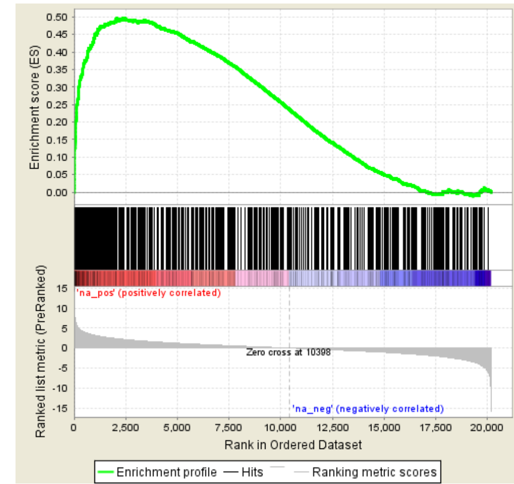

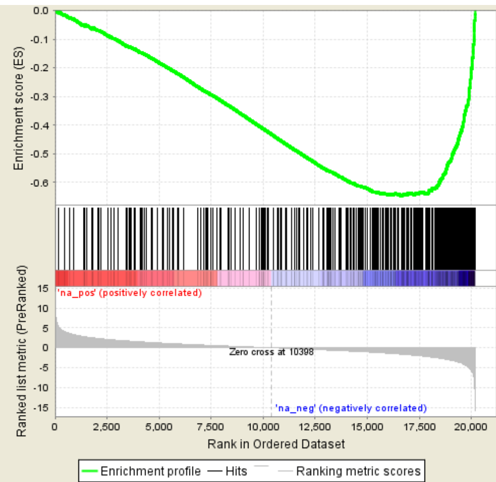

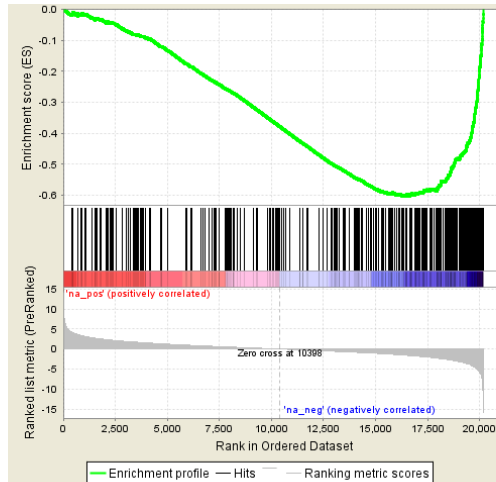

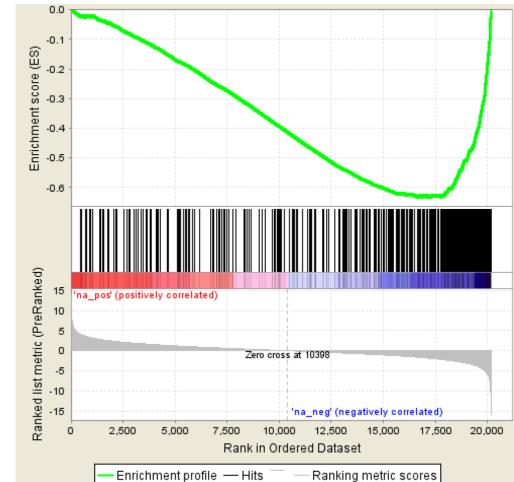

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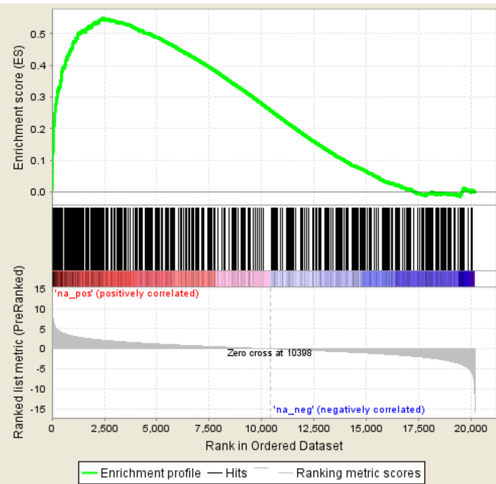

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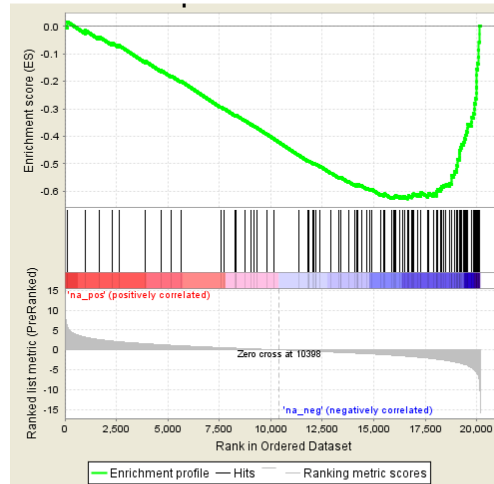

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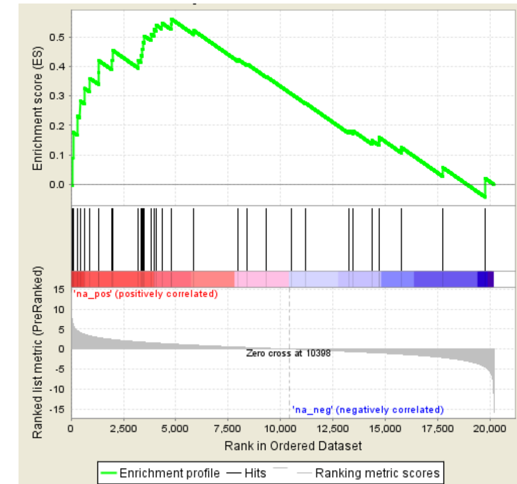

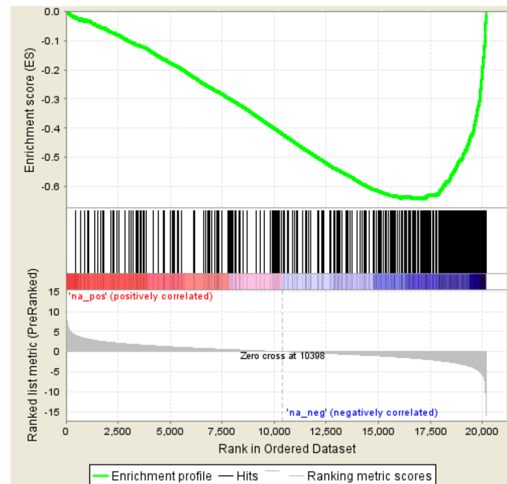

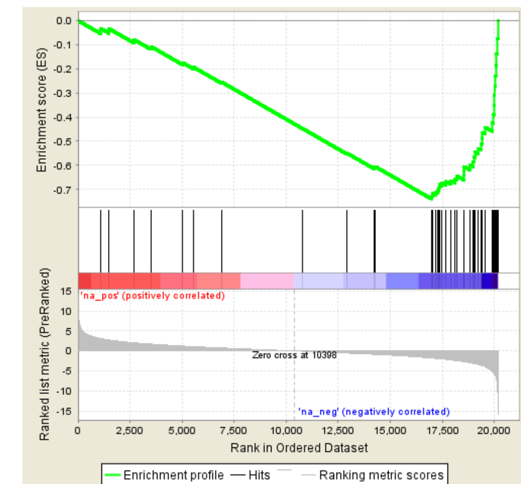

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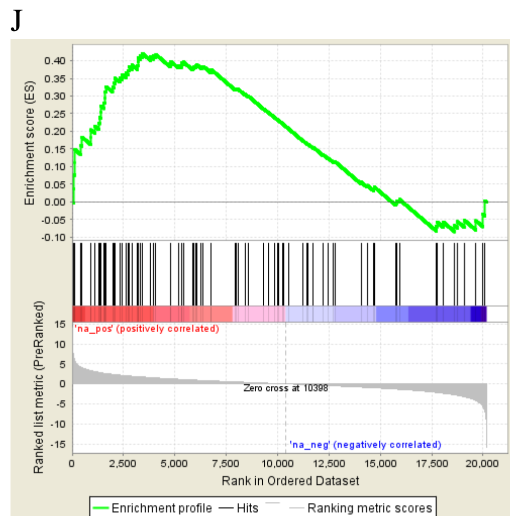

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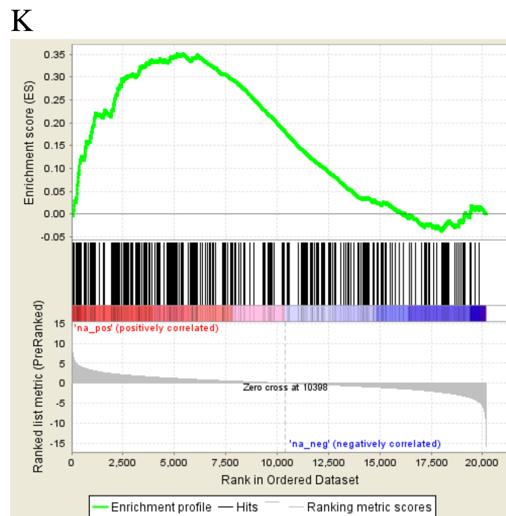

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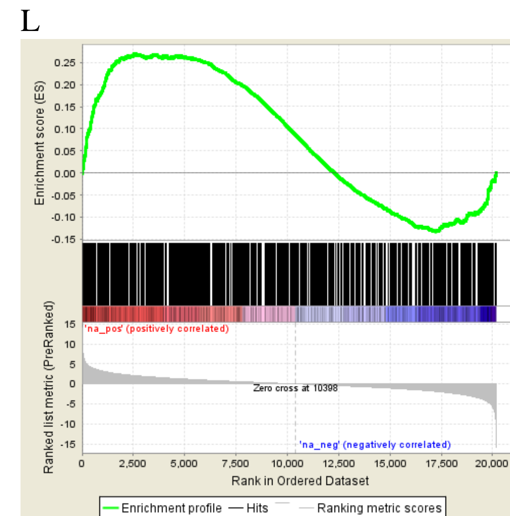

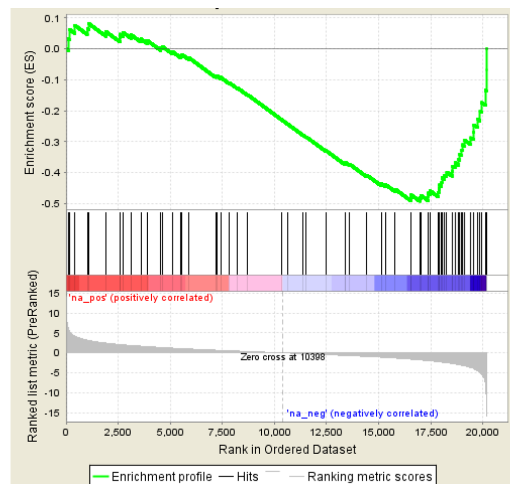

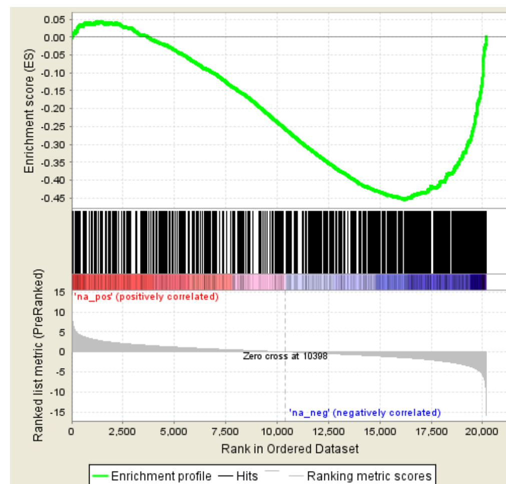

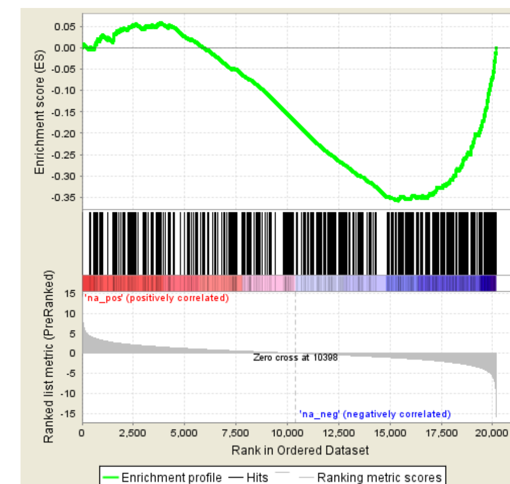

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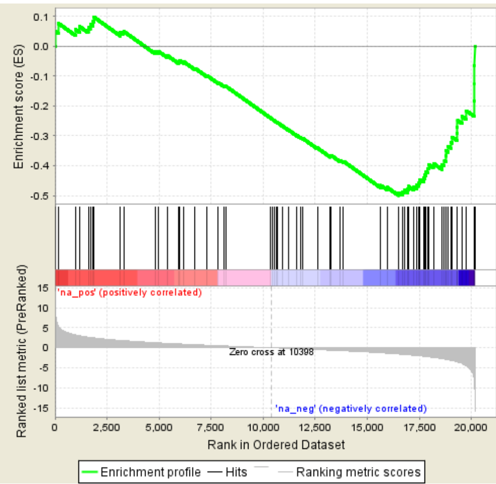

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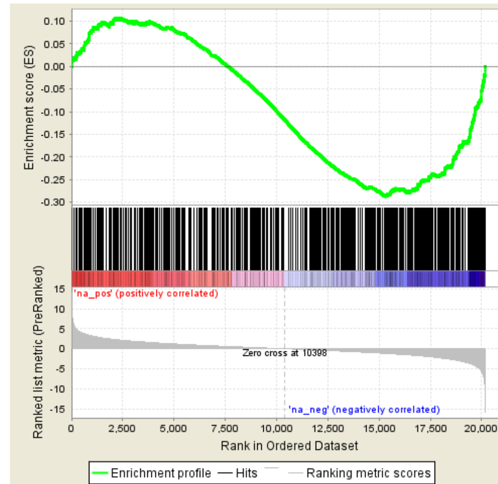

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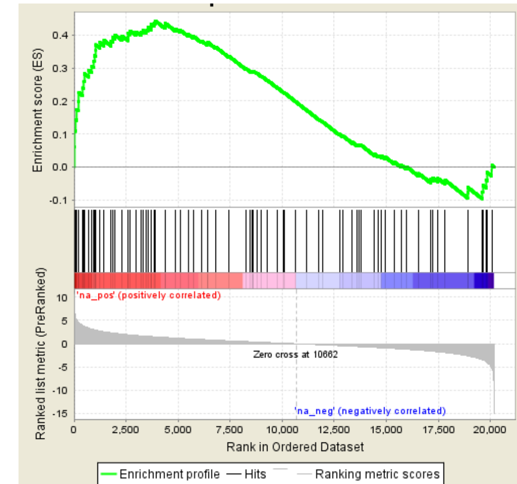

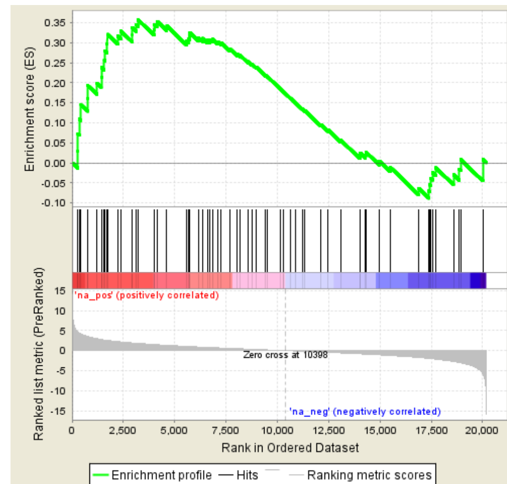

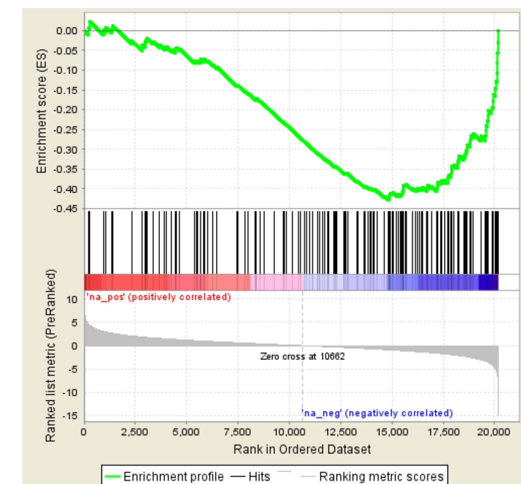

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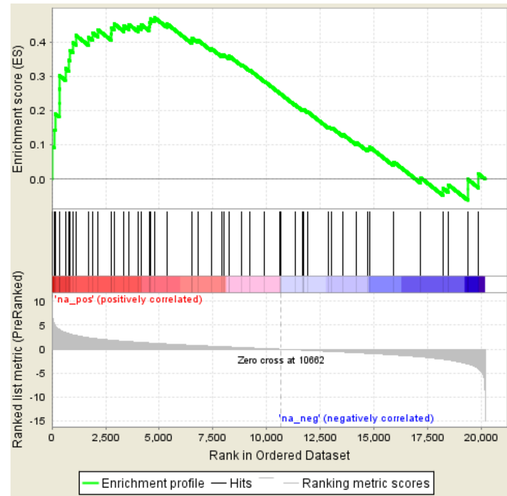

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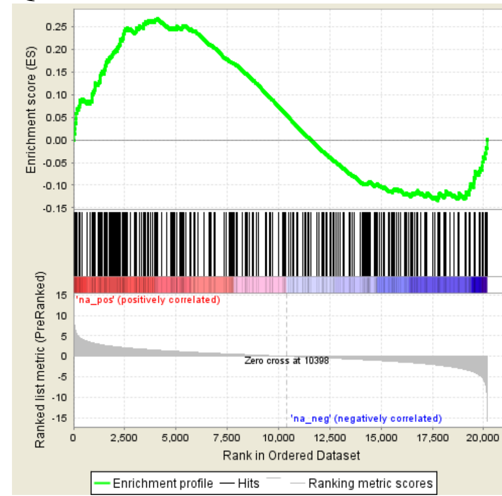

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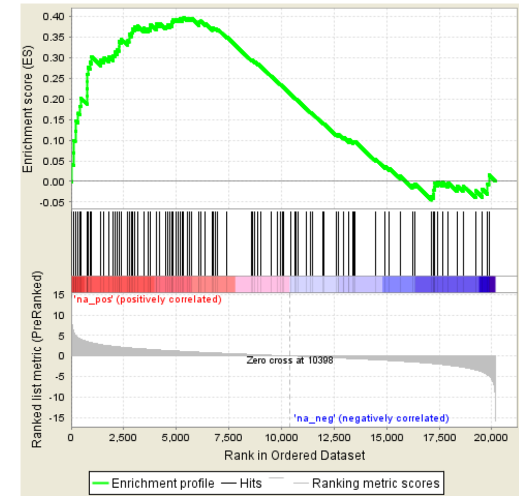
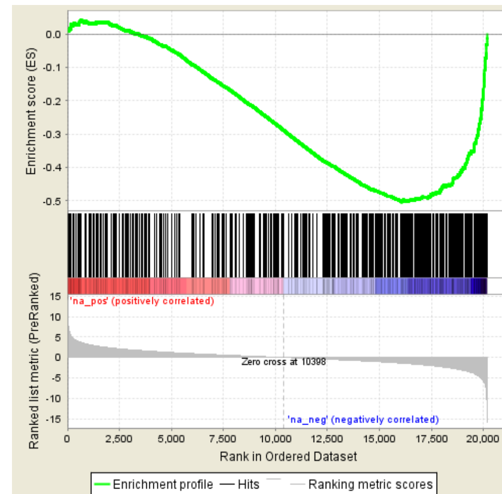




We also sought to determine whether there was enrichment in the ranked (all AIMAH *vs* normal) gene list of DEG sets from studies of other ACT (adrenocortical carcinoma, ACC and adrenocortical adenoma, ACA). In all studies, ACT were classified according to established histopathologic criteria (Weiss score) (Weiss, 1984). There was concordant enrichment (positive enrichment for upregulated genes; negative enrichment for downregulated genes) for DEG in ACC compared with normal adrenal gland/cortex or ACA, in ACC with an aggressive, malignant course and in paediatric ACT (Figure 4.9, Panels I-L, Q) (Appendix 4; Tables A.4.1 and A.4.2) (Giordano *et al.,* 2003; Soon *et al.,* 2009; de Reyniès *et al.,* 2009; West *et al.,* 2007). These data suggest a molecular signature of malignancy in the AIMAH nodules, which was not predictable based on the benign clinical course of treated AIMAH, with no known propensity for metastasis. The data also suggest that AIMAH shares molecular mechanisms with other ACT. This is surprising, since AIMAH is bilateral, with monoclonal and polyclonal nodules observed, whilst ACC and ACA are mostly unilateral and monoclonal in origin (Gicquel *et al.,* 1994).

MEN1 is a familial syndrome caused by mutations in the tumour suppressor gene, menin (*MEN1*) (Thakker *et al.,* 1989; Friedman *et al.,* 1989). Carriers of *MEN1* germline mutations are predisposed to develop various endocrine tumours, although a somatic (second-hit) mutation, is necessary (Knudson, Jr, 1971). Mutations in *MEN1* occur rarely in sporadic ACT (Görtz *et al.,* 1999; Heppner *et al.,* 1999). Since *men1* null mice show developmental defects and die at embryonic day 11.5 to 13.5; several groups have developed a *men1* conditional knockout mouse model (Bertolino *et al.,* 2003; Biondi *et al.,* 2004; Crabtree *et al.,* 2003).

Genes positively regulated by menin in a *men1* conditional null murine model, were enriched in the AIMAH data sets (Figure 4.9, Panel R) (Appendix 4; Tables A.4.1 and A.4.2) (Scacheri *et al.,* 2006). This could suggest a role for menin in the tumorigenesis of AIMAH, although, as already discussed, menin was not differentially expressed in our microarray analysis (Table

4.4). However, expression has only been studied at the mRNA level; we have not validated expression by RT-qPCR and have not examined protein expression. Nevertheless, the menin gene set we used is likely to represent only a small subset of all genes regulated by menin; had our comparison been of a larger list of menin-regulated genes, our findings may have been different. Also, the menin gene set was derived from DEG in pancreatic islet cells from conditional null mice (15 weeks of age) compared with wildtype mice; in contrast our ranked list was derived from DEG in human AIMAH compared with normal human adrenal cortex (Scacheri *et al.,* 2006). In these different tissues and species, the genes regulated by menin may also be different. Furthermore, GSEA is an analytical method, designed to generate hypotheses for further study, but not intended to otherwise substitute for the obtained biological data (Subramanian *et al.,* 2005).

The data sets were discordantly enriched (positive enrichment for downregulated genes; negative enrichment for upregulated genes) for genes regulated by ACTH in the murine adrenocortical tumour cell line, Y1 (Schimmer *et al.,* 2006) (Figure 4.9, Panel N). This may be due to the absence of ACTH in AIMAH and is consistent with the prevailing concept of the ACTH-independence of tumorigenesis in AIMAH, discussed in Chapter 1 (Antonini *et al.,* 2006). In contrast however, detection of intra-adrenal ACTH was reported in two cases of AIMAH; inciting speculation that locally produced ACTH may regulate adrenocortical steroidogenesis and/or proliferation (Lefebvre *et al.,* 2003; Mazzuco *et al.,* 2007).

In the first study, ACTH immunoreactivity was present in cells of the peripheral and central zones of hyperplastic nodules from a 46 year old male with Cushing's syndrome due to AIMAH; the organisation of the nodules around clusters of ACTH-containing cells suggested that ACTH may have stimulated proliferation *via* a paracrine or autocrine mechanism (Lefebvre *et al.,* 2003). *In situ* hybridization detected intracellular pro-opiomelanocortin (*POMC*) mRNA in a similar distribution to ACTH immunoreactivity, suggesting that intra-

adrenal ACTH was due to ectopic *POMC* expression (Lefebvre *et al.,* 2003). The estimated intra-adrenal interstitial space ACTH concentration was 1000-fold greater than normal plasma ACTH levels and the ACTH released from perifused hyperplasia fragments was correlated with cortisol levels (Lefebvre *et al.,* 2003). *In vivo*, and prior to adrenalectomy, there was a 270% increase in cortisol following administration of Cosyntropin (250μg, intravenously) (Lefebvre *et al.,* 2003). The data suggest that intra-adrenal ACTH, operating *via* a paracrine or autocrine mechanism, may stimulate cortisol production and/or, as already discussed, adrenocortical cell proliferation (Lefebvre *et al.,* 2003). The mechanism by which a subpopulation of adrenal cells produced ACTH was not elucidated (Lefebvre *et al.,* 2003).

In another study, conditioned medium from AIMAH cultured cells strongly increased cortisol production from normal cells (Mazzuco *et al.,* 2007). Using an immunoradiometric assay with a specific antiserum, ACTH was detected in AIMAH, but not in normal, cells; these findings were confirmed immunohistochemically (Mazzuco *et al.,* 2007). β-endorphin and α-melanocyte stimulating hormone were not detected in the AIMAH tissue, suggesting intra-adrenal production of ACTH, rather than POMC (Mazzuco *et al.,* 2007). The cortisol response to ACTH administration was negligible compared with control cells; however basal cortisol levels were 30-fold higher; it was suggested that intra-adrenal ACTH might have regulated basal cortisol secretion (Mazzuco *et al.,* 2007).

These are the only two reported cases of AIMAH where there is some evidence that intra-adrenal ACTH might regulate adrenocortical cell proliferation and/or steroidogenesis. On the contrary, and consistent with the prevailing theory that these tumours are truly ACTH-independent, we found discordant enrichment for genes regulated by ACTH in our ranked gene list. Our data do not provide evidence for intra-adrenal production of ACTH, in which case we would have expected concordant enrichment for genes regulated by ACTH.

Steroidogenic factor-1 (SF-1) is a nuclear receptor transcription factor with a critical role in adrenal development and steroidogenesis, as discussed in Chapter 1 (Ozisik *et al.,* 2003). Compared with ACT in adults, paediatric ACT have higher expression of *SF-1* (RT-qPCR) and gene amplification of *SF-1* is also more frequent (47% *vs* 10%; paediatric *vs* adult) (Almeida *et al.,* 2010). Increased *SF-1* dosage increases proliferation, decreases apoptosis of human adrenocortical cells and induces adrenocortical tumours in transgenic mice (Figueiredo *et al.,* 2005; Pianovski *et al.,* 2006; Doghman *et al.,* 2007). Our data do not suggest a role for SF-1 having a positive regulatory effect in AIMAH, since there was no enrichment for genes regulated by an increased SF-1 dosage (Appendix 4; Table A.4.1).

There was enrichment for DEG in ACA (*vs* normal) in the advanced *vs* early AIMAH ranked gene list (Figure 4.9, Panel O; Appendix 4; Tables A.4.3 and A.4.4) (Giordano *et al.,* 2009). These data suggest that there may be distinct molecular mechanisms acquired in advanced AIMAH, beyond merely a longer duration of disease.

The Steroidogenic Pathway in AIMAH-01

The adrenal steroid synthetic pathway has been discussed in Chapter 1 and is shown in Figure 4.10. As discussed in Chapters 1 and 3, steroidogenesis is inefficient in AIMAH. This is due to differential expression of steroidogenic enzymes in AIMAH cells and accounts for the discordance between the massive macronodular adrenal glands and the relatively mild hypercortisolism. Classically, the adrenal nodules of AIMAH are comprised of clear and compact cells (described in Chapters 1 and 2). *In situ* hybridisation and immunohistochemical studies have found that 3β-hydroxysteroid dehydrogenase 2 (3βHSD2) is expressed only in clear cells, whilst CYP17A1 is expressed predominantly in compact cells; this differential expression of steroidogenic enzymes renders these cells "co-dependent" for steroid synthesis (Sasano *et al.,* 1994; Wada *et al.,* 1996; Aiba *et al.,* 1991). This differential localization of steroidogenic enzymes is unique to AIMAH. The mechanism underlying reduced

steroidogenic enzyme expression in AIMAH is not known – although it may be due to de-differentiation of tumour cells. Alternatively, as discussed in Chapter 3, it could be the primary defect in AIMAH.

DEG and transcription factors predicted to have altered activity and involved in the adrenal steroid synthetic pathway in AIMAH-01 are shown in Figure 4.10. RT-qPCR of genes encoding steroidogenic enzymes and other proteins important in the steroidogenic synthetic pathway (steroidogenic acute regulatory protein (*StAR*) and the transcription factors Sp1 and steroidogenic factor-1 (*SF-1*)) was performed in the AIMAH-01 tumours and the normal adrenal glands. StAR has an important role in the acute regulation of steroidogenesis because it transports cholesterol to the inner mitochondrial membrane, the location of CYP11A1; the transport of cholesterol to CYP11A1 is a rate-limiting step of steroidogenesis (Clark *et al.,* 1994). The conversion of cholesterol to pregnenolone by CYP11A1 is also a rate-limiting step of steroidogenesis (Miller, 2008). SF-1 and Sp1 are important transcriptional regulators of the expression of *CYP11A1* and *StAR*; through their influence on the expression of *CYP11A1* and *StAR*, they are thus also important regulators of steroidogenesis overall (Sugawara *et al.,* 2000). Hence *StAR*, *SF-1* and *Sp1*, in addition to the steroidogenic enzymes, were selected for analysis by RT-qPCR.

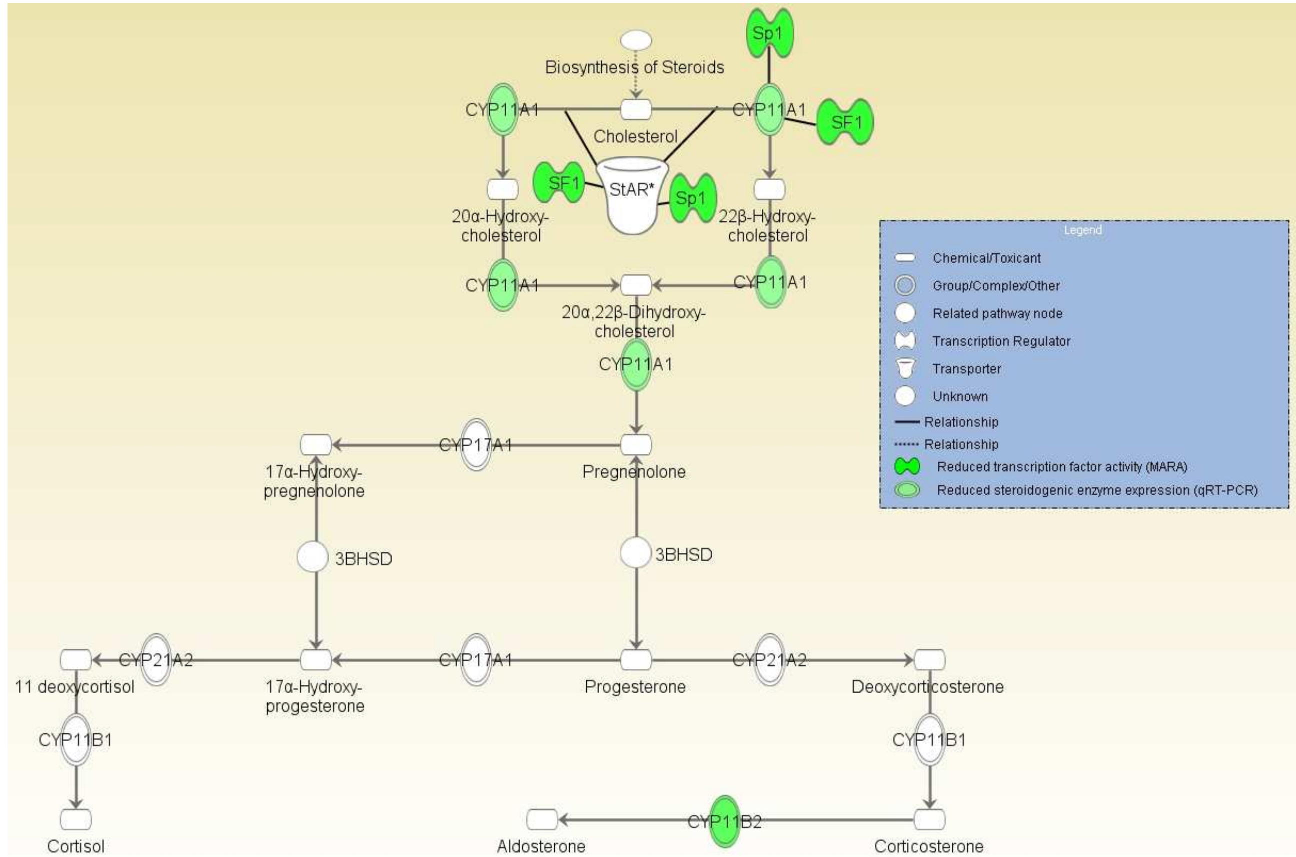


Figure 4.10. The adrenal steroid synthetic pathway in AIMAH-01.
Enzymes and transcription factors with altered expression or activity in AIMAH-01 are indicated in green. The cholesterol side-chain cleavage enzyme (CYP11A1) (7-fold; p=0.09) and aldosterone synthase (CYP11B2) (13-fold; p=0.004). There was a trend to downregulation of Steroidogenic Acute Regulatory (StAR) protein (2-fold; p=0.22); the transporter protein which facilitates delivery of cholesterol to the inner mitochondrial membrane, the site of CYP11A1. Transcription factor profiling using motif activity response analysis (MARA) predicted reduced activity of Steroidogenic factor 1 (SF1) and transcription factor Sp1 (Sp1); both are regulators of CYP11A1 and StAR expression. Figure generated using Ingenuity Pathway Analysis (Ingenuity Systems®; http://www.ingenuity.com/).

The RT-qPCR results are summarized in Table 4.5. The mean concentration (± standard error) of each gene studied for the AIMAH-01 and normal adrenals is shown in Figure 4.11. We measured basal and stimulated (after Synacthen®) steroid intermediates *in vivo* in III-2 and III-3 (AIMAH-01; Figure 2.1). These data have been presented in Chapter 3. We compared the steroidogenic enzyme defects suggested by measurement of the steroid intermediates with the gene expression and RT-qPCR data.

Table 4.5. Expression of enzymes and transcription factors involved in the steroidogenic pathway, in AIMAH-01.

Gene name	Log_2FC	FDR	FC (q-PCR)	*p*-value (qPCR)
Transcription factors				
Steroidogenic acute regulatory protein (StAR)	(-)0.55	0.11	-2.3	0.22
Steroidogenic factor 1 (SF-1, NR5A1)	(-)0.04	0.91	+1.1	0.8
Sp1 transcription factor (Sp1)	(+)0.06	0.87	+1.2	0.78
Steroidogenic enzymes				
Cholesterol side-chain cleavage enzyme (CYP11A1)	(-)1.4	0.02	-7.4	0.09
11β-hydroxylase (CYP11B1)	(-)0.31	0.35	-1.8	0.17
Aldosterone synthase (CYP11B2)	(-)2.1	0.0005	-13.4	0.004
17α-hydroxylase (CYP17A1)	(-)0.55	0.13	-1.5	0.38
21-hydroxylase (CYP21A2)	(-)0.3	0.38	+1.5	0.45
3β-hydroxysteroid dehydrogenase 2 (3βHSD2)	(-)0.26	0.36	+0.97	0.86

Log_2FC – according to microarray; FDR – false-discovery rate (*p*-value obtained after multiple test correction); FC (fold-change) obtained by quantitative reverse transcription-PCR

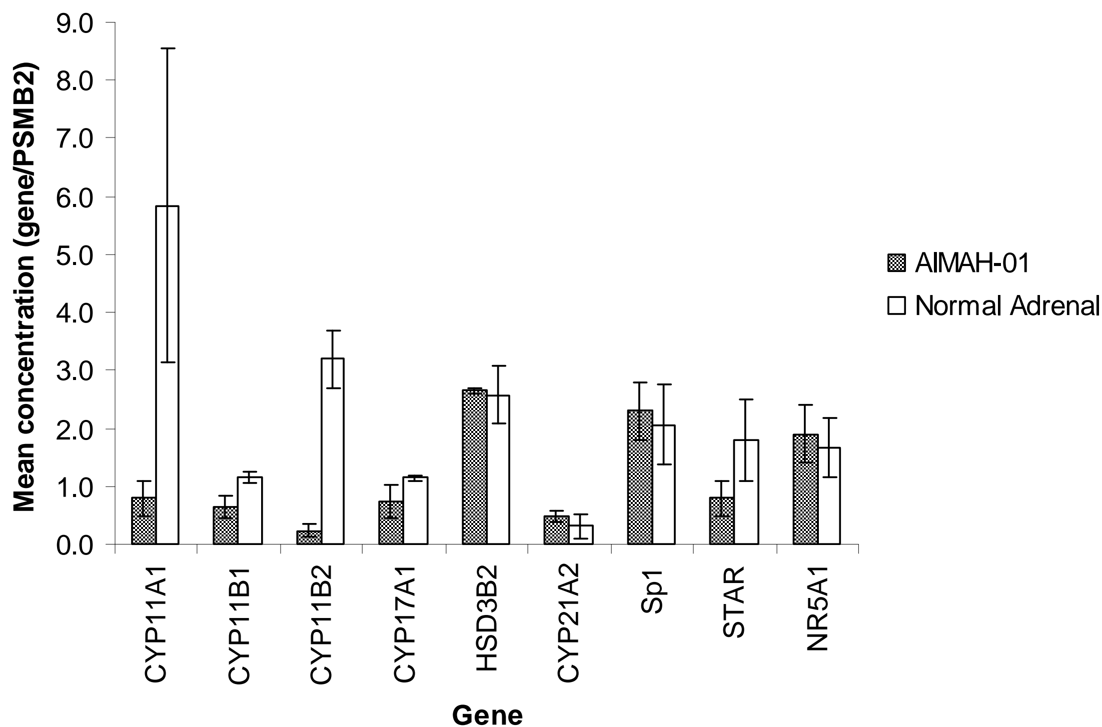


Figure 4.11. Expression of enzymes and transcription factors involved in the steroidogenic pathway in AIMAH-01 tumours. The expression in normal adrenal is also shown. (RT-qPCR)Gene expression was normalized to the housekeeping gene, proteasome subunit beta type-2 (*PSMB2*). The data are shown as mean ± standard error.
CYP11B2 was the only statistically significantly differentially expressed steroidogenic enzyme ($p < 0.05$). There was a trend to downregulation of *CYP11A1* in the AIMAH-01 nodules (p=0.09).
Abbreviations: CYP11A1 - cholesterol side chain cleavage enzyme; CYP11B1 - 11β-hydroxylase; CYP11B2 - aldosterone synthase; CYP17A1 - 17α-hydroxylase; CYP21A2 - 21-hydroxylase; 3βHSD2 - 3β-hydroxysteroid dehydrogenase 2; NR5A1 – steroidogenic factor-1 (SF-1); Sp1 – transcription factor Sp1; STAR – steroidogenic acute regulatory protein

There was a 13-fold reduction in aldosterone synthase (*CYP11B2*) expression (p=0.004) and a trend to downregulation (seven-fold) of *CYP11A1* (p=0.09) in the AIMAH-01 nodules compared with normal adrenal cortex. A seven-fold reduction in expression of *CYP11A1* may be biologically significant. *CYP11A1* catalyses the first, rate-limiting step of cortisol synthesis (the conversion of cholesterol to pregnenolone) (Figure 4.10), and is the critical determinant of the adrenal gland's chronic steroidogenic capacity (Miller, 2008). Hence, downregulation of *CYP11A1* would impair the cellular cortisol-producing capacity and could account for, at least in part, the discordance between the macronodular adrenal glands of III-2 and III-3 (AIMAH-01; Figure 2.1) and the relatively mild hypercortisolism. Furthermore,

there was a trend to downregulation (1.8-fold) of expression (FDR=0.06) of an adrenal-specific transcriptional regulator of *CYP11A1* (transcriptional-regulating protein 132; *TREP-132/TRERF1*), which interacts with SF-1 and CREB-binding protein (CBP/p300) to enhance *CYP11A1* promoter activity (Gizard *et al.,* 2001; Gizard *et al.,* 2002). Lower pregnenolone levels could be predicted as a result of downregulation of *CYP11A1* in AIMAH-01 (Figure 4.10). However, as discussed in Chapter 3, basal and stimulated pregnenolone levels in these individuals were similar to historical controls (Table 3.1) (Munabi *et al.,* 1986). Pregnenolone levels vary widely in normal individuals, and they reflect total adrenocortical, rather than individual cellular, CYP11A1 function (Munabi *et al.,* 1986). Thus, downregulation of *CYP11A1* in the AIMAH-01 nodules may have been relatively compensated for by adrenocortical hyperplasia, thereby maintaining normal pregnenolone levels.

Reduced expression of *CYP11B2* in the AIMAH-01 tumours is consistent with the low basal and stimulated aldosterone levels measured in these patients compared with historical controls, presented in Chapter 3 (Raff and Findling, 1990). However, also as discussed in Chapter 3, there were several confounding factors that could have altered aldosterone levels. Furthermore, *CYP11B2* is expressed exclusively in the zona glomerulosa of the adrenal cortex where it catalyses the production of aldosterone from corticosterone; the differential gene expression therefore likely reflects the presence and absence of zona glomerulosa in the normal and AIMAH-01 samples, respectively; the cortisol-secreting AIMAH-01 nodules are derived from the zona fasciculata (White, 1994). In addition, if *CYP11B2* expression was truly decreased in the zona glomerulosa of the AIMAH-01 tumours, then corticosterone (the immediate precursor of aldosterone) levels could be expected to be increased, due to reduced conversion to aldosterone (Figure 4.11). However, corticosterone levels were not significantly different from controls; although partial CYP11B1 deficiency could have abrogated an increase in corticosterone (Table 3.1; Figure 3.1) (Nishida *et al.,* 1977). Clarification of *CYP11B2* expression could be achieved by laser capture of zona glomerulosa

from the AIMAH-01 tumours followed by protein detection studies (e.g., immunohistochemistry).

Compared with historical controls, there was a nonsignificant trend to higher concentrations of 11-deoxycortisol, progesterone and 17-hydroxyprogesterone after Synacthen® administration, in III-2 and III-3 (Table 3.1) (Munabi *et al.,* 1986). This suggested partial abnormalities of the enzymatic steps regulated by CYP11B1, CYP17A1 and CYP21A2 (Figure 4.10). RT-qPCR suggested -1.8, -1.5 and +1.5 FC in mRNA expression, respectively ($p > 0.05$ for all) which is consistent with the biochemistry which suggested partial enzyme deficiency, although upregulation of *CYP21A2* is not. This latter discrepancy could be explained by cellular heterogeneity of enzyme expression in the AIMAH-01 nodules or the use of different control groups for the biochemical and molecular studies. Moreover, since Synacthen® testing was performed post-adrenalectomy, the *in vivo* steroid intermediate studies evaluated the physiology of the left adrenal, whilst the expression studies were performed on tumour nodules from the (resected) right adrenal glands; this could account for the discrepancy between the clinical and molecular data since heterogeneity between AIMAH adrenal glands has been observed, most obvious by morphological asymmetry between the glands (N'Diaye *et al.,* 1999).

Motif activity response analysis (MARA) predicted reduced activities of transcription factor Sp1 (Sp1) and steroidogenic factor-1 (SF-1/NR5A1) in AIMAH-01 (Figure 4.10). As already discussed, these transcription factors regulate gene expression of the enzymes or proteins that mediate the two rate-limiting steps of steroidogenesis. Firstly, they regulate the conversion of cholesterol to pregnenolone by regulating *CYP11A1* transcription (Liu and Simpson, 1997). Reduced activity of Sp1 and SF-1 could explain the seven-fold downregulation of *CYP11A1* in the nodules, in conjunction with a trend to reduced expression of *TRERF1*, discussed earlier. Secondly, SF-1 and Sp1 regulate *StAR* expression (Sugawara *et al.,* 2000). The

important role of StAR in the delivery of cholesterol to the inner mitochondrial membrane, the site of CYP11A1, has been discussed above and in Chapter 1. Hence reduced activity of SF-1 and Sp1 could also result in a defect in the mitochondrial transport of cholesterol. *Sp1* and *SF-1* were not differentially expressed in the AIMAH-01 tumours compared with normal adrenal gland (RT-qPCR, FC 1.2 and 1.1, respectively). Hence, reduced activity of these transcription factors can not be explained by reduced gene expression. We postulate that reduced transcription factor activity may be due to altered post-translational modification of these transcription factor proteins (e.g., lowering their activity, increasing their degradation or altering their interactions with coactivators) or due to reduced activity of an altered, common transcriptional coactivator.

There was a trend to a two-fold downregulation (RT-qPCR) of *StAR* expression in AIMAH-01 (p=0.22) (Table 4.5). The downregulation of *StAR* in the AIMAH-01 tumours, although not statistically significant, may have biological significance for the function of the steroidogenic pathway in AIMAH, particularly in conjunction with the downregulation of *CYP11A1*. Furthermore, AIMAH is heterogeneous; therefore there may be more severe defects in *StAR* expression in other AIMAH cells that we have not studied. Defective transport of cholesterol to CYP11A1, due to downregulation of *StAR* is a novel mechanism of inefficient steroidogenesis in AIMAH. Until now, we have known that steroidogenesis in AIMAH is inefficient due to the reduced expression of various enzymes, but the precise mechanisms have not been further elucidated. Since all of these genes are regulated by ACTH, these data of altered activity of transcription regulators of critical steroidogenic enzymes or other proteins involved in the steroidogenic pathway may be explained by undetectable circulating ACTH levels in AIMAH.

The promoter of the GIP receptor gene has six consensus binding sites for Sp transcription factors (Baldacchino *et al.,* 2005). Co-regulator of Sp1 transcription factor, subunit 9

(CRSP9; an important regulator of Sp1 activity) and Sp3 transcription factor were overexpressed in GIP-dependent AIMAH (Ryu *et al.,* 1999; Baldacchino *et al.,* 2005). However, these transcription factors were also overexpressed in non-GIP sensitive adrenal adenomas and Cushing's disease; thus, their overexpression alone is not sufficient to explain ectopic GIP receptor expression (Baldacchino *et al.,* 2005). In contrast, predictive transcription factor activity modelling (MARA) of the non-GIP-dependent AIMAH-01 tumours, suggested reduced activity of Sp1. If GIP sensitivity and hence ectopic GIP receptor expression is (even partly) Sp1 dependent, then reduced activity of Sp1 could account for the non-GIP-sensitivity of the AIMAH-01 tumours.

Genes regulated by increased *SF-1* dosage were enriched in our advanced *vs* early AIMAH ranked gene list (GSEA) (Figure 4.9, Panel P) (Doghman *et al.,* 2007). Furthermore, RT-qPCR showed a trend towards a two-fold upregulation of *SF-1* in advanced AIMAH, compared with the early AIMAH tumours. These data suggest a possible role for *SF-1* in disease progression in AIMAH-01. The distinction between early and advanced AIMAH forms was based on the clinical phenotype, and this was ultimately dependent on the cortisol-producing capacity, and hence total adrenocortical cellular mass, of the adrenal glands. Increased SF-1 dosage is present in childhood ACT and in some adult ACT, it augments proliferation and diminishes apoptosis of an ACT cell line (H295R) *in vitro* and stimulates ACT development in transgenic mice; hence a role in progression from early to advanced AIMAH is biologically plausible (Almeida *et al.,* 2010; Figueiredo *et al.,* 2005; Pianovski *et al.,* 2006; Doghman *et al.,* 2007).

Transforming growth factor β 2 (TGFβ2) belongs to the TGFβ superfamily of structurally related proteins that are involved in the regulation of a wide array of biological functions including cell growth, differentiation and angiogenesis (Massagué *et al.,* 2000). *TGFβ2* was five-fold (RT-qPCR; p=0.004) upregulated in the AIMAH-01 nodules (Figure 4.12).

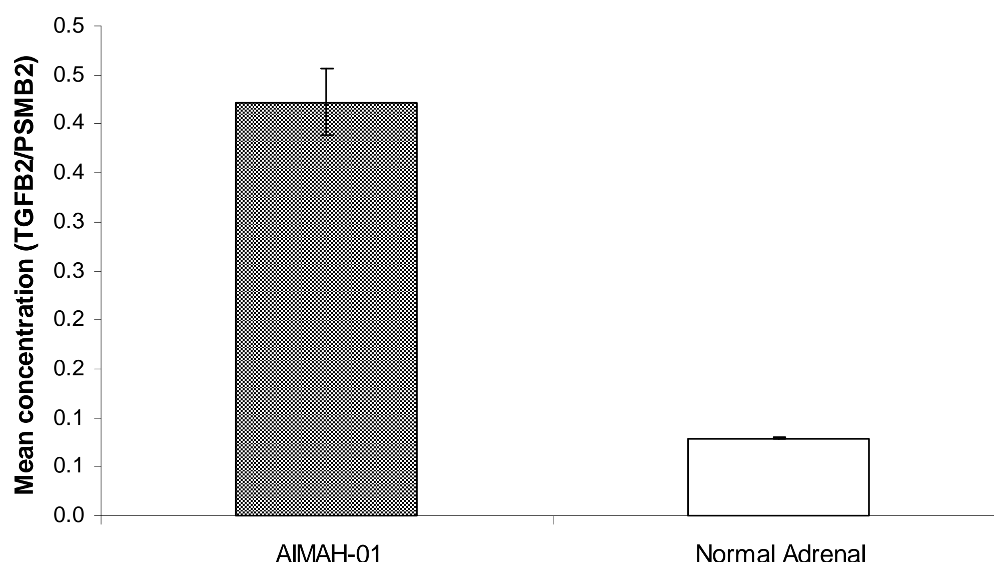


Figure 4.12. Expression of *TGFB2* in AIMAH-01 tumours. The expression in normal adrenal is also shown. Gene expression was normalized to the housekeeping gene, proteasome subunit beta type-2 (*PSMB2*). The data are shown as the mean ± standard error. *p*=0.004 for a difference between the two groups.

TGFβ (TGFβ1) is produced by bovine adrenocortical cells and may operate via a paracrine or autocrine mechanism involving binding to the TGFβ receptors expressed by the cells (Feige *et al.,* 1991). *In vitro*, TGFβ partially (50-60%) inhibits basal and acute steroidogenesis in bovine adrenocortical cells; TGFβ2 had a similar effect (Feige *et al.,* 1991). The effects on steroidogenesis were dose-dependent, cAMP-independent and non-cytotoxic (Feige *et al.,* 1986). TGFβ acts at several points in the steroidogenic pathway. It inhibits cortisol, corticosterone, androstenedione and aldosterone production; that the steroidogenic defect could be circumvented by addition of 25-hydroxycholesterol, pregnenolone or progesterone, suggested that inhibition of steroidogenesis occurred prior to the common precursor, progesterone (Figure 4.10) (Hotta and Baird, 1986). Also, steroidogenesis is fully restored by exogenous 17α-hydroxypregnenolone or 17α-hydroxyprogesterone, suggesting that impairment of CYP17A1 activity is an important mechanism by which TGFβ impairs steroidogenesis (Figure 4.10) (Feige *et al.,* 1986; Perrin *et al.,* 1991).

Since LDLs do not completely restore steroidogenesis, TGFβ may impair conversion of cholesterol esters to cholesterol; an effect on cholesterol transport to the inner mitochondrial membrane via reducing StAR expression has also been demonstrated *in vitro* (Hotta and Baird, 1986; Brand *et al.,* 1998). In a recent *in vitro* study in murine adrenal tumour Y1 cells, TGFβ reduced *SF-1* transcription; a similar effect in the H295R cell line has also been demonstrated (Lehmann *et al.,* 2005). The minimum lag-time of 16 hours of treatment of adrenocortical cells with TGFβ before steroidogenesis is impaired suggests that TGFβ does not operate as a direct enzyme inhibitor.

TGFβ also inhibits steroidogenesis in the normal human adrenal cortex, although neither basal nor ACTH-stimulated cortisol production is reduced (Lebrethon *et al.,* 1994a). Thus, the *in vitro* steroidogenic enzyme defects induced by TGFβ in bovine adrenocortical cells can not be directly extrapolated to the normal human adrenal cortex. TGFβ decreases *CYP17A1* expression resulting in a decrease in DHEA production (Lebrethon *et al.,* 1994a). DHEAS levels were significantly lower in AIMAH-01 (III-2, III-3; Figure 2.1) than in historical controls (Chapter 3), and there was a trend to a 1.5-fold (RT-qPCR; $p > 0.05$) downregulation of *CYP17A1* in AIMAH-01 (Table 4.5). The data of reduced *SF-1* gene transcription in H295R cells by TGFβ suggests that the effect of TGFβ on steroidogenesis may differ between tumour and normal adrenal cells (Lehmann *et al.,* 2005).

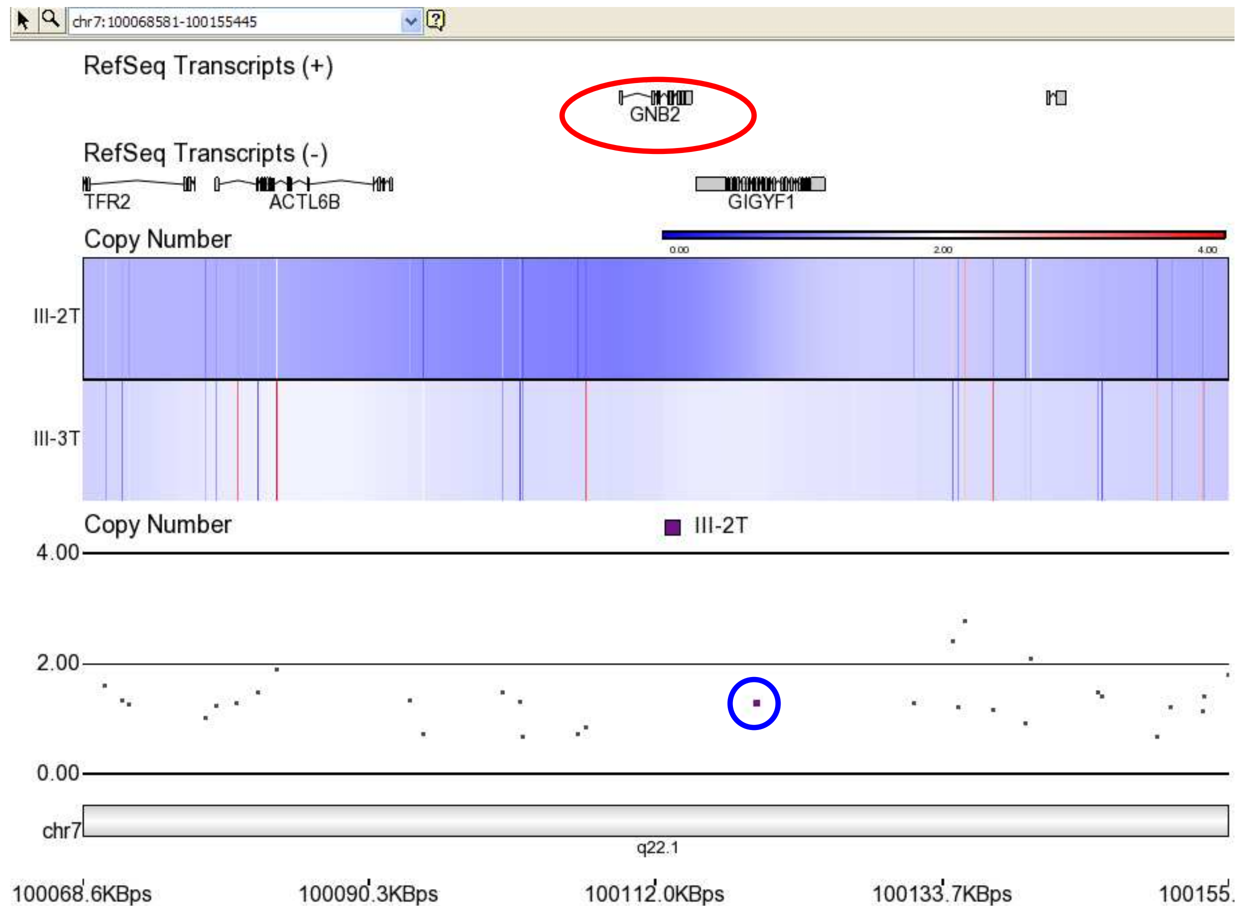
The functions of TGFβ2 are less well characterized, although it does bind to the same receptors as TGFβ1, signals through similar intracellular proteins and has been reported to have similar inhibitory effects on adrenocortical steroidogenesis (de Fraipont *et al.,* 2005). Thus, TGFβ2 may play a role in the steroidogenic enzyme defects of the adrenocortical cells of AIMAH and we suggest further studies in this area are needed.

The Protein Kinase A pathway in AIMAH-01

The Protein kinase A (PKA) pathway has been discussed in Chapter 1. PKA is comprised of four regulatory subunits (PRKAR1A, PRKAR1B, PRKAR2A, PRKAR2B) bound to two catalytic (C) subunits which have kinase activity (Figure 1.11). In humans, PKA is the main mediator of cAMP signalling (Daniel *et al.,* 1998). When cAMP binds to the four regulatory subunits of PKA, it causes them to dissociate from the catalytic subunits, which are then active and able to mediate PKA signalling. The PKA/cAMP pathway may be dysregulated in ACT – ACA, ACC, AIMAH and primary pigmented nodular adrenocortical disease (Libé *et al.,* 2005; Bertherat *et al.,* 2003; Bourdeau *et al.,* 2006; Peri *et al.,* 2001; Rosenberg *et al.,* 2003).

The PKA pathway was one of the most highly overrepresented (IPA) in the (all AIMAH *vs* normal) comparisons (Appendix 3; Table A.3.3). As shown in Figure 4.13, PKA pathway activation may occur independently of cAMP, and *via* TGFβ signalling. In the AIMAH-01 tumours, the expression of adenylate cyclase isoform 5 (*ADCY5*) was reduced (Figure 4.13). This could result in reduced intracellular cAMP production, and hence, reduced cAMP-dependent PKA pathway activation. However, since there are many isoforms of adenylate cyclase (*ADCY*), reduced expression of *ADCY5* may not alter the overall ability of the AIMAH cell to generate cAMP. The G-protein beta gamma (Gβγ) complex which activates ADCY is comprised of two subunits, G-protein beta (Gβ) and G-protein gamma (Gγ) (Figure 4.13). Although there was increased expression of the *Gγ* subunit, there was a putative tumour copy number (CN) loss in the region containing the Gβ subunit gene (Figure 4.14). The CN loss may be sufficient to affect the abundance of the Gβγ complex, thus impairing activation of ADCY and hence intra-adrenal production of cAMP. Thus, if PKA is activated in these AIMAH tumours, these data suggest this may not be *via* a cAMP-dependent mechanism.

Figure 4.13. Cyclic AMP-independent protein kinase A (PKA) activation involving TGFβ.
TGFβ binds to its receptor, resulting in dimerisation of the TGFβR 1 and 2 receptors, resulting in phosphorylation of SMAD proteins. SMAD proteins are able to activate PKA by binding to the regulatory subunits (PKAr) of the molecule, which frees the catalytic subunits (PKAc) of PKA to be biologically active. TGFβ2 was overexpressed in AIMAH-01. Gene expression changes in the cAMP-dependent pathway of PKA activation, detected by microarray analysis in the AIMAH-01 tumors, are shown. Abbreviations: AC – adenylate cyclase, Gγ – guanine nucleotide binding protein (G protein), gamma 11, PLC – phospholipase. Figure generated using Ingenuity Pathway Analysis (Ingenuity Systems®; http://www.ingenuity.com/).



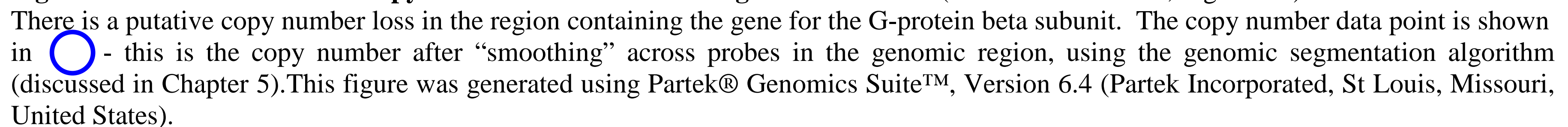


Figure 4.14. Putative somatic copy number variation involving *GNB2* in III-2. (III-2T: AIMAH-01, Figure 2.1).
There is a putative copy number loss in the region containing the gene for the G-protein beta subunit. The copy number data point is shown in ◯ - this is the copy number after "smoothing" across probes in the genomic region, using the genomic segmentation algorithm (discussed in Chapter 5).This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

There was also a putative CN loss of the 2A regulatory subunit of PKA (*PRKAR2A*) in one tumour (Figure 4.15). Although, *PRKAR2A* was not detected as being differentially expressed in the AIMAH-01 tumours, a CN loss may be sufficient to reduce gene expression. This would result in an imbalance between the regulatory and catalytic subunits of PKA, resulting in unbound, and therefore, active, catalytic subunits (Figure 1.11). In some cortisol-secreting adenomas, and compared with non-secretory adenomas, ACC and normal adrenal, defective expression of the PRKAR2B (R2B) protein in association with increased basal PKA activity has been observed (Vincent-Dejean *et al.,* 2008; Mantovani *et al.,* 2008). In the absence of a somatic *PRKAR2B* mutation and preserved gene transcription (mRNA expression is unchanged), deficiency of the PRKAR2B protein suggested increased protein degradation or another post-transcriptional defect.

The (all AIMAH *vs* normal) list was discordantly enriched (positively enriched for genes downregulated and negatively enriched for genes upregulated) for DEG in a primary pigmented nodular adrenocortical disease (PPNAD) tumour from an individual with a known *PRKAR1A* mutation (Horvath *et al.,* 2006c) (Figure 4.9, Panel M; Appendix 4; Tables A.4.1 and A.4.2). Sporadic PPNAD or PPNAD associated with Carney complex is frequently associated with mutations in the regulatory subunit, R1A of PKA (*PRKAR1A*) (Casey *et al.,* 2000; Lionel *et al.,* 2002; Groussin *et al.,* 2002a; Groussin *et al.,* 2002b; Kirschner *et al.,* 2000a). These inactivating mutations result in dysregulated cAMP-mediated PKA signalling. PPNAD is characterized by micronodular adrenal hyperplasia, florid clinical and biochemical hypercortisolism and the onset of Cushing's syndrome by the third decade. Thus, this disorder can be considered diametrically opposite AIMAH – a disorder characterized by macronodular adrenal hyperplasia, inefficient steroidogenesis with mild clinical and biochemical hypercortisolism causing late-onset Cushing's syndrome. Hence, it is not surprising that these disorders with their distinct clinical presentations and adrenal morphologies have a distinct molecular basis.

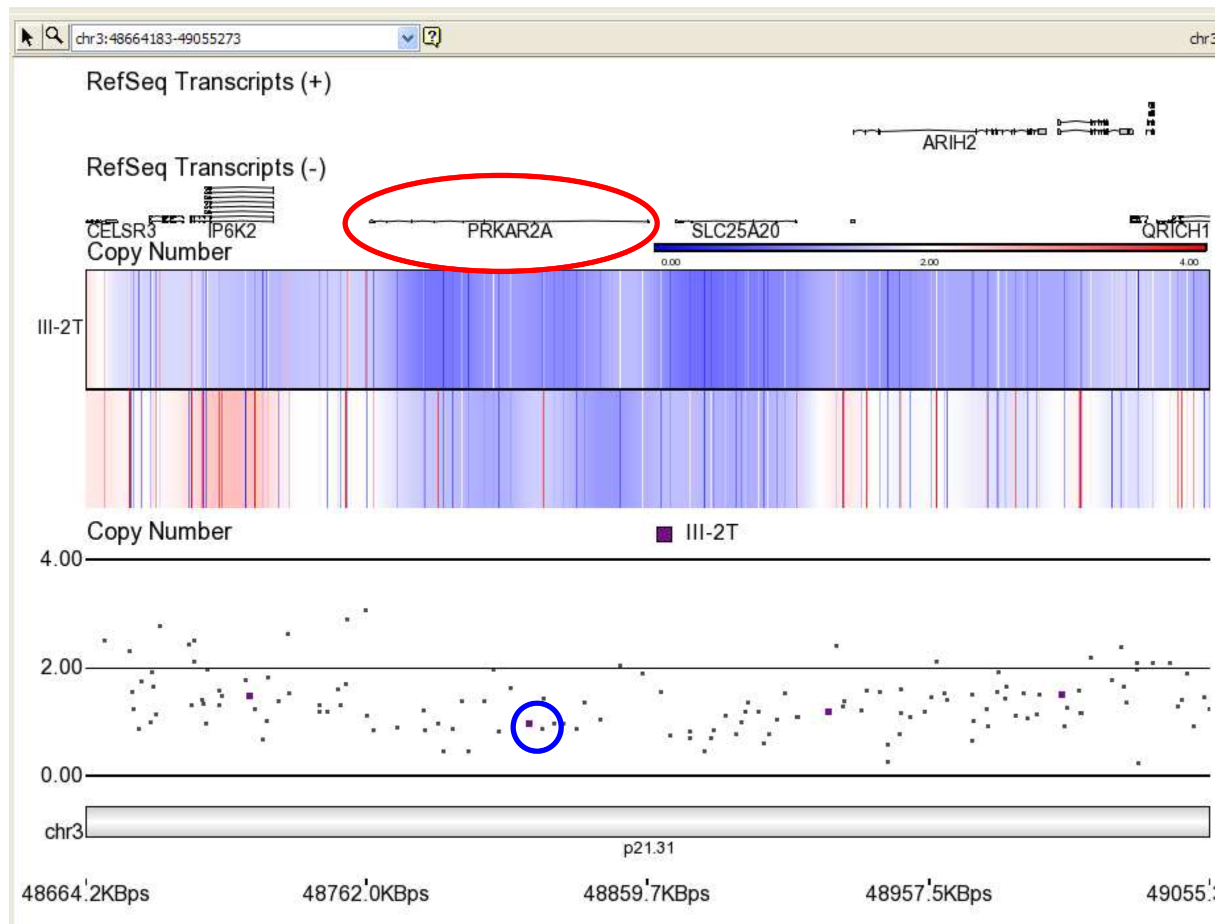
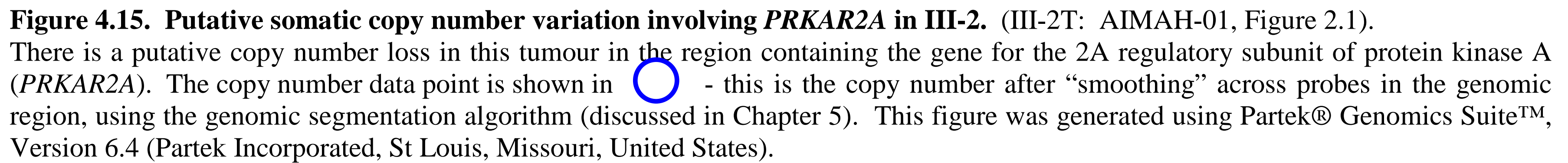


Figure 4.15. Putative somatic copy number variation involving *PRKAR2A* in III-2. (III-2T: AIMAH-01, Figure 2.1). There is a putative copy number loss in this tumour in the region containing the gene for the 2A regulatory subunit of protein kinase A (*PRKAR2A*). The copy number data point is shown in  - this is the copy number after "smoothing" across probes in the genomic region, using the genomic segmentation algorithm (discussed in Chapter 5). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

Although the PPNAD gene set was based on a tumour from only one patient, the data were validated using adrenocortical tissue from 14 other PPNAD patients (Horvath *et al.,* 2006c). However, the data were obtained using a different technique (serial analysis of gene expression – SAGE; which is based on generating, cloning and sequencing short sequence tags) from that which we used in our study, and caution in the comparison is therefore necessary. These data suggest at least that the cAMP-dependent PKA pathway may not be activated in AIMAH-01 tumours. Cyclic AMP-independent PKA activation could initiate different intracellular signalling cascades, with distinct cellular consequences in AIMAH compared with cAMP-dependent PKA activation in PPNAD.

The mechanisms by which cAMP-independent PKA activation occurs have not been fully elucidated, although the available data suggest they are various. There are many *in vitro* examples which provide evidence for cAMP-independent activation of PKA in endocrine and other cell models (Dulin *et al.,* 2001; Gwosdow *et al.,* 1993; Gwosdow *et al.,* 1994; Kim *et al.,* 2009; Kohr *et al.,* 2010; Zieger *et al.,* 2001). In mouse anterior pituitary cells (AtT-20 cells), interleukin-1 (IL-1) activates PKA and stimulates ACTH release without any detectable increase in intracellular cAMP; cAMP-independence was confirmed by unchanged PKA activation by IL-1 in the presence of an ADCY inhibitor and a phosphodiesterase inhibitor (Gwosdow *et al.,* 1993; Gwosdow *et al.,* 1994).

Some cAMP-independent mechanisms of PKA activation may involve direct activation of the catalytic (C) subunit of PKA (Dulin *et al.,* 2001; Zieger *et al.,* 2001; Wu *et al.,* 2002). The natural inhibitor of nuclear factor kappa beta (NFκB), inhibitor of κB (IκB), may be complexed with the C subunit, retaining it in an inactive state. Activation of the NFκB pathway involves degradation of IκB, enabling the release and hence activation of the C subunit (Zhong *et al.,* 1997). c-Myc (v-myc myelocytomatosis viral oncogene homolog (avian)) is a proto-oncogene which encodes a transcription factor ubiquitously expressed in

somatic cells and is involved in the control of cell growth and differentiation. *In vivo*, c-Myc directly activates PKA by promoting transcription of the Cβ subunit (Wu *et al.,* 2002).

TGFβ2 was five-fold upregulated (RT-qPCR; p=0.004) in the AIMAH-01 nodules (Figure 4.12). TGFβ is able to activate PKA independently of cAMP in Mv1Lu (lung epithelial cell line) cells (Zhang *et al.,* 2004). As shown in Figure 4.13, intracellular signalling of TGFβ is mediated by SMAD proteins (Attisano and Wrana, 2000). TGFβ binds to the cell-surface type II TGFβ (TGFβRII) receptor, which phosphorylates the TGFβ type I receptor (TGFβRI). This phosphorylation activates the kinase activity of RI, phosphorylation and activation of SMAD proteins results (Attisano and Wrana, 2000). Activated SMAD3 forms a complex with SMAD4 and activates PKA by binding to either PRKAR1B or PRKAR2A (Zhang *et al.,* 2004) (Figure 4.13). Since TGFβ2 may have similar functions to TGFβ, its effect on PKA and cAMP levels in AIMAH warrants further evaluation.

TGFβ itself is also both a suppressor and a promoter of tumorigenesis (Massagué *et al.,* 2000). The tumour suppressor function of TGFβ is lost in many tumour-derived cell lines (Gold, 1999). In human cancer, high levels of TGFβ are correlated with advanced tumour stage; disease progression may be favoured by reduced immune surveillance and angiogenic factors (Gold, 1999). Although TGFβ inhibits human adult and fetal adrenocortical cell proliferation *in vitro,* this effect may be lost in AIMAH (Zatelli *et al.,* 2000; Ragazzon *et al.,* 2009; Riopel *et al.,* 1989). Whilst TGFβ overexpression may not be the initiating event for tumorigenesis, it may perpetuate cellular proliferation in AIMAH-01.

In the comparison of advanced *vs* early AIMAH there was concordant enrichment for genes upregulated in PPNAD (GSEA). This suggests a possible role for involvement of the cAMP-dependent PKA pathway in the progression from early to advanced AIMAH. cAMP-

dependent PKA activation promotes adrenocortical tumorigenesis and hyperplasia and, we postulate, could drive the progression from early to advanced AIMAH.

Wnt/β-catenin signalling in AIMAH-01

The Wnt signalling pathway has been described in Chapter 1. This pathway is activated in sporadic benign and malignant ACT and in PPNAD (Tadjine *et al.,* 2008a; Tadjine *et al.,* 2008b; Polakis, 2000; Gaujoux, Tissier *et al.,* 2008; Kikuchi, 2003; Gordon and Nusse, 2006). The differential expression of Wnt pathway genes in AIMAH has been previously reported (Bourdeau *et al.,* 2004; Almeida *et al.,* 2011; Table 4.1).

We found a two-fold downregulation of the lipoprotein receptor-related protein, *LRP5*, receptor in the AIMAH-01 tumours (Figure 4.16). Since this is a necessary co-receptor for Wnt signalling, its reduced expression may impair pathway activation. However, cadherin 12 (N-cadherin 2; *CDH12*) expression was two to three-fold upregulated in AIMAH-01 (Figure 4.16); overexpression has recently been reported in sporadic AIMAH (Almeida *et al.,* 2011). We also found increased activity of a number of transcription factors (MARA) predicted to regulate *CDH12* transcription (Figure 4.16). Furthermore, *in vitro* data has shown that TGFβ, of which the TGFβ2 isoform was upregulated in the AIMAH-01 tumours, increases the expression and function of N-cadherins in chondroblasts (Pötter *et al.,* 1999; Tsonis *et al.,* 1994). CDH12 binds phosphorylated β-catenin, stabilizing it in the cytoplasm and preventing it from degradation.

Protein phosphatase 2A (PP2A) is involved in the dephosphorylation of β-catenin; the dephosphorylated protein subsequently translocates to the nucleus to function as a transcriptional coactivator (Kikuchi, 2003). Expression of PP2A was two-fold downregulated in the AIMAH-01 tumours (Figure 4.16).

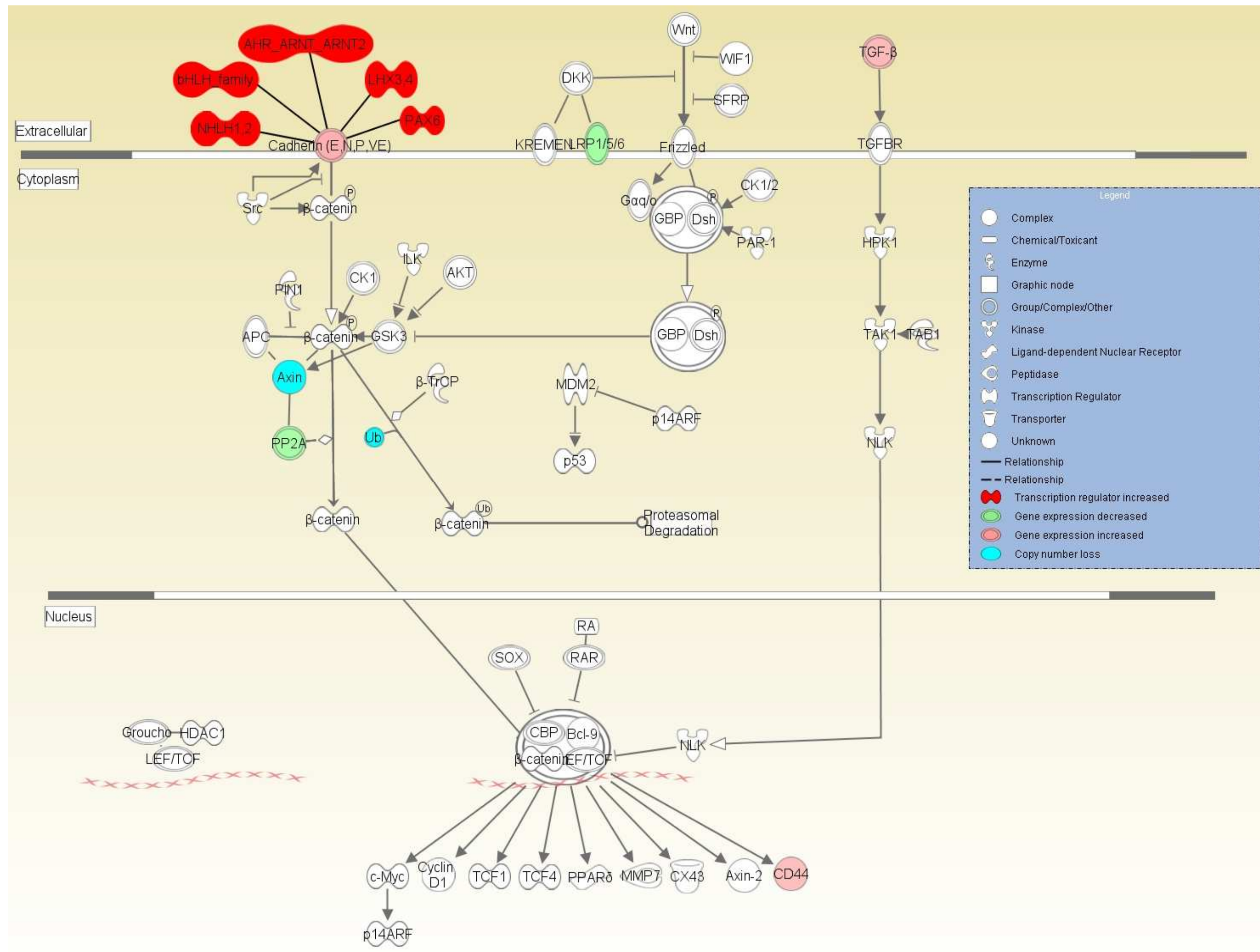


Figure 4.16. The Wnt/β-catenin signalling pathway in AIMAH-01. (Figure legend is on page 200).

Abbreviations: AHR – aryl hydrocarbon receptor; ARNT/2 – aryl hydrocarbon receptor nuclear translocator/2; AXIN – axin; bHLH_family – basic helix-loop-helix transcription factor; LHX3/4 – LIM homeobox 3/4; LRP5/6 - lipoprotein receptor-related protein 5/6; NHLH1/2 – nescient helix loop helix 1/2; PAX6 – paired box 6; PP2A – protein phosphatase 2A; TGFβ – transforming growth factor β; Ub – ubiquitin B. Figure generated using Ingenuity Pathway Analysis (Ingenuity Systems®; http://www.ingenuity.com/).

Two genes involved in this pathway were in regions of putative somatic CN loss (III-2, AIMAH-01; Figure 2.1). These were axin-1 (*AXIN1*) and Ubiquitin B (*UBB*) (Figures 4.17 and 4.18). AXIN1 is involved in the degradation of β-catenin and UBB, ubiquitination of phosphorylated β-catenin which is subsequently degraded. Although CN loss of *UBB* and *AXIN1* may have no significant consequences on pathway function, both act to degrade β-catenin and hence a CN loss could provide another means of intracellular β-catenin accumulation.

Despite differential expression of genes which could result in β-catenin accumulation, in the absence of Wnt signalling, intracellular β-catenin levels are low. Wnt signalling molecules were not differentially expressed; furthermore the necessary coreceptor for Wnt signalling *LRP5* was downregulated in the AIMAH-01 tumours. Overall, our data do not provide evidence for Wnt/ β-catenin pathway activation in AIMAH-01.

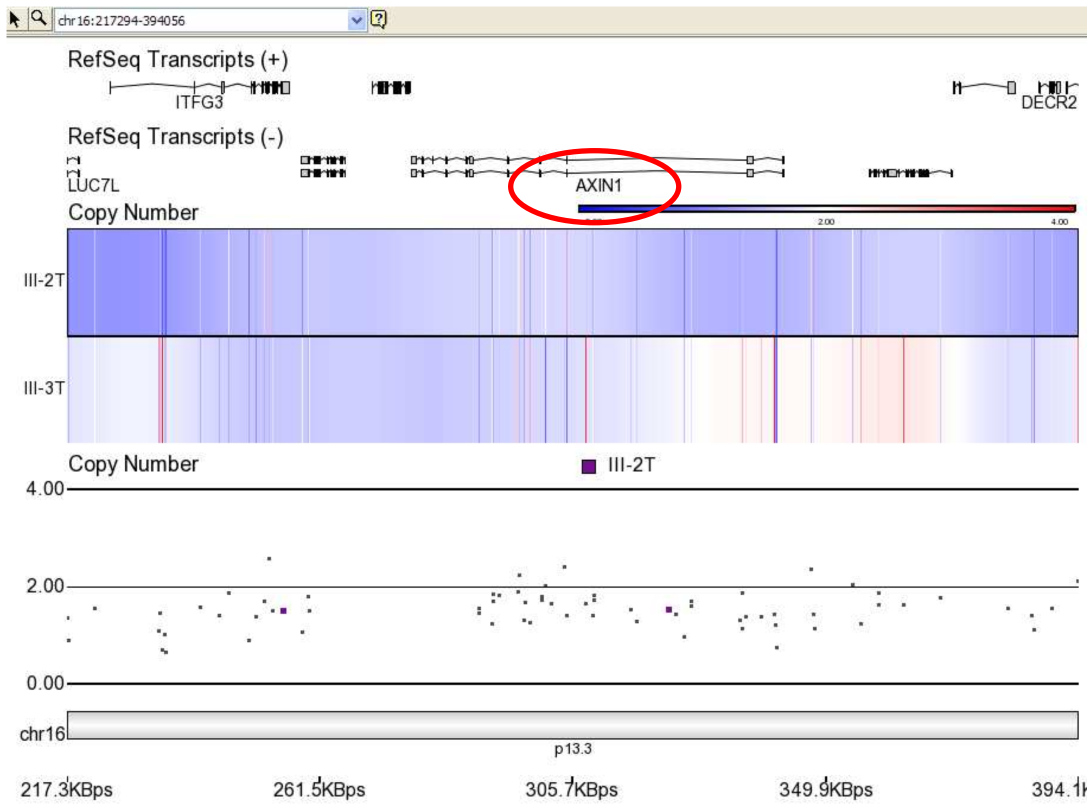


Figure 4.17. Putative somatic copy number variation involving *AXIN* in III-2. (III-2T: AIMAH-01, Figure 2.1). There is a putative copy number loss in the region containing *AXIN*. This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

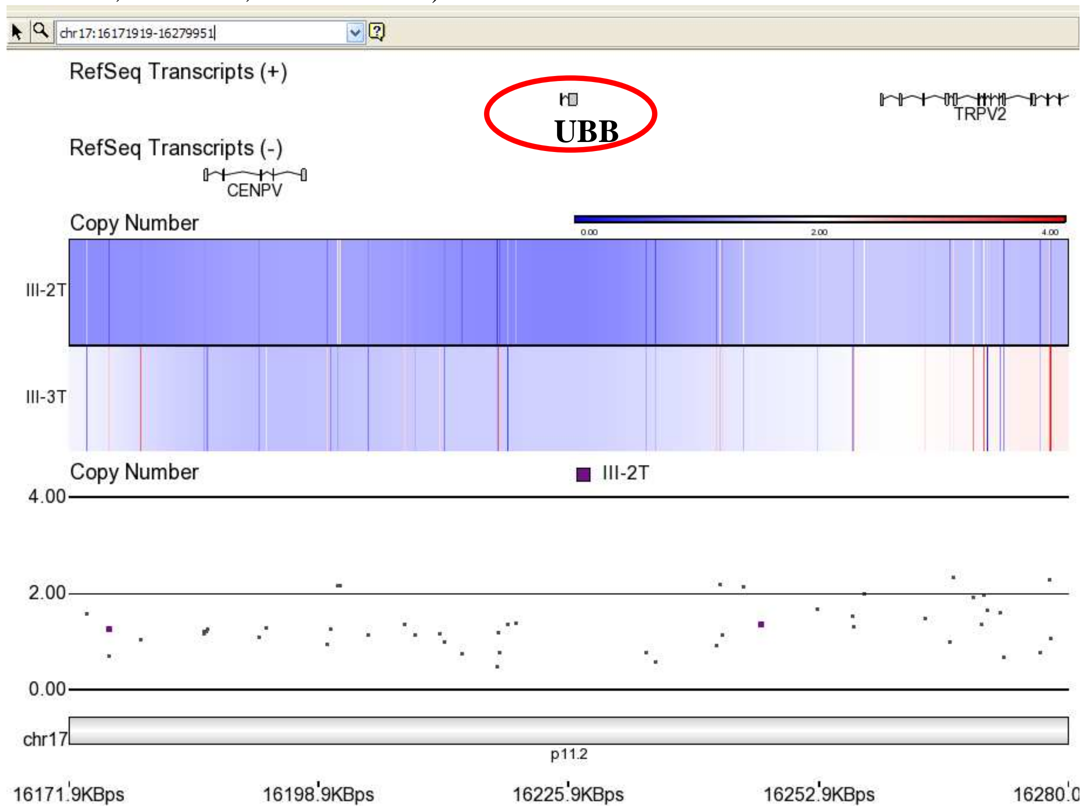


Figure 4.18. Putative somatic copy number variation involving *UBB* in III-2. (III-2T: AIMAH-01, Figure 2.1). There is a putative copy number loss in the region containing ubiquitin B. This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

4.4 Conclusion and Future Studies

Genome-wide gene expression analyses of adrenal nodules from AIMAH-01 have identified novel mechanisms of inefficient steroidogenesis. Our data reveal defects in the rate-limiting steps of steroidogenesis; a trend to downregulation of: (1) *CYP11A1*, which catalyses the conversion of cholesterol to pregnenolone, the first steroid intermediate; and (2) *StAR*, the carrier protein for cholesterol to the inner mitochondrial membrane, the site of CYP11A1. Our data also suggest that downregulation of steroidogenic enzyme expression may be due to reduced activity of transcription factors (SF-1, Sp1). Our analyses suggest that SF-1 may drive the progression from early to advanced AIMAH; a biologically plausible possibility since SF-1 stimulates adrenocortical cell proliferation. Inefficient steroidogenesis is a unique property of AIMAH tumours which is not observed in other adrenocortical tumours; thus whether this is the primary defect driving macronodular adrenal hyperplasia in AIMAH is a hypothesis worthy of future study. To date, PKA pathway activation in ACT has been shown to be cAMP-dependent. Our studies suggest that PKA signalling in AIMAH may occur via a cAMP-independent mechanism involving TGFβ2. Future studies should explore the effects of overexpression of TGFβ2 on AIMAH cell proliferation. The Wnt/β-catenin pathway was not activated in AIMAH-01.

These data have generated new hypotheses for the mechanisms of involvement of pathways already implicated in AIMAH or other ACT. Further studies are needed to firstly confirm differential protein expression (e.g., Western blot, immunohistochemistry), and then investigate the pathways that we have implicated here. These studies will require the ability to grow AIMAH cells in culture. Alternatively in the current genomic era, the ultimate identification of the genetic basis of familial AIMAH will allow the generation of cell and animal models of AIMAH, for further study.

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

The genetic basis of familial ACTH-independent macronodular adrenal hyperplasia (AIMAH) is not known. In isolated cases, the genetic basis of sporadic AIMAH has been elucidated; that mutations in several genes have already been observed, strongly suggests that AIMAH is likely to be genetically heterogeneous. These mutations have been identified by sequencing candidate genes; the selection of candidates has been guided by: (1) genes known to be associated with non-endocrine tumours occurring in patients with AIMAH (e.g., fumarate hydratase in hereditary leiomyomatosis and renal cell cancer syndrome; adenomatous polyposis coli in colon cancer); and (2) genes with known involvement in adrenal or other endocrine tumours and/or Cushing's syndrome (CS) (e.g., menin – multiple endocrine neoplasia 1 syndrome; phosphodiesterase 11A – adrenal hyperplasia; GNAS complex locus – McCune-Albright syndrome) (Matyakhina *et al.,* 2005; Hsiao *et al.,* 2009; Fragoso *et al.,* 2003). The genes and mutations associated with sporadic AIMAH have been discussed in Chapter 1 and are summarised in Table 5.1.

In addition, a kindred (an affected father and two daughters) with primary aldosteronism manifesting as severe hypertension and hypokalaemia in childhood, has recently been reported (Geller *et al.,* 2008). The phenotype was accompanied by non-suppressibility of cortisol to dexamethasone and massive adrenal hyperplasia; bilateral adrenalectomy restored normotension and normokalaemia (Geller *et al.,* 2008). The causative genetic mutation for

Table 5.1. Mutations associated with sporadic ACTH-independent Macronodular Adrenal Hyperplasia.

Reference	Case Nō	Gene	Germline Variant# Amino acid change§	Tumour tissue	Clinical syndrome
Sporadic					
Matyakhina *et al.*, 2005	1	Fumarate hydratase (FH)	c.782_788del7bp; p.P261X	Loss of heterozygosity of the FH locus (1q42.3-43)	Hereditary leiomyomatosis and renal cell cancer syndrome - cutaneous and uterine leiomyomas
Aloi *et al.*, 1995	1	ACTH receptor (MC2R)	c.833T>G;* p.F278C	No data	None
Hsiao *et al.*, 2009	1	Adenomatous polyposis coli	c.4393_4394delAG	Same as germline	Polyps and desmoid tumours
Hsiao *et al.*, 2009;	1	GNAS complex locus (GNAS)	None	c.602G>A; p.R201H	None
Fragoso *et al.*, 2003	1 2	GNAS	None	c.601C>A; p.R201S c.602G>A; p.R201H	None
Hsiao *et al.*, 2009	1	Menin (MEN1)	c.1481C>T p.P494L	Same as germline	Hyperparathyroidism
Familial					
Hsiao *et al.*, 2009	1	PDE11A**	c.2599C>G** p.R867G	Same as germline	

*homozygous for the mutation; **the variant found in PDE11A is a SNP (rs61306957; from National Centre for Biotechnology Information SNP database (dbSNP129))

#Variant is given in Human Genome Variation Society (HGVS) notation as follows: NCBI mRNA reference sequenced ID: {nucleotide interval}{changed nucleotide>new nucleotide}; unless otherwise stated – all mutations/variants occurred in heterozygosity
Nucleotide abbreviations: A – adenine; C – cytosine; G – guanine; T - thymine
§Amino acid change given in HGVS notation as follows: (1) p.{code first amino acid changed}(amino acid interval}{code new amino acid}
Abbreviations: C – cysteine; F – phenylalanine; G – glycine; L – leucine; P – proline; R – arginine; X – STOP codon

this autosomal dominant form of familial adrenal hyperplasia and hyperaldosteronism has recently been identified – a novel germline missense mutation in a gene encoding a potassium channel (*KCNJ5*) (Choi *et al.,* 2011). Other somatic missense mutations in *KCNJ5* were found in eight of 22 sporadic aldosterone-producing adenomas studied (Choi *et al.,* 2011). The mutations occur in highly conserved residues of the potassium channel and alter its function (Choi *et al.,* 2011). The end consequence of altered potassium channel function due to these mutations is intracellular calcium accumulation, which stimulates aldosterone production and cellular proliferation (Choi *et al.,* 2011). It remains to be determined whether other endocrine tumours, including AIMAH, have related mutations that account for concomitant cell proliferation and hormone secretion. As regards the potential involvement of potassium channel mutations in the pathogenesis of AIMAH, cortisol production and secretion does not require intracellular calcium accumulation (Figure 1.4). It is intriguing however, that potassium channel activity may be augmented by activation of G protein-coupled receptors, which are frequently aberrantly expressed by AIMAH tumours (Krapivinsky *et al.,* 1995; Gregerson *et al.,* 2001).

In this chapter, I will discuss the genome-wide linkage studies we have performed with the aim of identifying the chromosomal locus, or loci, harbouring the disease-causing gene(s) for AIMAH in our three families (AIMAH-01, -02 and -03) (Chapter 2), and a fourth previously published family, AIMAH-04, from whom germline DNA samples were available for several family members (Gagliardi *et al.,* 2009; Nies *et al.,* 2002).

We hypothesised that AIMAH was inherited as a Mendelian disorder in these four unrelated families, although not necessarily due to the same gene (genetic or locus heterogeneity) or mutation (allelic heterogeneity) between families; hence linkage analysis for each family was performed independently of the other families. Due to the small size of, and number of affected individuals in, AIMAH-02 and AIMAH-03 (Chapter 2; Figure 2.10 and 2.12), neither

kindred was considered to have sufficient statistical power for independent linkage analysis. These families were SNP-typed, so that we could study the haplotype in affected individuals, of any potential locus that may have arisen from the linkage analyses of the two more statistically powerful kindreds, AIMAH-01 and AIMAH-04. Since, when we performed these studies, and even at the time of writing this thesis, the genetic basis of familial AIMAH was not known, we used genome-wide studies in order to identify, in an unbiased manner, all possible candidate genomic loci and genes, and then applied bioinformatics analyses to prioritise the candidates for further study. Moreover, prior to our studies, and to the best of our knowledge, linkage studies of familial AIMAH had not been performed – thus there were no chromosomal loci that had been previously associated with familial AIMAH which we could have selected to study.

The linkage studies described in this chapter, using single nucleotide polymorphism (SNP) genotyping and the phenotyping data presented in Chapter 2, constitute our first approach towards discovery of the causative gene in familial AIMAH. The theoretical basis of linkage analysis has been discussed in Chapter 1. Linkage analysis aims to localise the mutation responsible for a particular inherited disease to a chromosomal locus by examining the relationship between genotype and phenotype data. As discussed in Chapter 1, regions of copy number variation (CNV) or loss of heterozygosity (LOH) in tumours may harbour the disease-causing allele (Knudson, Jr, 1971). Therefore we also studied the available resected AIMAH tumours (AIMAH-01; Chapter 2) for somatic CNV and LOH. Specifically, we sought to identify regions of CNV or LOH in either tumour that corresponded to any possible loci arising from our linkage analysis of AIMAH-01, since this would have assisted in restricting the number of candidate genes within a given locus for further study. We also sought to identify any regions of CNV or LOH that were common to both tumours, and which may have therefore directed us to other potential disease allele-harbouring loci.

5.2 Research Methods

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee.

Nucleic acid extraction

DNA was extracted from leukocytes and fresh frozen adrenal gland (normal and AIMAH) using the Qiagen QIAamp® DNA microkit (Qiagen, Germany), according to the manufacturer's instructions. RNA extraction from lymphocytes was performed using the Qiagen QiAamp® RNA Blood Mini Kit (Qiagen).

Genotyping

All phenotyped individuals were genotyped (Figures 2.1, 2.10 and 2.12). AIMAH-04 is shown in Figure 5.1. Germline DNA from AIMAH-04 was provided by Dr Constantine A. Stratakis (Section on Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, Maryland, United States).

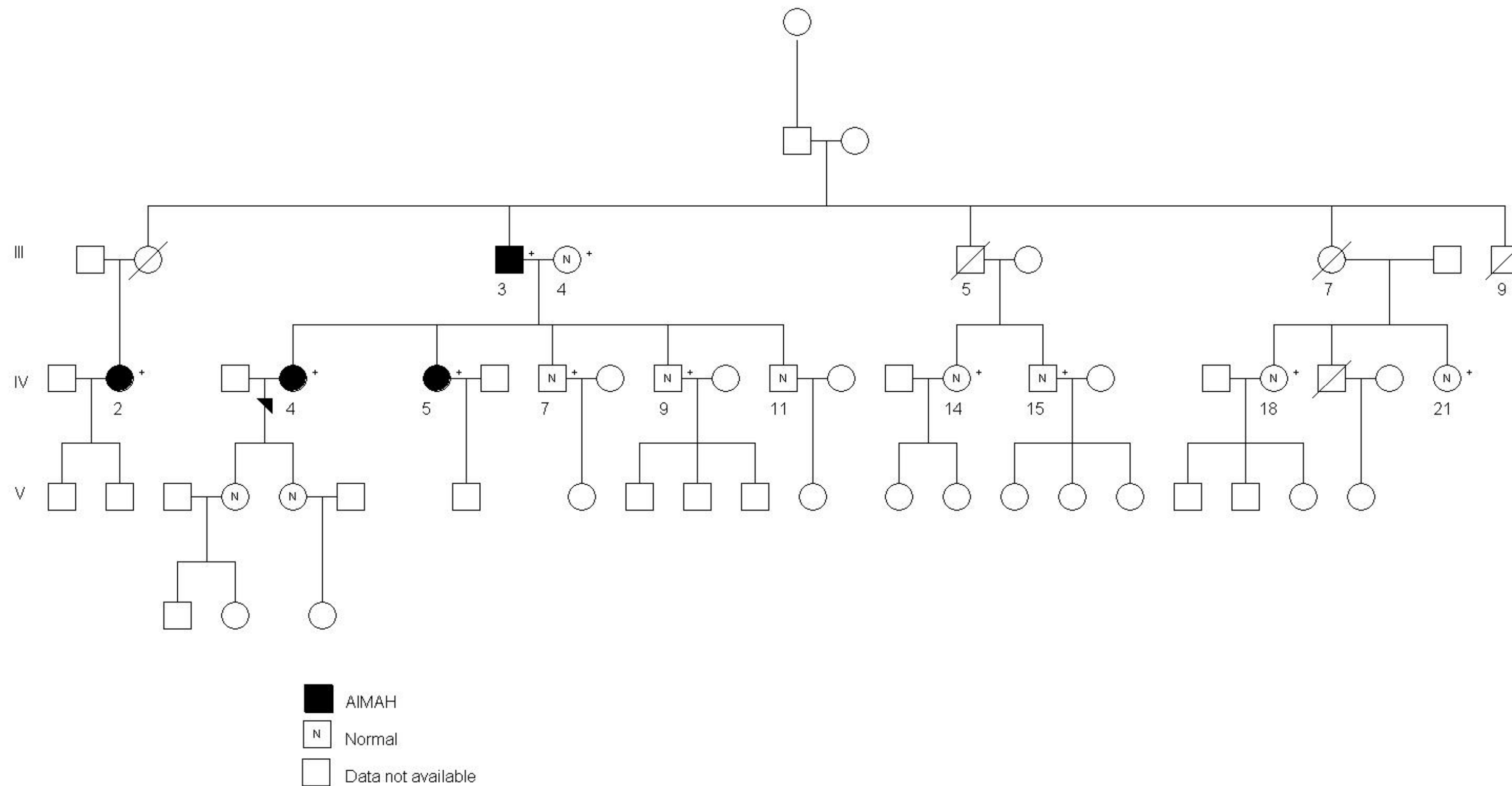


Figure 5.1. Pedigree of kindred AIMAH-04.
+ indicates individuals from whom DNA was available and who were genotyped.
In addition, individuals I-1, III-2, III-7 were reported to have had Cushingoid features.

Germline and adrenal tumour (III-1, III-2, III-3 – AIMAH-01; Figure 2.1) DNA samples were processed by the Australian Genome Research Facility (AGRF), Parkville, Victoria. All DNA samples processed were of moderate to high-quality (260/280: 1.7–1.9 and 260/230: 1.6-2.2), and showed minimal to no degradation on a 2% agarose gel. DNA was hybridized to the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix Inc, Santa Clara, California, USA) and processed according to the manufacturer's protocol (Affymetrix, 2008).

The Affymetrix SNP array 6.0 has 1.8 million markers – 900,000 each of SNP and CNV probes, providing amongst the most comprehensive genome coverage commercially available at the time of the study. The high density of SNPs in the human genome makes them powerful linkage markers, although this is somewhat diminished by their limited heterozygosity, and hence informativeness, for linkage, discussed in Chapter 1. Genotypes were called using the Birdseed Algorithm (http://www.affymetrix.com/products/software/specific/birdseed_algorithm.affx).

Linkage Analysis

The genotyping data were uploaded to LINKDATAGEN. LINKDATAGEN is a program (perl script) that generates input files for a number of linkage analysis software tools (Bahlo and Bromhead, 2009; http://bioinf.wehi.edu.au/software/linkdatagen/index.html). The data were preprocessed by LINKDATAGEN as follows: (1) error checks, including removing Mendelian errors by removing the entire SNP; (2) selection of SNPs to fulfil linkage equilibrium conditions; and (3) removal of uninformative markers based on their heterozygosity in the reference (HapMap) population (The International HapMap Consortium, 2003).

LINKDATAGEN was used to select a sparser subset of approximately 12,000 autosomal SNPs. The SNPs with the highest heterozygosity (more informative for linkage) were chosen

for the marker subset, with a minimum genetic distance of 0.15 centiMorgans (cM) between markers (1cM is the genetic distance between two loci with a recombination frequency of 1% - 1 crossover per 100 meioses; corresponds to approximately one million base pairs) (Strachan and Read, 2011).

Nonparametric linkage analysis

Nonparametric linkage (NPL) analysis was performed on each AIMAH family using the MERLIN program (Abecasis *et al.,* 2002). Computational constraints precluded analysis of AIMAH-01 in its entirety, so a subset was used (III-1, -2, -3; IV-1 – IV-6; V-1 – V4, -8, -11, -12: AIMAH-01, Figure 2.1). These individuals were selected because they were key members of the family and deemed to increase the statistical power to detect linkage. The Whittemore and Halpern NPL pairs statistic was used to test for allele sharing among affected individuals. As discussed in Chapter 1, NPL is a powerful model-free method of analysis when the mode of inheritance is not known. When the distribution of the phenotype suggests a pattern of inheritance then parametric linkage analysis, in which the genetic model is specified, is more powerful, provided the model is correct.

Parametric linkage analysis

Parametric multipoint linkage analysis was performed in MERLIN for each AIMAH family. The following disease model assumptions were made:

1. autosomal dominant inheritance
2. broadest phenotype possible – i.e., affected = any one of: (1) suppressed ACTH (ACTH < 10ng/L or < 2.2pmol/L (SI units)); (2) elevated 24 hour urinary free cortisol (> 300 or > 350nmol/day, depending on the assay); (3) nonsuppression to dexamethasone (defined as cortisol > 50nmol/L at 0900h after 1mg oral dexamethasone at 2300h the night prior); (4) morphological adrenal changes on CT scan; or (5) absent or aberrant vasopressin (VP) response (described in Chapter 2).

3. penetrance of the disease allele, *a* (normal allele, A):

    a. P(affected | AA) = 0.01

    b. P(affected | A*a*) = 0.8

    c. P(affected | *aa*) = 0.8

4. P(*a*) = 0.0001 (assuming *a* to be a very rare allele within the population).

Liability class modelling (AIMAH-01)

Liability classes were used to better model the disease in AIMAH-01. A particular focus was put on finding the model that would best enhance the linkage signal on chromosome 14, which had been identified by parametric linkage analysis as harbouring a region of possible linkage. The P(affected | A*a*) assigned to each individual was varied according to the generation of which they were a member; since the P(affected | A*a*) increases with increasing age. The final model used, where *a* represents the disease allele and A, the normal allele was:

P(*a*) = 0.0001.

Gen III (Figure 2.1): P(affected | AA) = 0.05, P(affected | A*a*) = P(affected | *aa*) = 0.9.

Gen IV (Figure 2.1): P(affected | AA) = 0.05, P(affected | A*a*) = P(affected | *aa*) = 0.5.

Gen V (Figure 2.1): P(affected | AA) = 0.05, P(affected | A*a*) = P(affected | *aa*) = 0.35.

Otherwise: P(affected | AA) = 0.05, P(affected | A*a*) = P(affected | *aa*) = 0.35.

Copy number variation analysis

A paired analysis was performed for each individual (III-2 and III-3, AIMAH-01; Figure 2.1) using tumour (III-2T, III-3T) and the respective individual's germline DNA. The CEL files were imported using the Partek® Genomics Suite™ (Partek Inc, St Louis, Missouri, United States) default settings. These were:

Probes to import: Interrogating probes

Probe filtering: Skip

Normalisation configuration was performed using the following parameters:

Prebackground adjustment: adjust for fragment length; adjust for probe sequence

Quantile normalization: skip

Copy number was created from the intensities.

CN analysis was performed using two statistical methods with the following parameters:

(1) Hidden Markov Modelling (HMM) region detection to detect deletions and amplifications.

The parameters for HMM smoothing were the Partek preset defaults:

States to detect: 0.1, 1, 3, 4, 5
States to ignore: 2
Max probability: 0.98
Genomic Decay parameter: 0
Sigma: 1

HMM was run using log of data and states.

CNV regions to be reported required a minimum of 3 genomic markers in the region.

(2) Genomic segmentation

Minimum genomic markers: 10
p-value threshold: 0.001
signal to noise: 0.3

Region report
Expected range: 0.3
p-value threshold: 0.01

An arbitrary threshold for amplification was defined as a region average or mean of at least 2.7 and for deletion less than 1.3.

Loss of heterozygosity analysis

The Affymetrix Power tools program was used to create CHP files from CEL files. Genotype calls were imported.

Parameters:

Max probability: 0.99
Genomic decay: 0
Genotype error: 0.01

Candidate gene screening

The coding regions (including splice sites) of selected candidate genes from the possible locus of linkage (AIMAH-01) were directly sequenced. The methods and primers are listed in Appendix 1. These candidates were prioritised on the basis of the tumour CNV/LOH data, gene function, tissue expression (Unigene), known human disease associations (OMIM) and involvement in pathways (IPA) overexpressed in the tumour expression data (Chapter 4).

5.3 Results and Discussion

Linkage Analysis

(i) *Nonparametric linkage (NPL) Analysis*

Autosomal NPL scores are plotted in Figures 5.2 and 5.3. Using this model, there was no evidence for linkage, at any locus, on any chromosome, for any of the four kindreds.

Chromosome 1

Chromosome 2

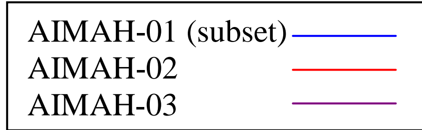
Chromosome 3

Chromosome 4

Chromosome 5

Chromosome 6

Figure 5.2. Autosomal nonparametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Non-parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any chromosomal locus in any of the families. The AIMAH-01 (subset) comprised individuals III-1, -2, -3; IV-1 – IV-6; V-1 – V-4, -8, -11, -12; Figure 2.1.



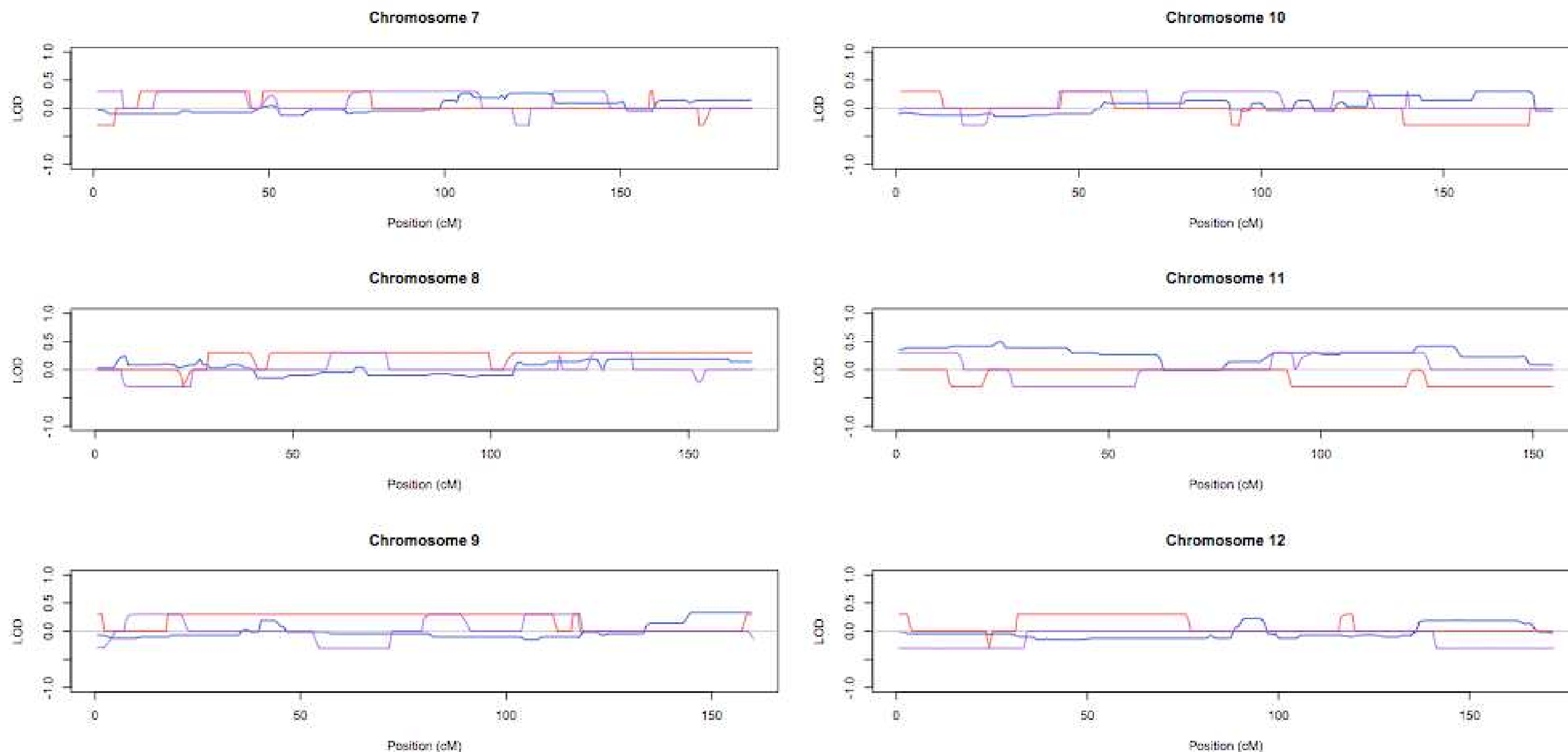


Figure 5.2 (continued). Autosomal nonparametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Non-parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any chromosomal locus in any of the families.
The AIMAH-01 (subset) comprised individuals III-1, -2, -3; IV-1 – IV-6; V-1 – V-4, -8, -11, -12; Figure 2.1.

AIMAH-01 (subset)
AIMAH-02
AIMAH-03

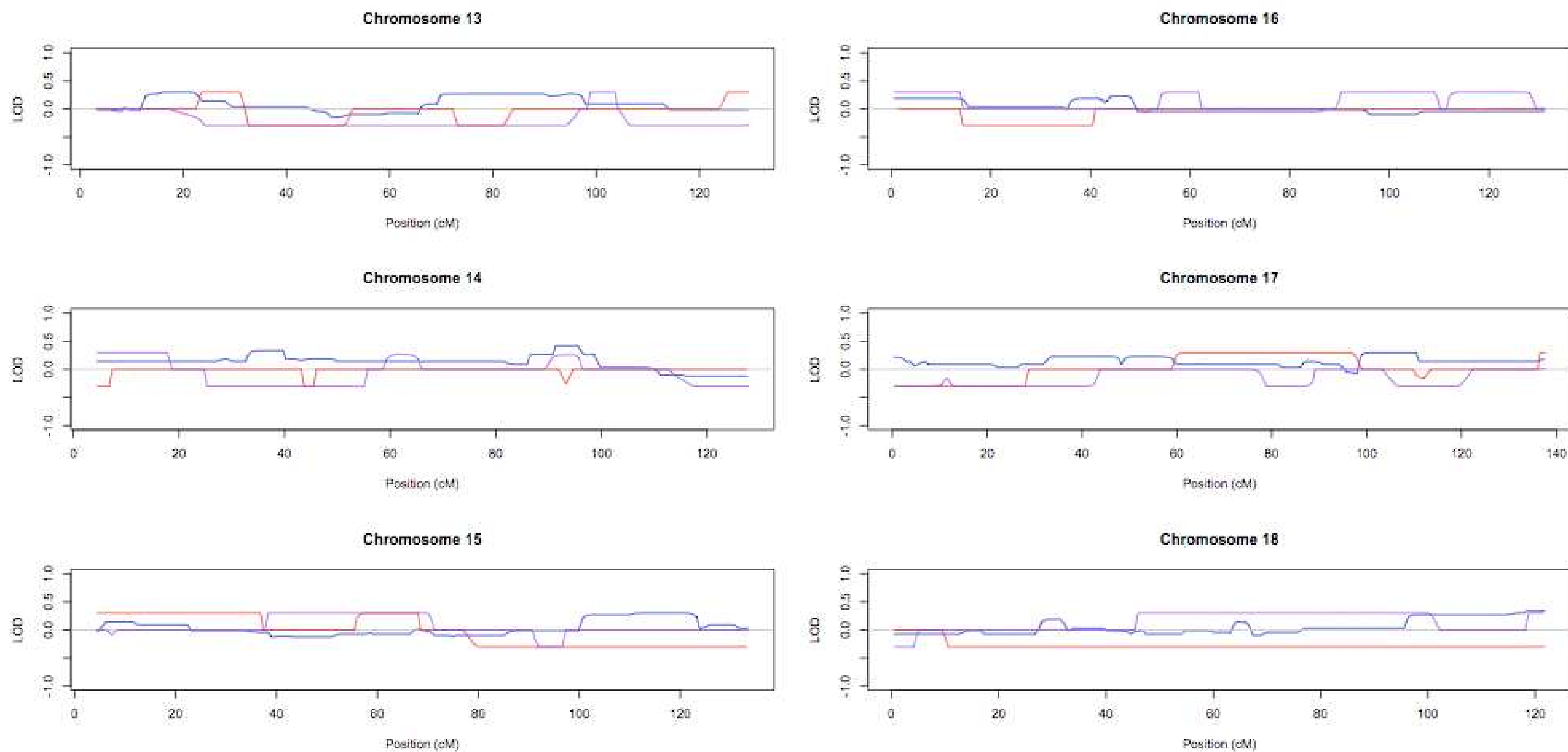


Figure 5.2 (continued). Autosomal nonparametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Non-parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any chromosomal locus in any of the families.
The AIMAH-01 (subset) comprised individuals III-1, -2, -3; IV-1 – IV-6; V-1 – V-4, -8, -11, -12; Figure 2.1.

AIMAH-01 (subset)
AIMAH-02
AIMAH-03

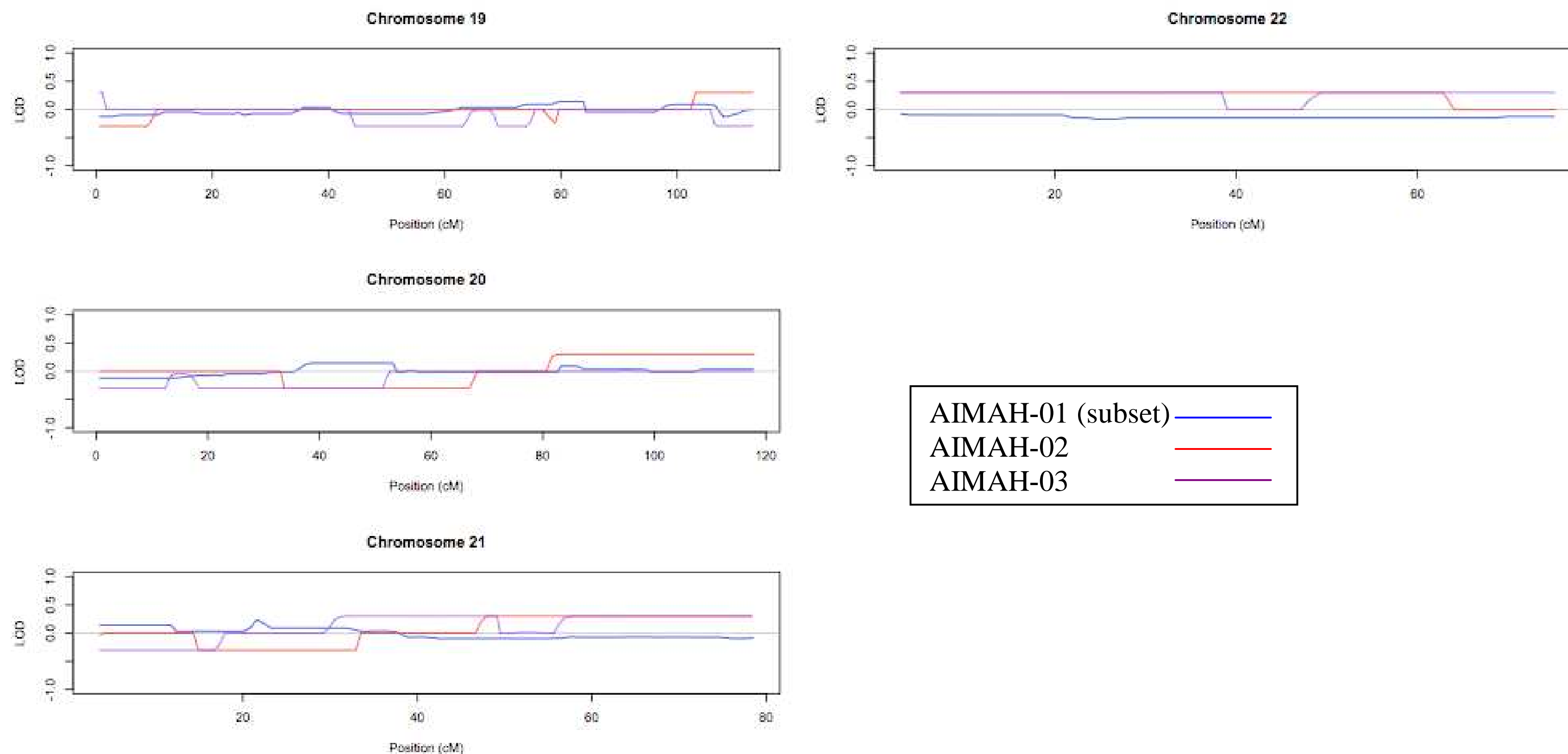


Figure 5.2 (continued). Autosomal nonparametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Non-parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any chromosomal locus in any of the families.
The AIMAH-01 (subset) comprised individuals III-1, -2, -3; IV-1 – IV-6; V-1 – V-4, -8, -11, -12; Figure 2.1.

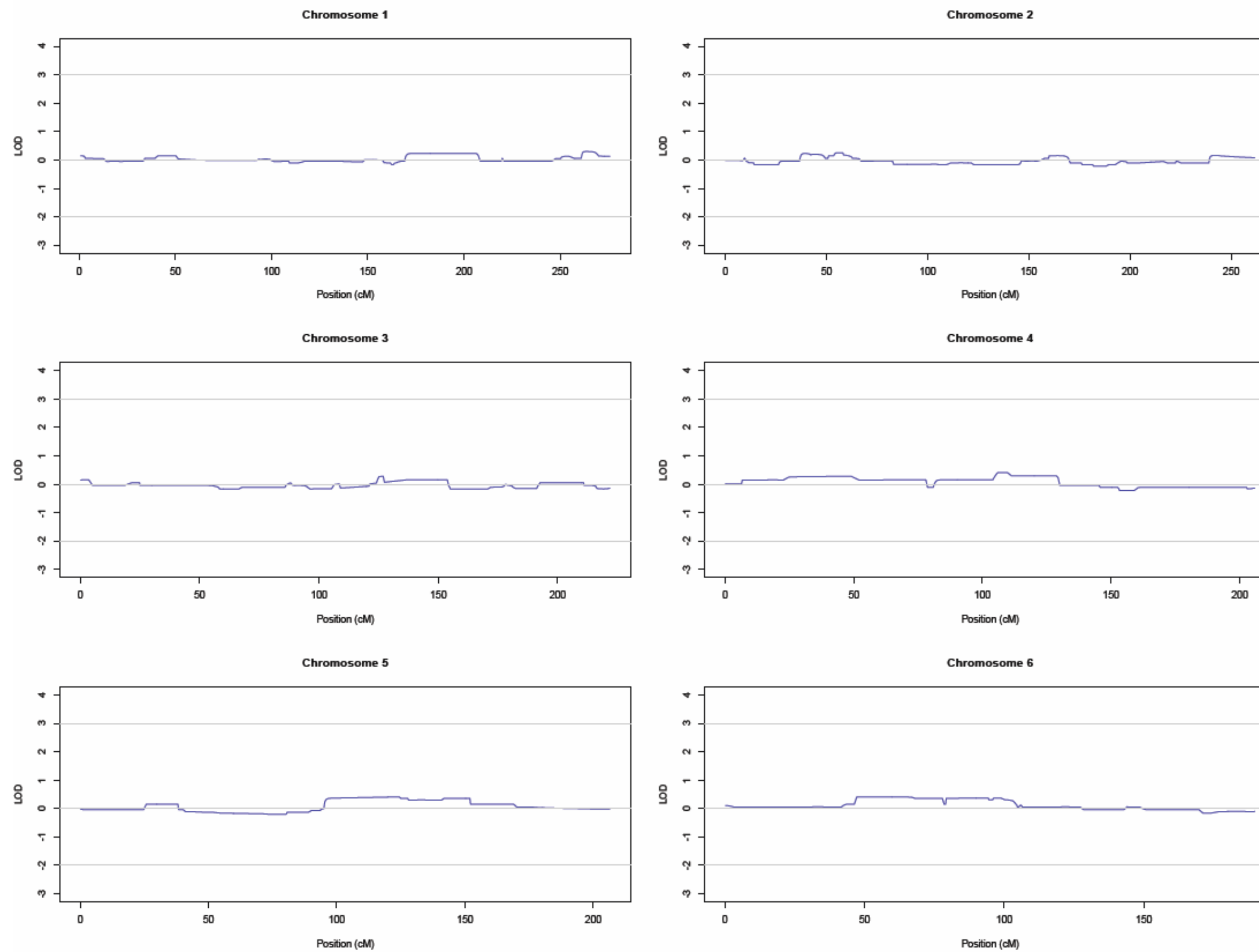


Figure 5.3. Autosomal nonparametric linkage analysis scores for kindred AIMAH-04.
Non-parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any chromosomal locus in AIMAH-04.

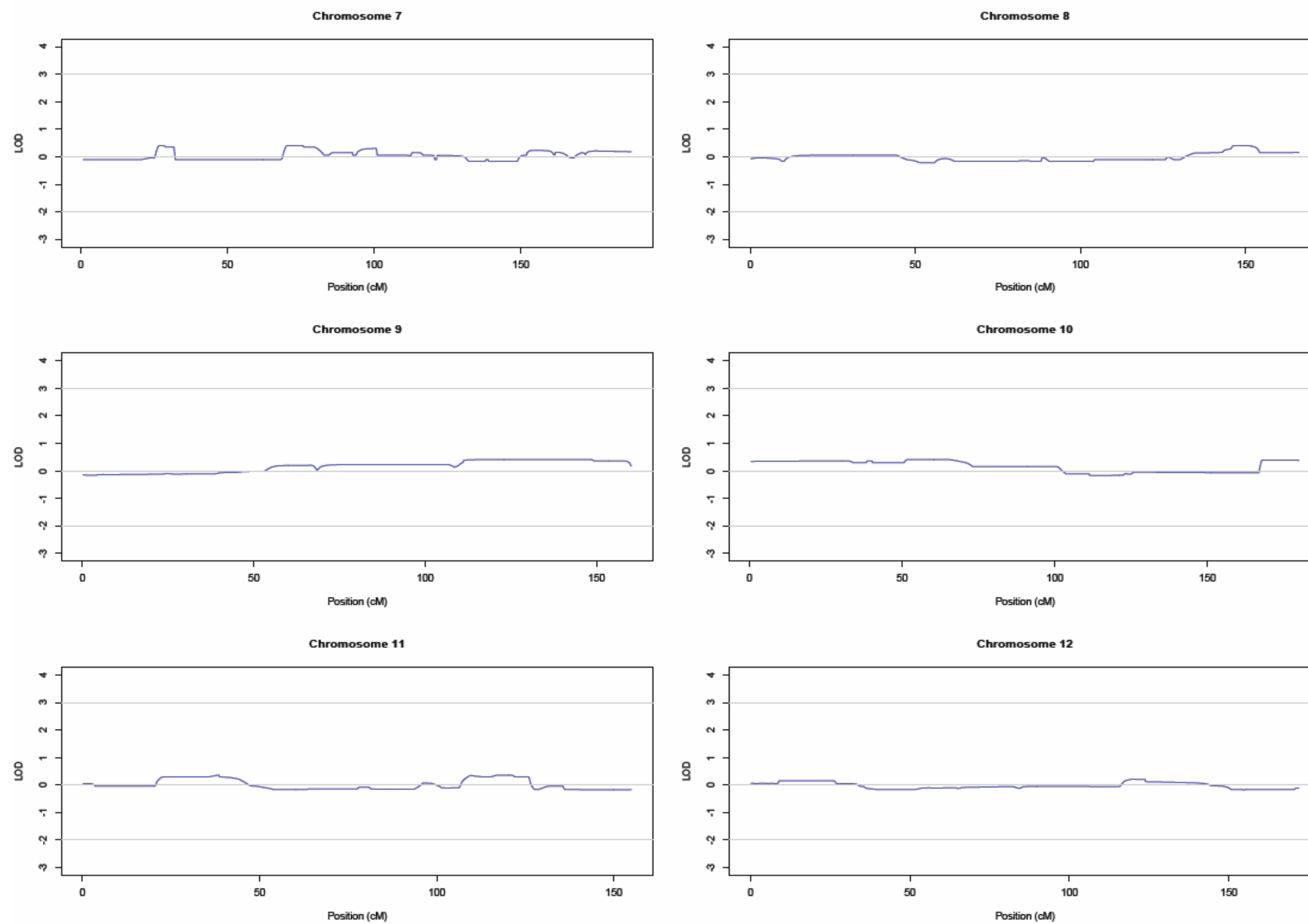


Figure 5.3 (continued). Autosomal nonparametric linkage analysis scores for kindred AIMAH-04.

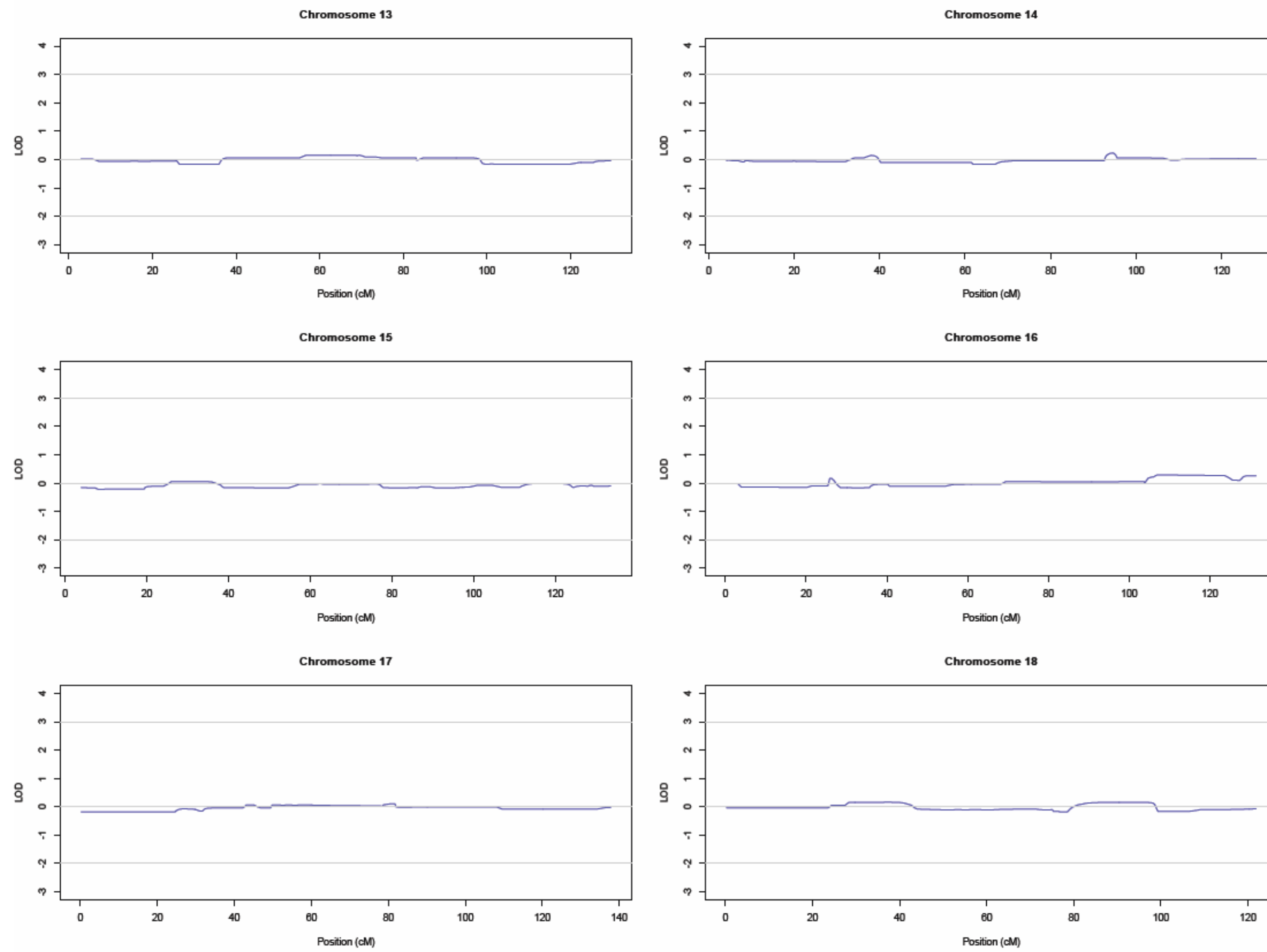


Figure 5.3 (continued). Autosomal nonparametric linkage analysis scores for kindred AIMAH-04.

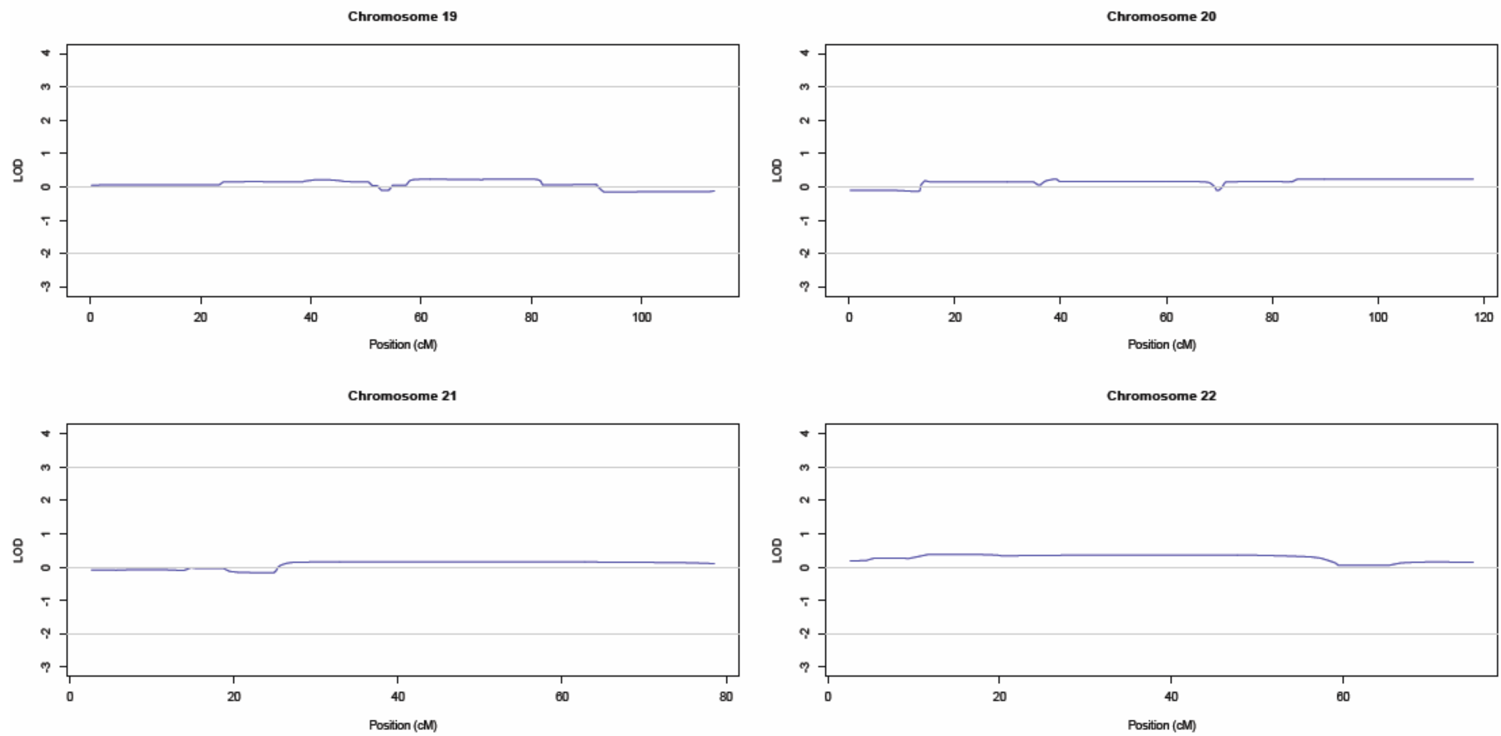


Figure 5.3 (continued). Autosomal nonparametric linkage analysis scores for kindred AIMAH-04.

(ii) *Parametric Linkage Analysis*

The autosomal parametric LOD scores are shown in figures 5.4 and 5.5. The peak LOD score for AIMAH-01 was 1.38, on chromosome 14; this increased to 1.83 using liability class modelling. This is equivalent to odds of 68 to 1 in favour of linkage at this locus. The flanking SNP markers were SNP_A-2060976 (Reference cluster ID - rs9026) and SNP_A-8402383 (rs12434958), corresponding to 14q32.11-14q32.12 (89333253-92665838; Human Genome Assembly 18 – NCBI36/hg18, March 2006; University of California, Santa Cruz (UCSC) Genome Browser - http://genome.ucsc.edu/) (Figure 5.6). The locus of possible linkage in AIMAH-01 was a 3.3Mb region containing 29 unique and annotated genes (UCSC; National Centre for Biotechnology Information; NCBI). This was a locus of *possible* linkage because the LOD score was < 3 (a LOD score > 3 is defined as genome-wide significant evidence of linkage). The genes in this locus have been annotated and are listed in Table 5.2. There were no other loci of possible linkage in AIMAH-01 (Figure 5.4). We note that a gene encoding a potassium channel (*KCNK13*) is located within the possible locus. This is of interest due to the recent discovery of potassium channel mutations in familial adrenal hyperplasia and hyperaldosteronism (Choi *et al.,* 2011).

We performed haplotype analysis of the AIMAH-01 locus in the other families, AIMAH-02, AIMAH-03 and AIMAH-04. Although the affected individuals from AIMAH-02 shared a haplotype in the locus, this was also shared by unaffected individuals (Figure 5.7). The affected individuals from AIMAH-03 and an unaffected sibling shared the entire locus identical by descent (Figure 5.8). In AIMAH-04, the AIMAH-01 locus, or part thereof, was shared by four out of five affected individuals (Figure 5.9). Thus, we were not able to implicate this locus in the other AIMAH families.

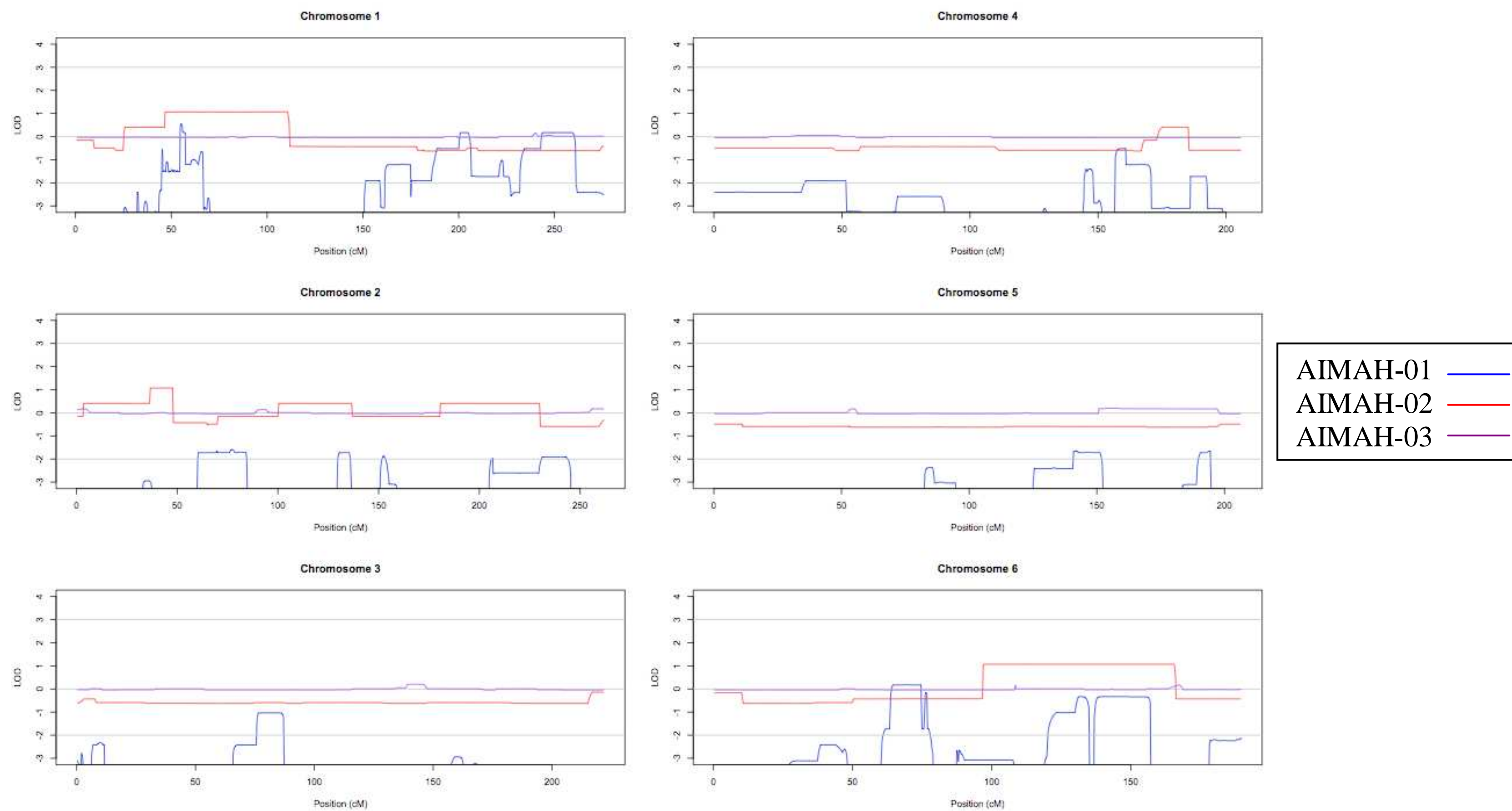


Figure 5.4. Autosomal parametric linkage analysis scores for kindreds AIMAH-01, -02 and -03.
Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any locus for chromosomes 1-6 for AIMAH-02 or -03.

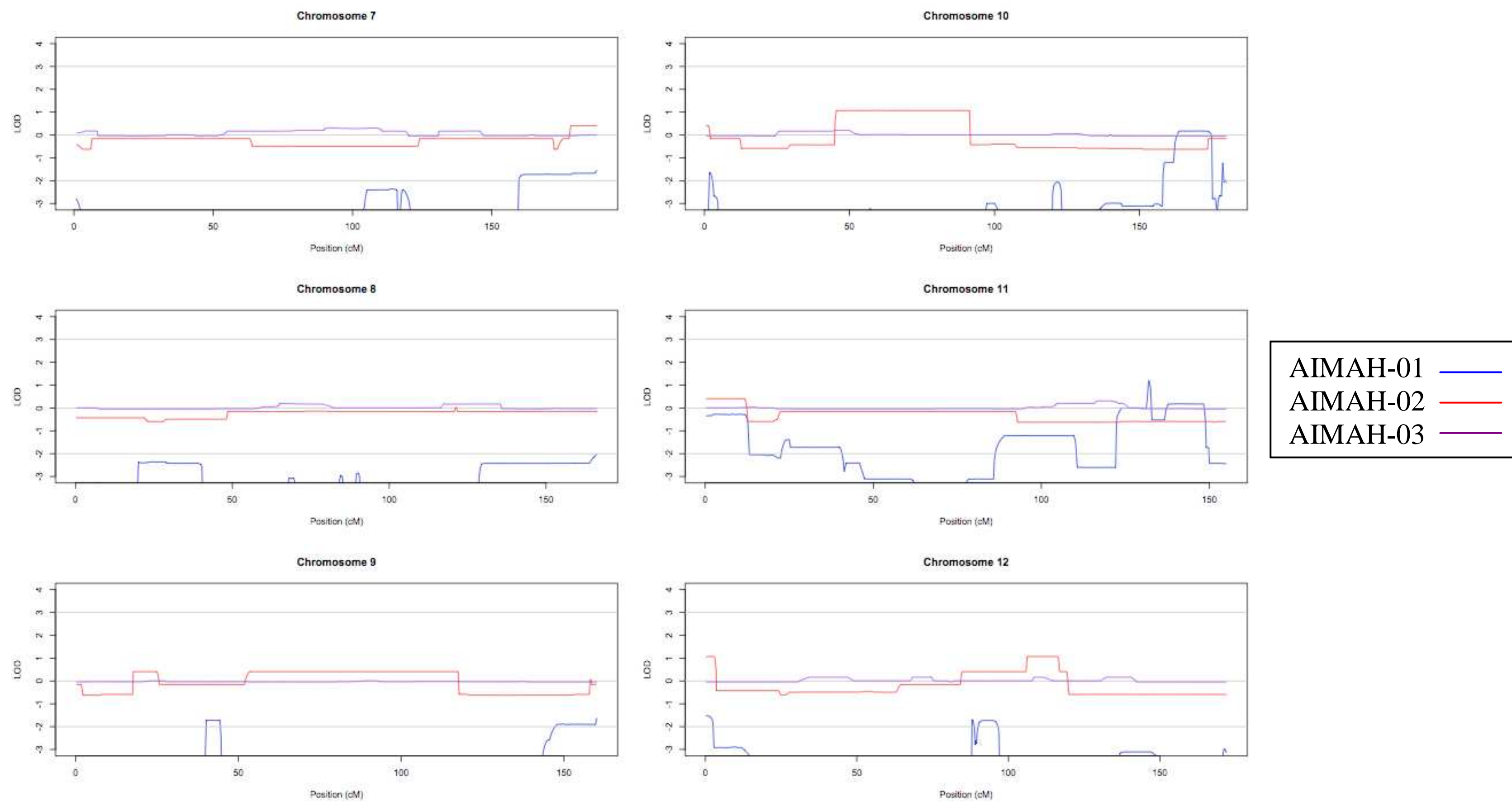


Figure 5.4 (continued). Autosomal parametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for any locus for chromosomes 7-12 for AIMAH-02 or -03.

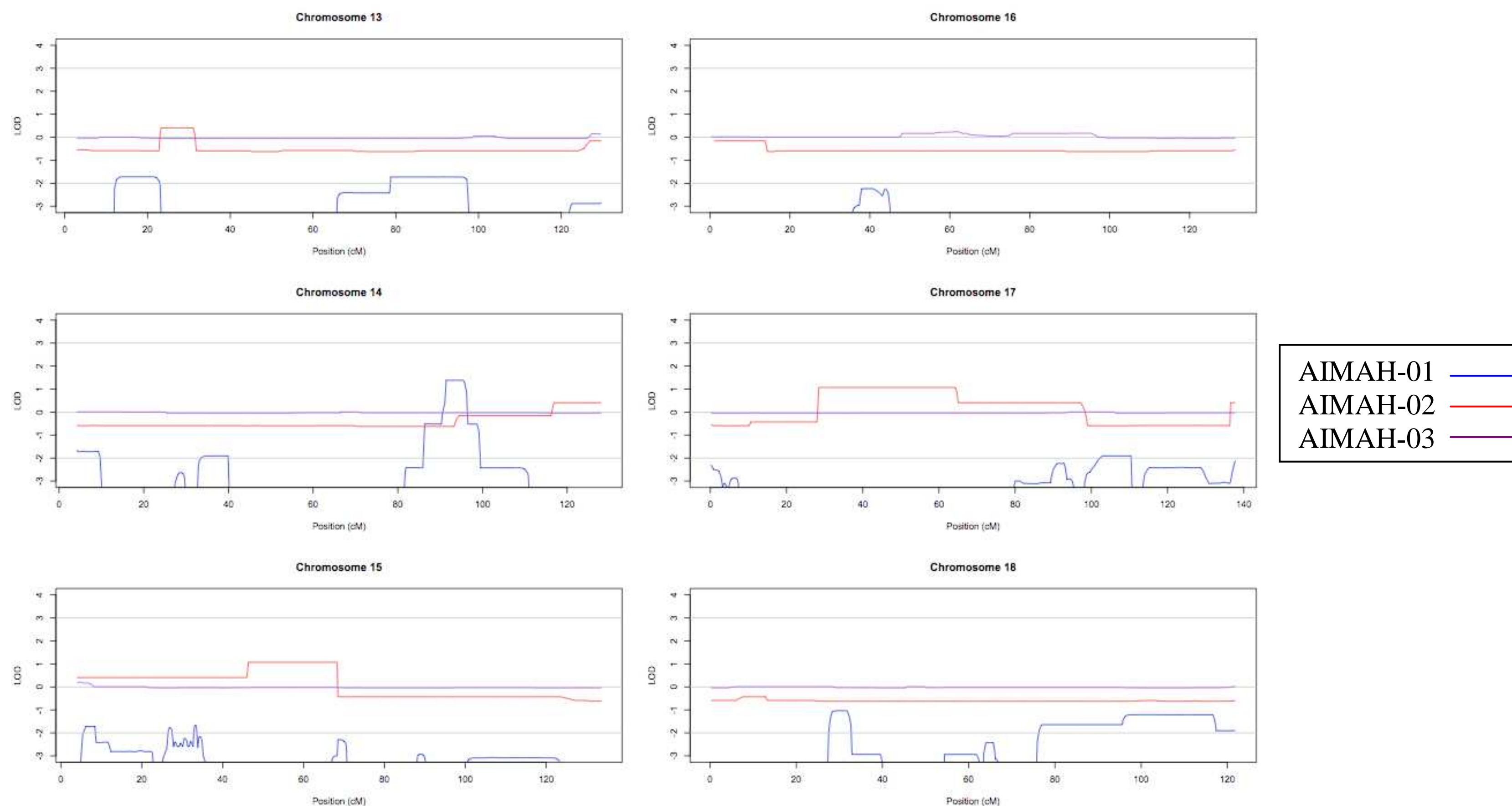


Figure 5.4 (continued). Autosomal parametric linkage analysis scores for kindreds AIMAH-01, -02 and -03.
Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD score (1.83) peak on chromosome 14 for AIMAH-01 is shown. The LOD scores did not strongly support or exclude linkage for any locus for chromosomes 13-18 in AIMAH-02 or AIMAH-03.

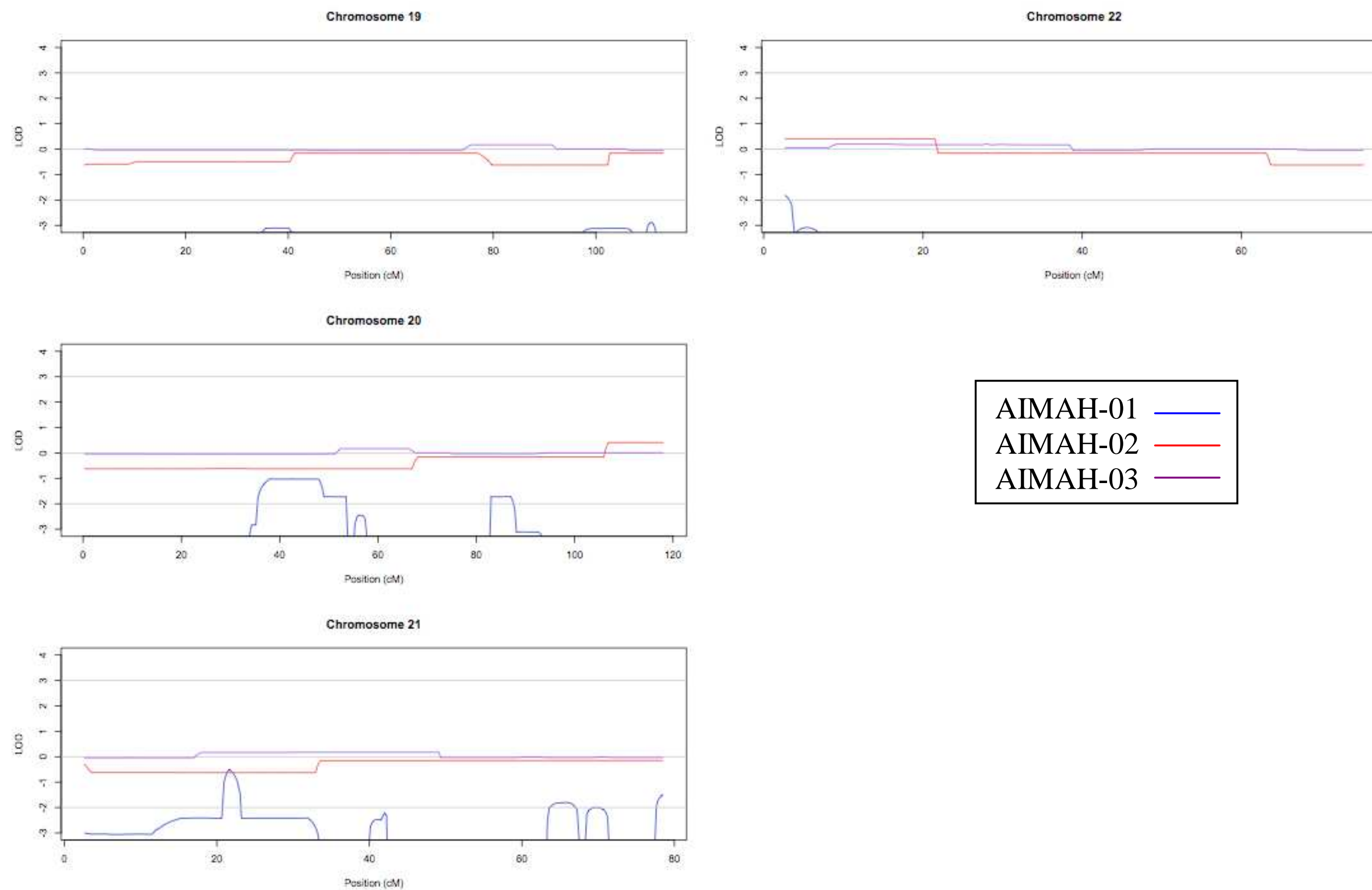


Figure 5.4 (continued). Autosomal parametric linkage analysis scores for kindreds AIMAH-01, -02 and -03. Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the x axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support or exclude linkage for chromosomes 19 to 22 for AIMAH-02 or AIMAH-03.

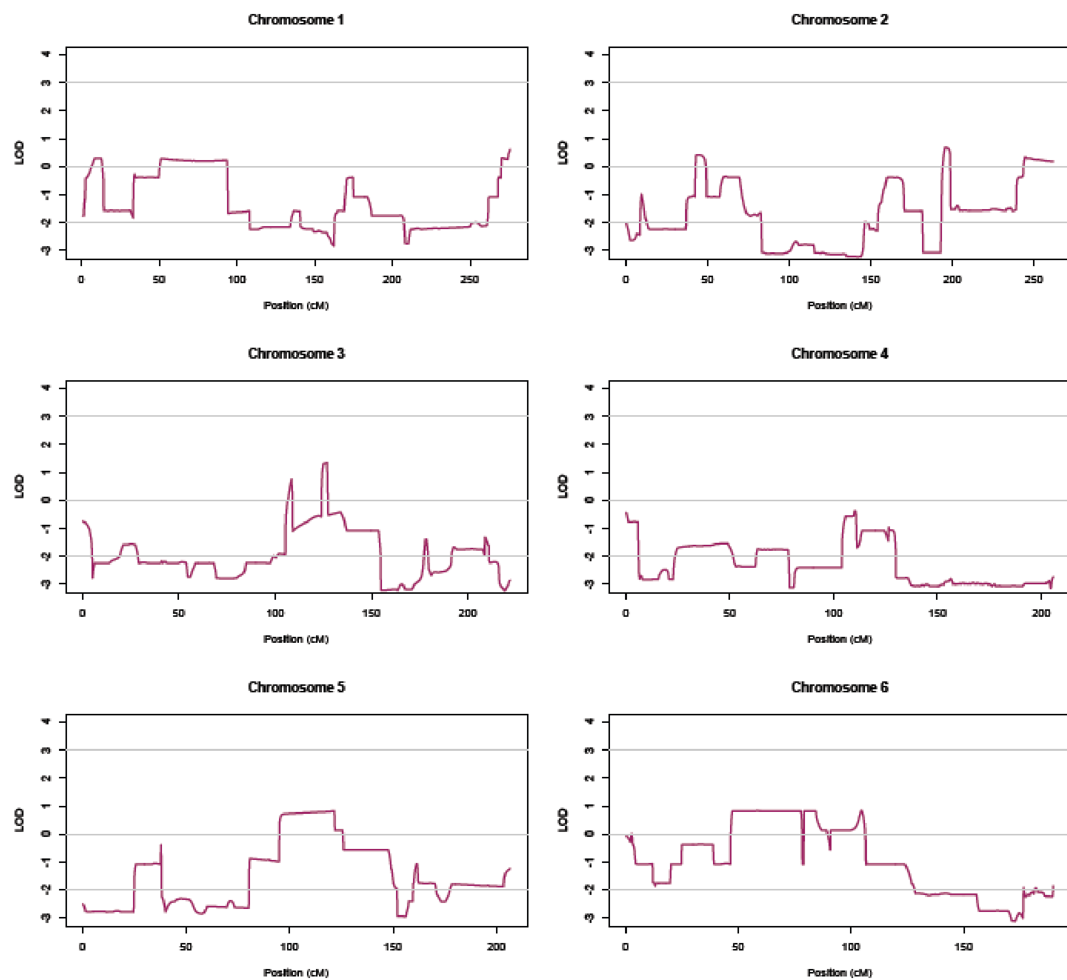


Figure 5.5. Autosomal parametric linkage analysis scores for kindred AIMAH-04.
Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support linkage for any locus on chromosomes 1-6.

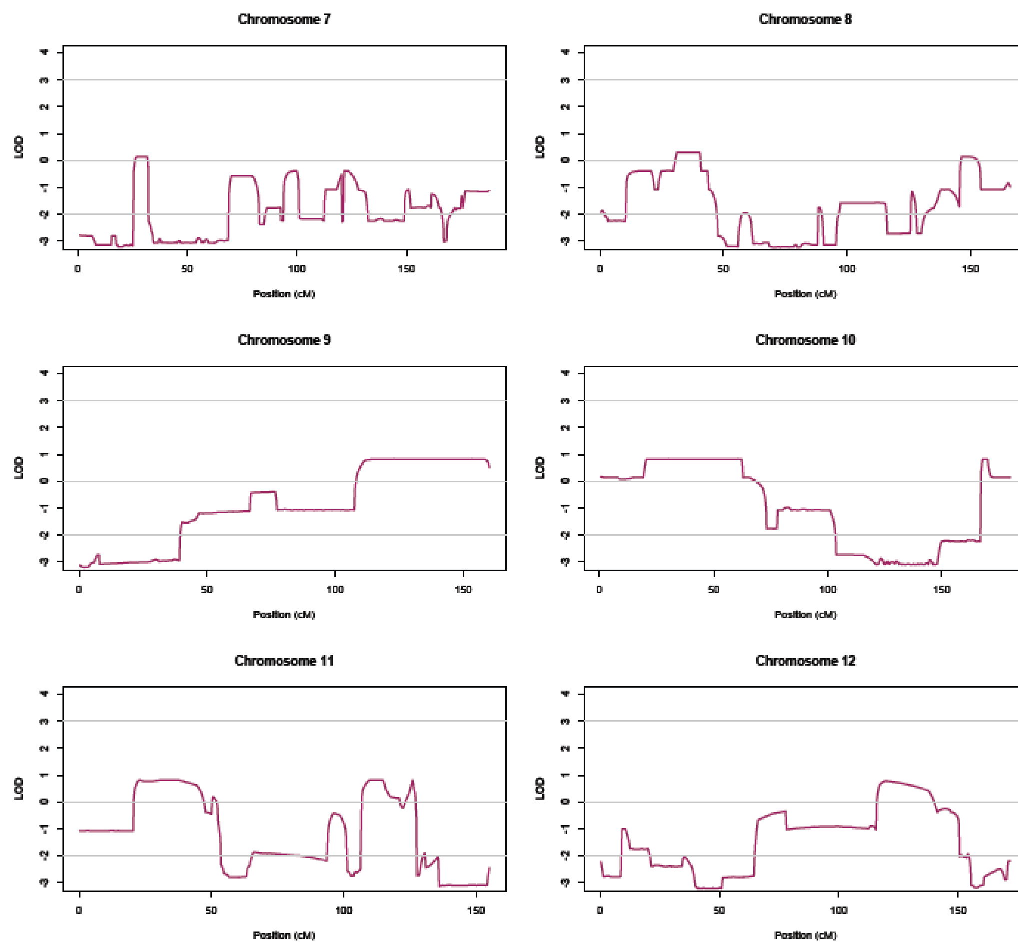


Figure 5.5 (continued). Autosomal parametric linkage analysis scores for kindred AIMAH-04.
Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support linkage for any locus on chromosomes 7-12.

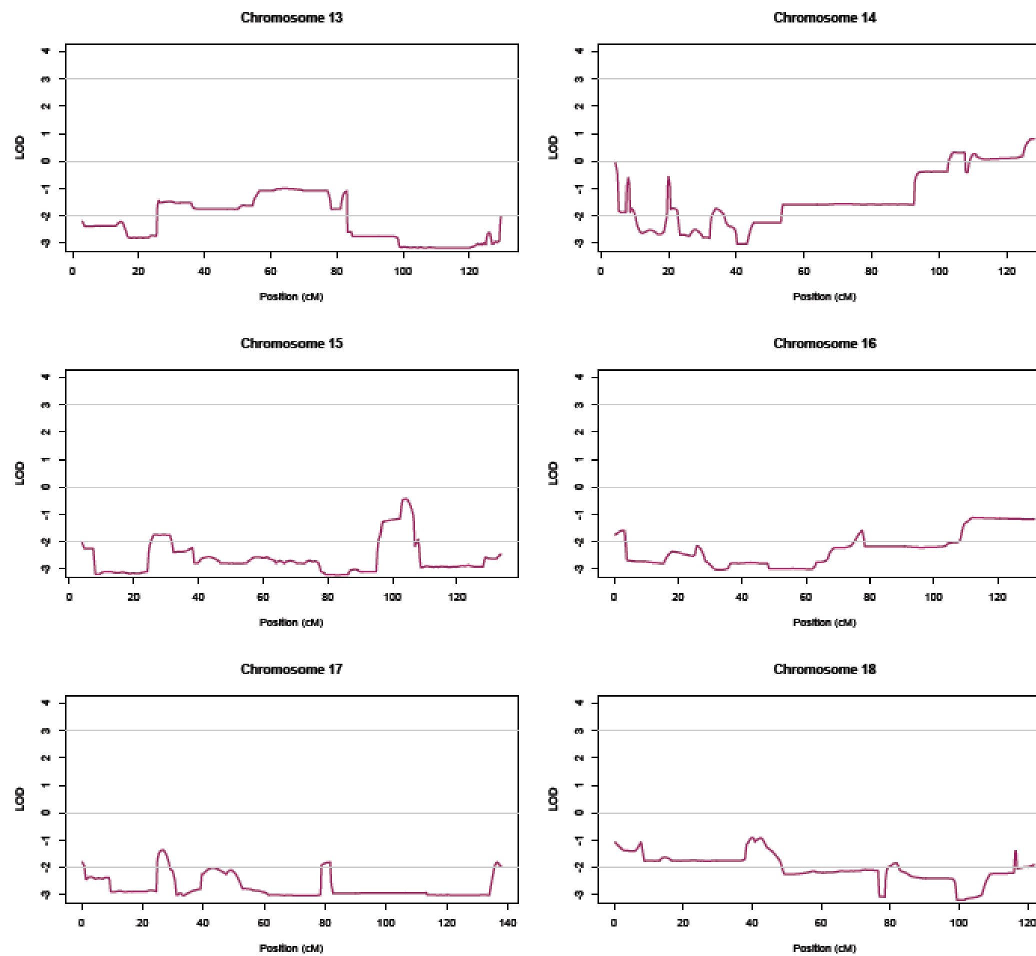


Figure 5.5 (continued). Autosomal parametric linkage analysis scores for kindred AIMAH-04. Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD scores did not strongly support linkage for any locus on chromosomes 13-18.

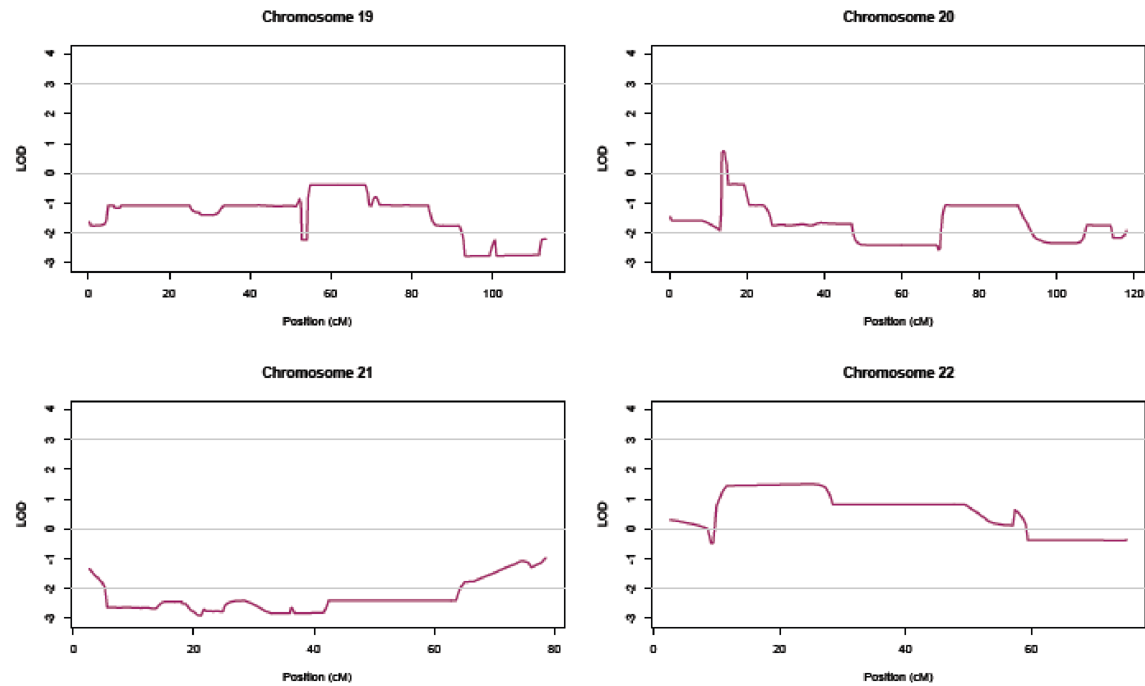


Figure 5.5 (continued). Autosomal parametric linkage analysis scores for kindred AIMAH-04. Parametric LOD scores are indicated on the *y* axis for each chromosome. Along the *x* axis is the chromosomal position in centiMorgans. The LOD score peak (1.50) on chromosome 22 for AIMAH-04 is shown. The LOD scores did not strongly support linkage for any other locus on chromosomes 19-22.

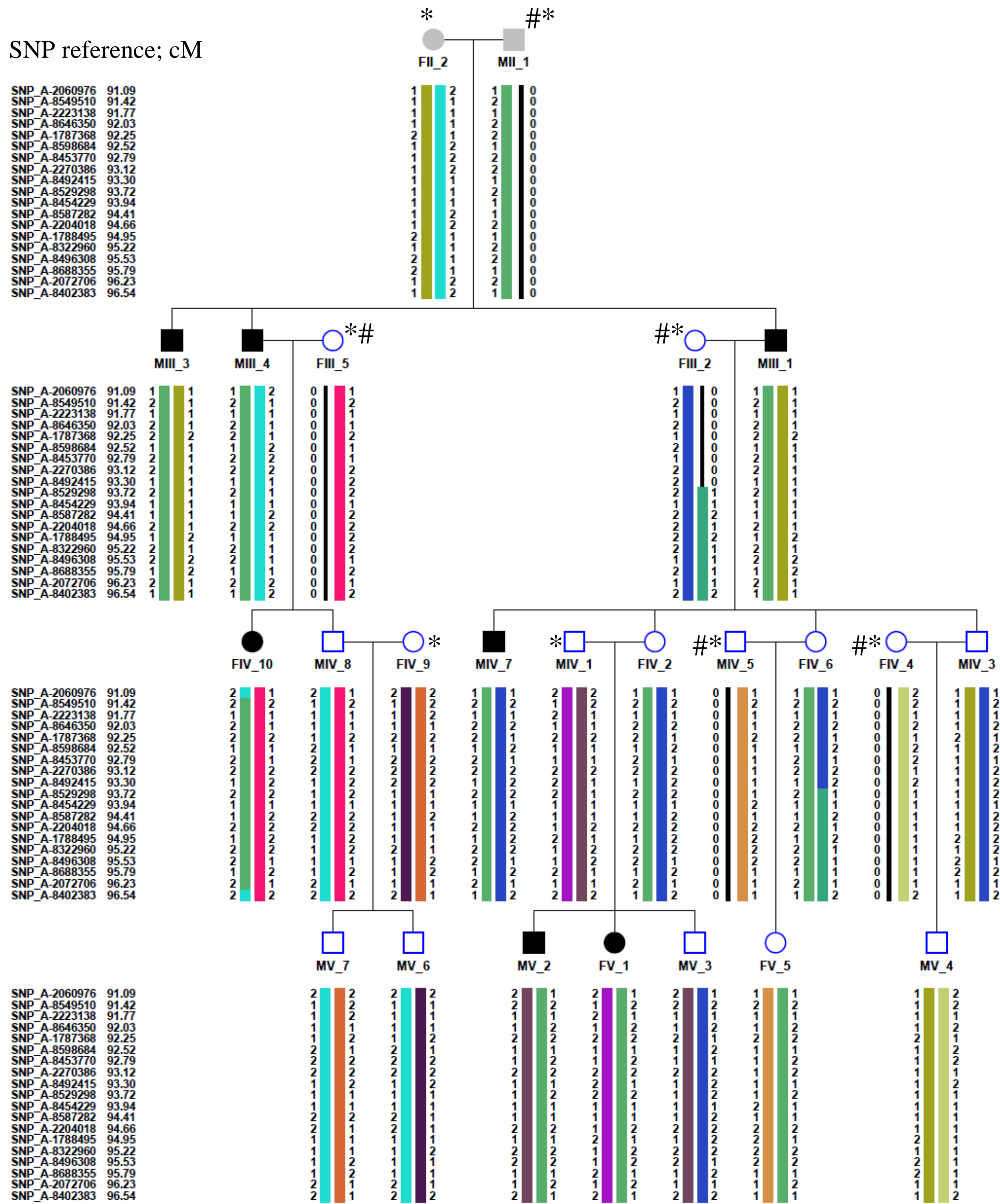


Figure 5.6. Possible locus in AIMAH-01.
This pedigree illustrates the region of possible linkage (dark green) on chromosome 14q32.11-14q32.12 in AIMAH-01. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. The genotype calls are given as binary coding "1" and "2". Spouses of family members shown in the diagram have not been genotyped (indicated by * on pedigree) – their genotype is inferred from the genotype of their children, and knowledge of one parental genotype, where available. "0" for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

Table 5.2. Genes in locus 14q32.11-14q32.12. The genes have been annotated with gene function, human disease association (OMIM), tissue expression (Unigene), Ingenuity Pathway Analysis, and whether there was evidence for copy number variation (CNV) or loss of heterozygosity (LOH) in the AIMAH-01 tumours. The genes selected for direct sequencing have been indicated in bold.

Name; symbol	Function	IPA	Unigene	OMIM	CNV	LOH
Ataxin 3; ATXN3	deubiquitinase activity		adrenal	spinocerebellar ataxia 3	yes	no
Calmodulin 1 (phosphorylase kinase, delta); CALM1	calcium-binding protein; growth, cell cycle, signal transduction	cAMP-mediated signalling; protein kinase A signalling	widespread; includes adrenal	?hip osteoarthritis	no	no
Cation channel, sperm-associated, beta; CATSPERB	trafficking or formation of a stable channel complex		not adrenal		no	no
Chromogranin A; CHGA	Regulates catecholamine release and storage		adrenal	?autonomic dysfunction syndromes	no	no
Chromosome 14 open reading frame 102; C14orf 102			not adrenal		no	no
Chromosome 14 open reading frame 143; C14orf143			not adrenal		no	no
Chromosome 14 open reading frame 159; C14orf159			widespread; includes adrenal		no	no
Chromosome 14 open reading frame 184; C14orf184			not adrenal		no	no

Table 5.2 (continued). Genes on Chromosome 14q32.11-14q32.12.

Name; symbol	Function	IPA	Unigene	OMIM	CNV	LOH
Cleavage and polyadenylation specific factor 2, 100kDa (CPSF2)	cleaves and polyadenylates mRNA precursors	tight junction signalling	not adrenal		yes	no
Coiled-coil domain containing 88C; CCDC88C	inhibits Wnt3a-induced accumulation of β-catenin and Wnt3a-dependent activation of T cell transcription		not adrenal		no	no
Fibulin 5; FBLN5	endothelial cell adhesion via integrins		widespread; includes adrenal	hereditary cutis laxa, age-related macular degeneration	no	no
G protein-coupled receptor 68; GPR68	stimulates inositol phosphate formation	G-protein coupled receptor signalling	not adrenal		no	no
Golgin A5; GOLGA5	maintains Golgi structure		widespread; includes adrenal		no	no
Inositol 1,3,4-triphosphate 5/6 kinase; ITPK1	phosphorylates inositol		not adrenal		no	no
Legumain; LGMN	lysosomal cysteine protease; antigenic peptide processing		widespread; includes adrenal		no	no
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1, 7kDa, NDUFB1	mitochondrial electron transport chain		widespread; includes adrenal		yes	no
ITPK1-antisense RNA (non-protein coding); ITPK1			no data		no	no

Table 5.2 (continued). Genes on Chromosome 14q32.11-14q32.12.

Name; symbol	Function	IPA	Unigene	OMIM	CNV	LOH
Potassium channel, subfamily K, member 13; KCNK13	potassium channel		not adrenal		no	no
Ras and Rab interactor 3; RIN3	guanine nucleotide exchange factor		not adrenal		no	no
Ribosomal protein S6 kinase, 90kDa, polypeptide 5; RPS6KA5	cAMP signalling	Fibroblast growth factor, ILK and p38 mitogen-activated protein kinase signalling	not adrenal		no	no
Small nucleolar RNA, H/ACA box 11B (retrotransposed); SNORA11B			no data		no	no
SMEK homolog 1, suppressor of mek1 (Dictyostelium); SMEK1	interacts with protein phosphatases		widespread; includes adrenal		no	no
Solute carrier family 24 (sodium/potassium/ calcium exchanger), member 4; SLC24A4	potassium-dependent ion exchanger		not adrenal		no	no
Tandem C2 domains, nuclear; TC2N			not adrenal		no	no
Tetratricopeptide repeat domain 7B; TTC7B			not adrenal		no	no

Table 5.2 (continued). Genes on Chromosome 14q32.11-14q32.12.

Name; symbol	Function	IPA	Unigene	OMIM	CNV	LOH
Thyroid hormone receptor interactor 11; TRIP11	interacts with thyroid hormone receptor beta	aryl hydrocarbon receptor signalling	widespread; includes adrenal		no	no
Tyrosyl-DNA phosphodiesterase 1; TDP1	repairs covalent topoisomerase I-DNA complexes		not adrenal	spinocerebellar ataxia (autosomal recessive)	no	no
RAB42, member RAS oncogene family, pseudogene 1; RAB42P1					no	no
hypothetical LOC400238; LOC400238			testis; not adrenal		no	no
BC039357			testis; not adrenal		no	no
C14orf159 variant protein					no	no
BC028746			testis, prostate; not adrenal		no	no
BC039675					no	no
AX721199					no	no
AX747894					no	no
AK093301			parathyroid; not adrenal		no	no

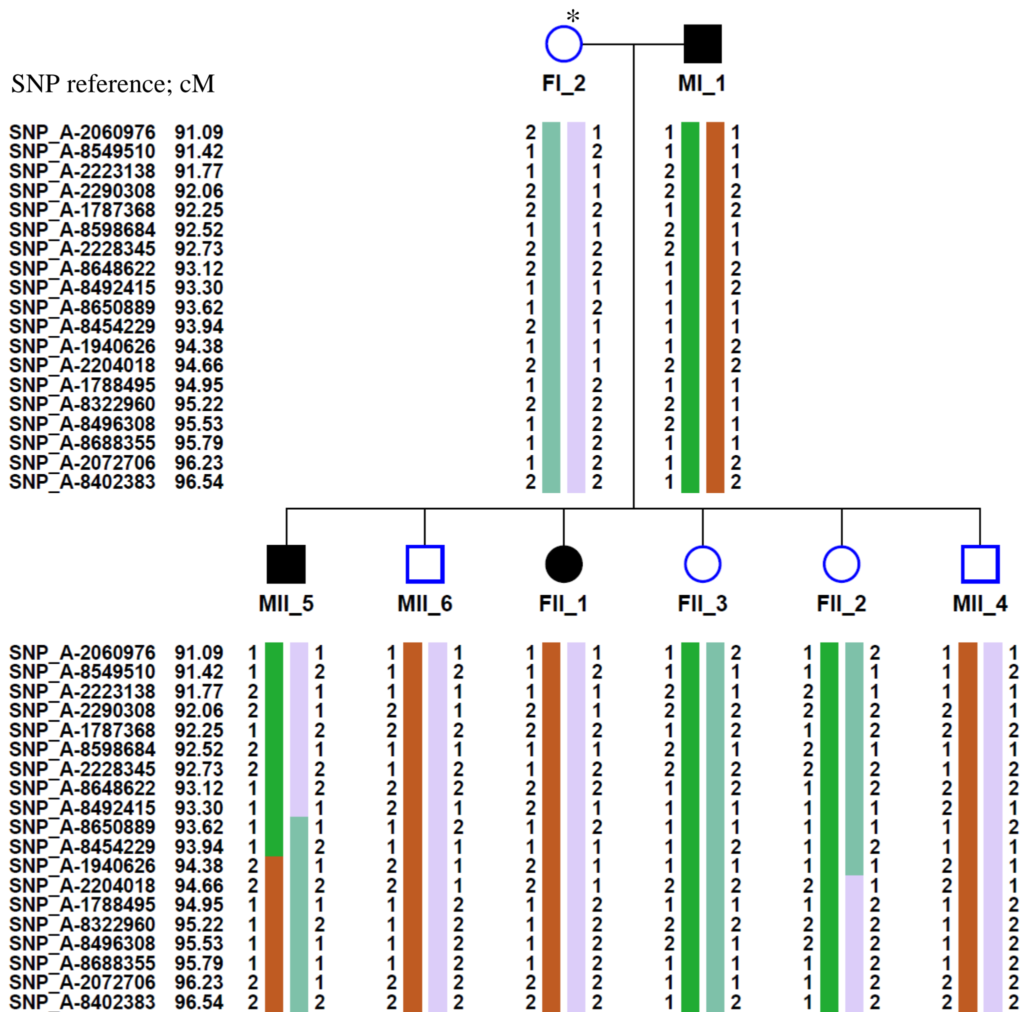


Figure 5.7. Haplotype analysis of AIMAH-02 (AIMAH-01 locus).
This pedigree shows the region of possible linkage (orange) on chromosome 14q32.11-14q32.12 in AIMAH-01 is shared by affected and unaffected individuals from AIMAH-02. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding "1" or "2". The genotype of FI_2 (indicated by *) is inferred from the genotype of her children and knowledge of the paternal genotype.

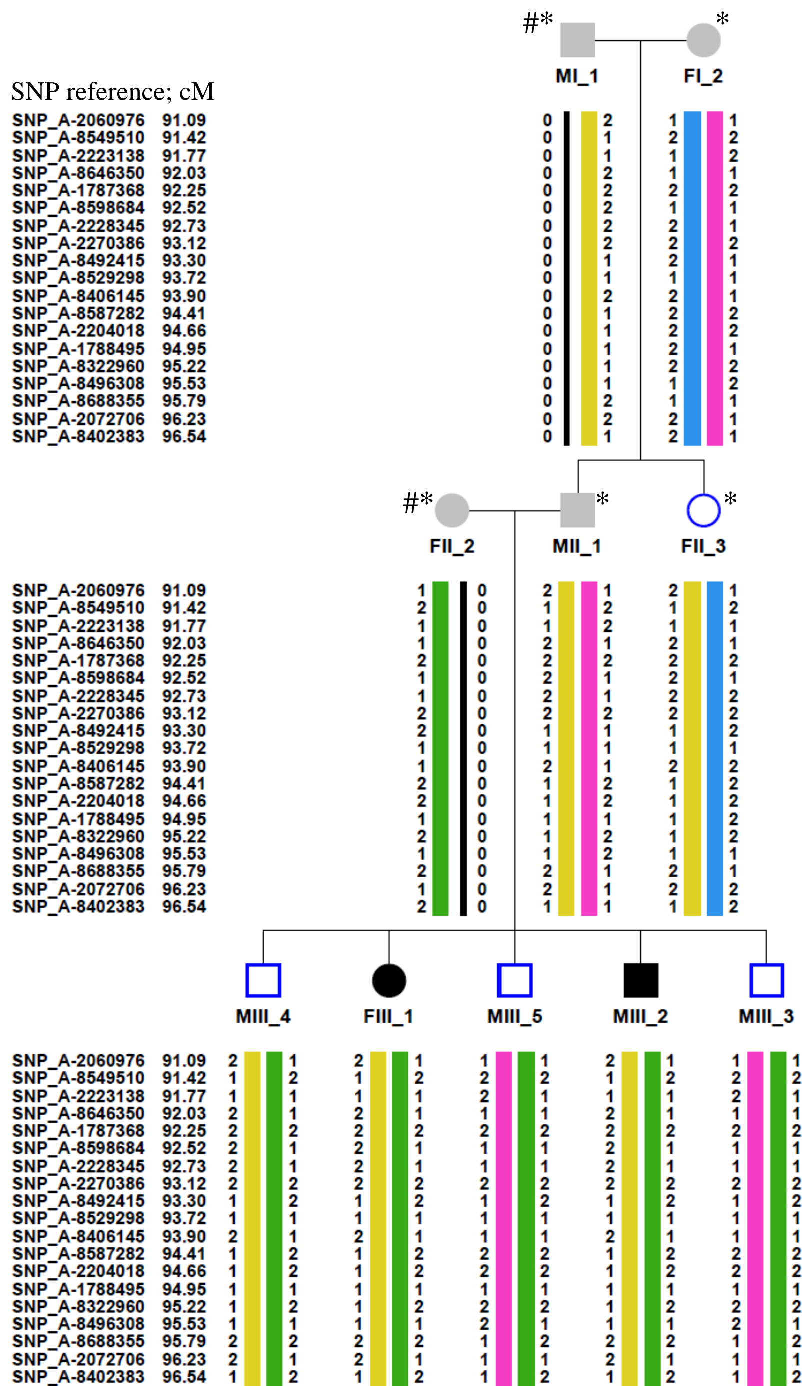


Figure 5.8. Haplotype analysis of AIMAH-03 (AIMAH-01 locus).
This pedigree shows the region of possible linkage (green) on chromosome 14q32.11-14q32.12 in AIMAH-01 is shared identical by descent in individuals from AIMAH-03. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding “1” or “2”. The parental genotypes (FII_2, MII_1, MI_1, FI_2; indicated by *) have been inferred from the genotypes of their children. “0” for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

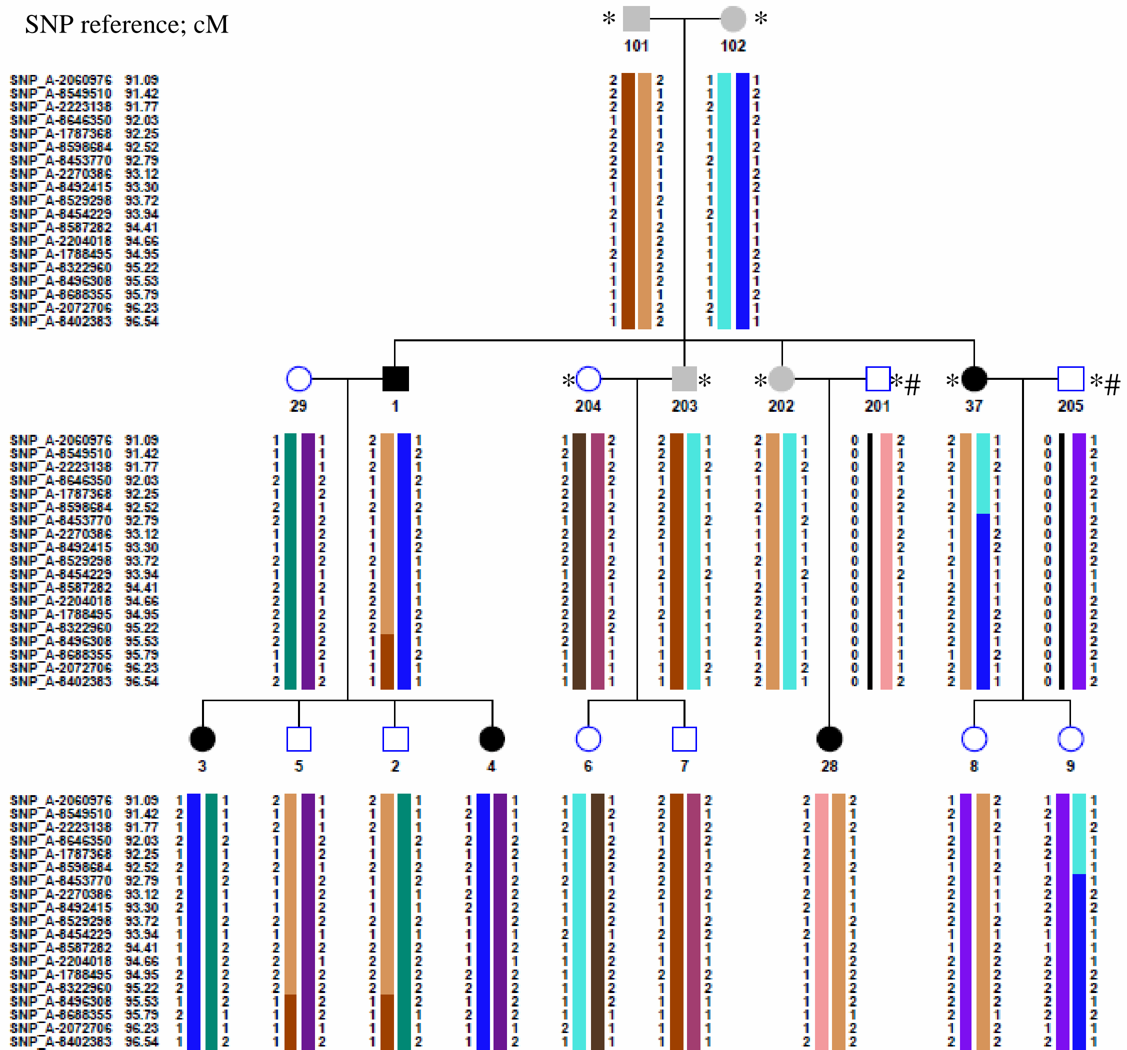


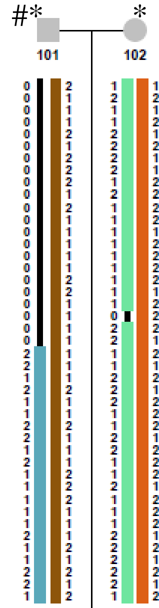
Figure 5.9. Haplotype analysis of AIMAH-04 (AIMAH-01 locus).
This pedigree shows the region (or part thereof) of possible linkage (royal blue) on chromosome 14q32.11-14q32.12 in AIMAH-01 is shared by four of the five affected individuals from AIMAH-04. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding “1” or “2”. Some genotypes (indicated by *) have been inferred from the genotypes of their children. “0” for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

The peak parametric LOD score for AIMAH-02 was 1.07, on chromosomes 1, 2, 6, 10, 12, 15 and 17. The peak parametric LOD score for AIMAH-03 was 0.31, on chromosomes 7 and 11. Hence, there was no region of linkage in AIMAH-02 or -03. Several characteristics of these kindreds limited the statistical power to detect linkage. These include the small pedigree size, lack of multiplex cases within each kindred and lack of affected individuals in multiple generations. Also, we believed it was important to assume genetic heterogeneity between kindreds, so each was analysed independently.

The peak LOD score for AIMAH-04 was 1.50, on chromosome 22. The flanking SNP markers were SNP_A-8630968 (rs4995261) and SNP_A-2115311 (rs5761946), corresponding to 22q11.21-22q12.1 (18676540-25809173) (Figure 5.10). The locus of possible linkage in AIMAH-04 was a 7.1Mb region containing 101 unique and annotated genes (UCSC, NCBI). The haplotype did not segregate perfectly with the phenotype, since it was shared by an unaffected individual (5, Figure 5.10). In AIMAH-01 and AIMAH-02, there was no haplotype in this region shared by affected, but not unaffected, individuals (Figures 5.11 and 5.12). Thus we can not implicate this locus in AIMAH-01 nor in AIMAH-02. In AIMAH-03, affected and unaffected individuals shared the entire region of possible linkage in AIMAH-04 (Figure 5.13).

SNP reference; cM

SNP_A-8460695 9.50
SNP_A-8630968 9.99
SNP_A-2133087 10.96
SNP_A-8655220 11.67
SNP_A-1795361 12.13
SNP_A-8356313 12.37
SNP_A-1814043 12.62
SNP_A-8551302 13.08
SNP_A-4259020 13.57
SNP_A-1880487 14.37
SNP_A-8335295 14.71
SNP_A-8672538 14.94
SNP_A-8432629 15.44
SNP_A-8376328 16.03
SNP_A-4230624 16.60
SNP_A-8649425 17.09
SNP_A-8386129 17.41
SNP_A-1813600 17.65
SNP_A-8495803 18.22
SNP_A-8323318 18.89
SNP_A-1903524 19.22
SNP_A-8686759 19.80
SNP_A-2075573 20.25
SNP_A-8434593 20.60
SNP_A-4195085 21.01
SNP_A-8579654 21.40
SNP_A-2186409 21.89
SNP_A-8426274 22.48
SNP_A-8655867 22.77
SNP_A-2103440 23.36
SNP_A-2056652 23.61
SNP_A-8405181 24.25
SNP_A-8429979 24.61
SNP_A-8546497 24.89
SNP_A-2060664 25.21
SNP_A-8396731 25.78
SNP_A-2067818 26.21
SNP_A-8587739 26.84
SNP_A-8521784 27.26
SNP_A-8473576 27.76
SNP_A-8594251 28.12
SNP_A-2115311 28.44
SNP_A-8674282 29.06



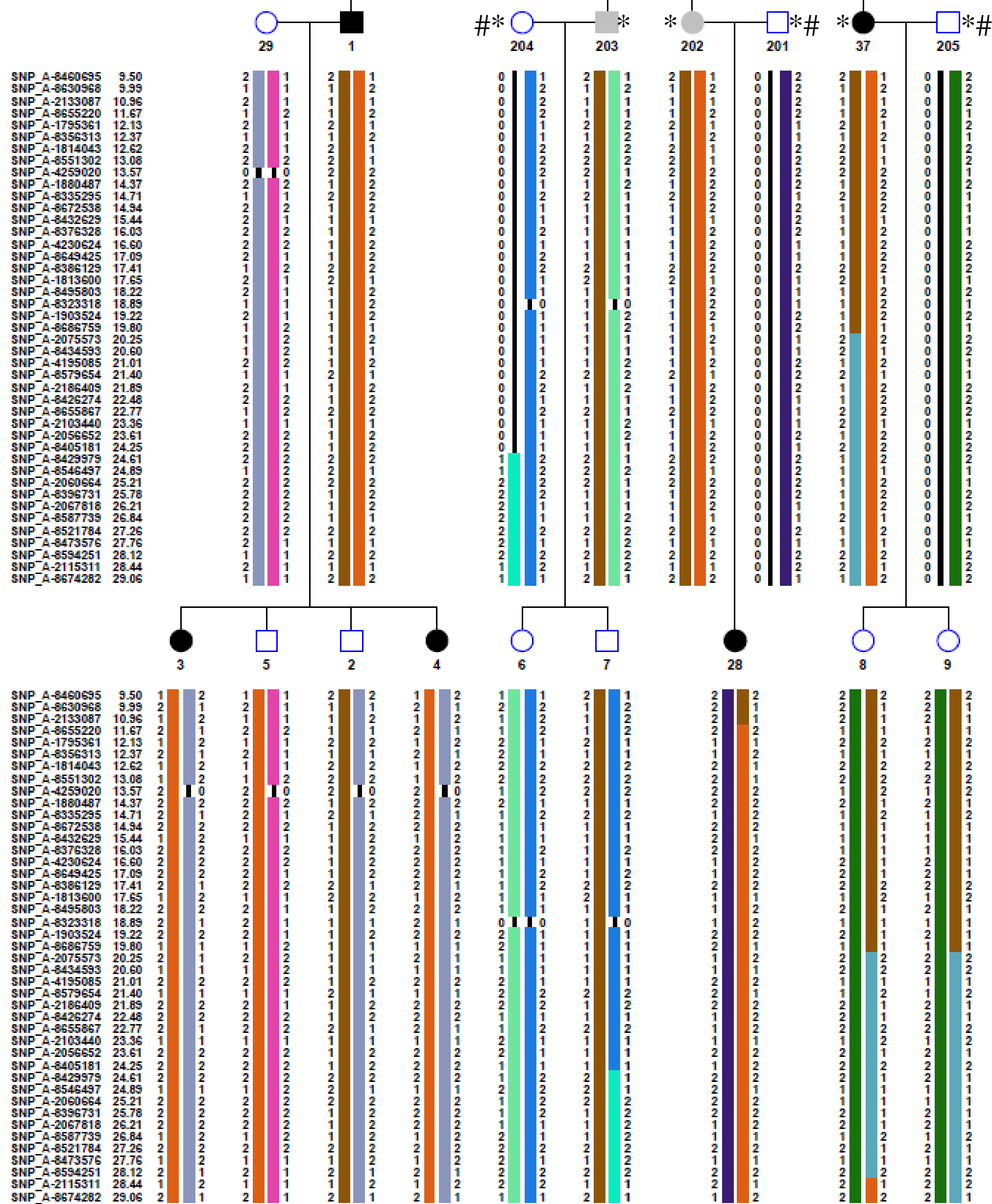


Figure 5.10. Possible locus in AIMAH-04 (Figure legend is on page 242).

Figure 5.10. Possible locus in AIMAH-04 (on page 241).
This pedigree illustrates the region of possible linkage (orange) on chromosome 22q11.21-22q12.1 in AIMAH-04. The region is shared by one unaffected individual (5). The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding “1” or “2”. Spouses of family members shown in the diagram have not been genotyped (indicated by * on pedigree) – their genotype is inferred from the genotype of their children, and knowledge of one parental genotype. “0” for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

Figure 5.11. Haplotype analysis of AIMAH-01 (AIMAH-04 locus) (on page 243).
This pedigree shows that there is no haplotype within the region of possible linkage on chromosome 22q11.21-22q12.1 (AIMAH-04) shared by the affected individuals of AIMAH-01. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding “1” or “2”. Spouses of family members have not been genotyped (indicated by * on pedigree) – their genotype is inferred from the genotype of their children, and knowledge of one parental genotype. “0” for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

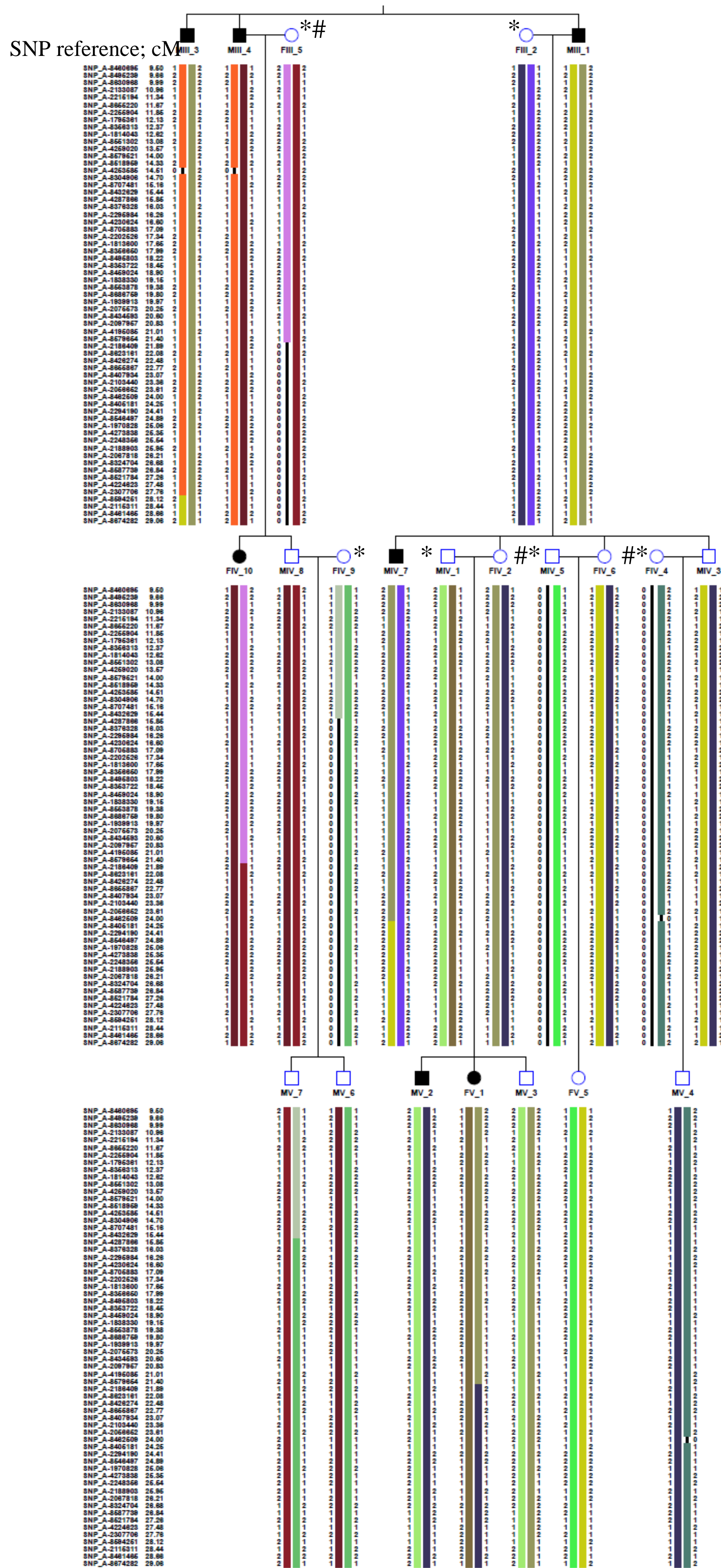


Figure 5.11. Haplotype analysis of AIMAH-01 (AIMAH-04 locus) (Figure legend is on page 242).

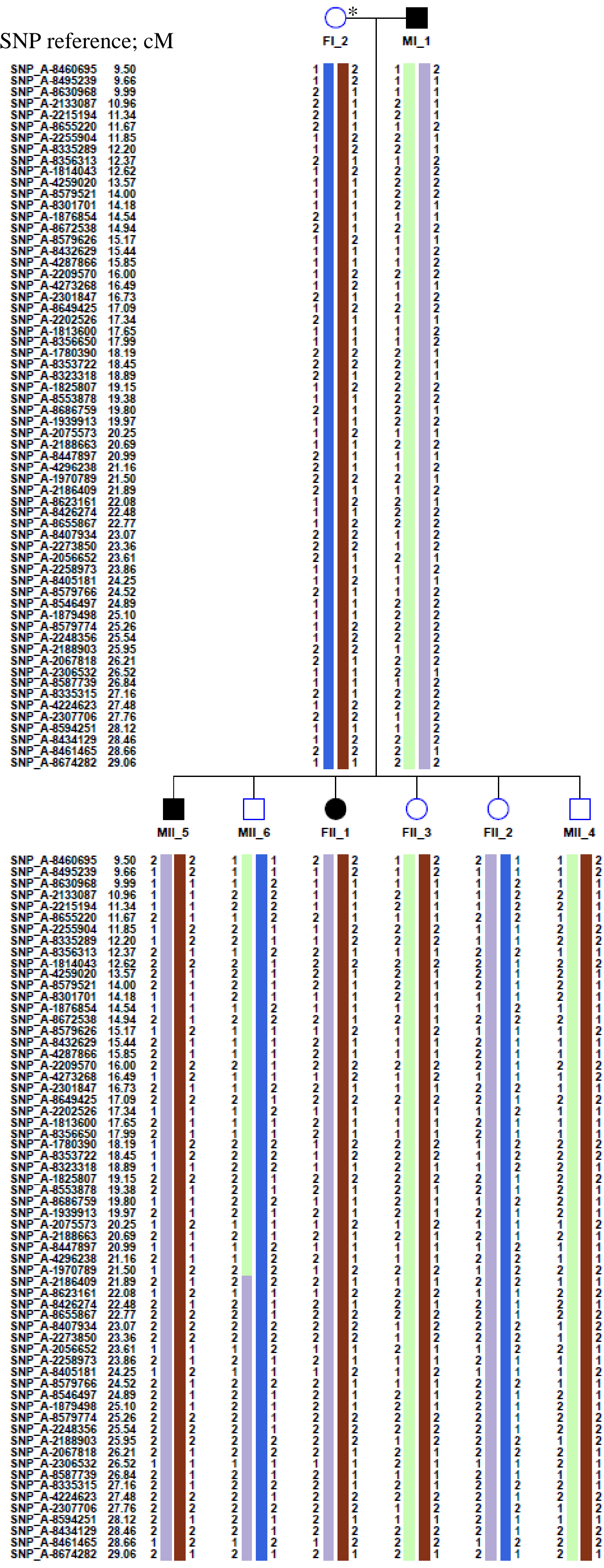


Figure 5.12. Haplotype analysis of AIMAH-02 (AIMAH-04 locus) (Legend is on page 245).

Figure 5.12. Haplotype analysis of AIMAH-02 (AIMAH-04 locus) (on page 244)
This pedigree shows that there is a haplotype within the region of possible linkage on chromosome 22q11.21-22q12.1 (AIMAH-04) shared by the affected individuals (lilac), but this is also shared by one unaffected individual (FII_2) and a part thereof is also shared by another unaffected individual (MII_6) of AIMAH-02. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding "1" or "2". The genotype of FI_2 (indicated by *) is inferred from the genotype of her children and knowledge of the paternal genotype.

Figure 5.13. Haplotype analyis of AIMAH-03 (AIMAH-04 locus) (on page 246)
This pedigree shows that the affected individuals of AIMAH-03 share both a paternal (purple) and a maternal (brown) haplotype within the region of possible linkage on chromosome 22q11.21-22q12.1 (AIMAH-04). The maternal haplotype is also shared by an unaffected individual (MIII_3), though the paternal is not. The SNP markers (Affymetrix code) and their genetic map distance (centiMorgans - cM) are indicated on the left of the diagram. Genotypes are given as binary coding "1" or "2". Spouses of family members shown in the diagram have not been genotyped (indicated by * on pedigree) – their genotype is inferred from the genotype of their children, and knowledge of one parental genotype. "0" for genotype indicates that there was insufficient information to infer a genotype (indicated by # on pedigree).

Figure 5.13. Haplotype analyis of AIMAH-03 (AIMAH-04 locus) (Figure legend is on page 245).

LOD scores < -2 are evidence against linkage. The SNPs flanking regions with LOD scores < -2, in AIMAH-01 and AIMAH-04, are presented in Appendix 5, Tables A.5.1 and A.5.2, respectively. These data excluded some of the genes which were mutated in sporadic AIMAH as causative in AIMAH-01: *MEN1*, *MC2R*, *GNAS* (Table 5.3).

Table 5.3. Selected LOD score data for AIMAH-01.
The LOD score within the region flanked by the SNPs listed, was < -2; which serves to exclude these genes as causative in AIMAH-01.

Gene symbol	Chromosomal coordinates (hg18)	rs name	Pos (bp)	LOD score
MC2R	Chr 18: 13872043-13905535	rs623152 rs7227395	10020105 40909586	-1.7189 -1.6549
GNAS	Chr 20: 56848190-56919645	rs6123669 rs6062682	55180999 62362264	-1.9297 -2.5063
MEN1	Chr 11: 64327562-64335342	rs550840 rs240569	63958215 64490254	-3.2718 -3.2666

Chromosomal coordinates are the reference start and end positions for the gene (UCSC). The rs value is the SNP reference number (dbSNP). Pos (bp) is the chromosomal position of the SNP (hg18).

Similar analysis for the AIMAH-04 linkage data excluded *FH* and *GNAS* (Table 5.4). Notably, *MEN1* was not excluded according to our linkage analysis since a LOD score < -2, by convention considered evidence against linkage, was not achieved. The actual LOD scores however are very close, and are shown in Table 5.4. Furthermore, prior mutation analysis of the coding regions of the *MEN1* gene had previously essentially excluded this gene as causative (Nies *et al.,* 2002).

Thus, after having performed linkage analysis studies of four AIMAH families, we arrived at a possible, and distinct, chromosomal locus in each of two of the families. This is suggestive, but not conclusive evidence, that familial AIMAH is, as has already been demonstrated anecdotally in sporadic AIMAH, genetically heterogeneous. This is not conclusive because genome-wide significant evidence of linkage was not achieved (Lander and Kruglyak, 1995).

Table 5.4. Selected LOD score data for AIMAH-04.
The LOD score within the region flanked by the SNPs listed, was < -2; which serves to exclude FH and GNAS as causative in AIMAH-04. For MEN1, the LOD score was -1.9.

Gene symbol	Chromosomal coordinates (hg18)	rs name	Pos (bp)	LOD score
FH	Chr 1: 239727480-239749708	rs528011	238084930	-1.9972
		rs2580238	240628728	-1.8968
GNAS	Chr 20: 56848190-56919645	rs99595	55423214	-1.9755
		rs944240	59632486	-1.7384
MEN1	Chr 11: 64327562-64335342	rs7101608	63128296	-1.8973
		rs320109	63436621	-1.9017
		rs1123251	63674177	-1.9015
		rs550840	63958215	-1.9016
		rs240569	64490254	-1.9032
		rs1195962	64697995	-1.9041
		rs2170082	65211371	-1.9075
		rs669371	65430729	-1.9064
		rs4930351	65823240	-1.9056
		rs11601870	66511185	-1.9053
		rs4930224	67357650	-1.9053
		rs2458267	67780538	-1.9057
		rs10501398	68088291	-1.9075
		rs4930243	68256450	-1.9101

Chromosomal coordinates are the reference start and end positions for the gene (UCSC). The rs value is the SNP reference number (dbSNP). Pos (bp) is the chromosomal position of the SNP (hg18).

Since display of the AIMAH phenotype is age-dependent, and we had genotyped three generations from AIMAH-01, liability classes were applied to better model the disease in this family. This was justified because the confidence that could be placed in the assigned phenotype was dependent on each individual's age. Apart from the age at presentation of the three siblings with CS, we do not know the youngest age at which the earliest manifestation of AIMAH occurs and nor do we know which is the earliest manifestation (adrenal nodules visible on CT scan or biochemical abnormalities). Although CS due to AIMAH typically presents in the 5th and 6th decades of life, adrenal nodules and subtle biochemical abnormalities are likely to antedate the clinical syndrome by several years, if not decades. This is because AIMAH is frequently subclinical, CS presents insidiously, in part due to the inefficient steroidogenesis of the tumours (discussed in Chapter 3) and prospective follow-up

of affected patients indicates that adrenal tumorigenesis progresses slowly (N'Diaye *et al.,* 1999). Hence, the older the individual, the more confident we can be that an "unaffected" individual will not develop AIMAH later. Conversely, in younger individuals, we are less confident of an "unaffected" status remaining so.

Any linkage analysis study is critically reliant upon accurate phenotyping. Three of the seven "affected" individuals from AIMAH-01 have subtle abnormalities of the HPA axis, but not definite AIMAH (Figure 2.1). A fourth "affected" individual has, in addition, a single left adrenal nodule, which has been stable on imaging over two years (Figure 2.1). Although adrenal nodules are uncommon (autopsy prevalence 0-0.36%) in her age group (late teenage years), this could be an adrenal incidentaloma (Kloos *et al.,* 1995). In addition, there was no morphological abnormality of the right adrenal gland - yet AIMAH is a bilateral adrenal disease. However, asynchronous development of adrenocortical hyperplasia in AIMAH has been observed; and it could be expected that this is more likely to be detected in early AIMAH (N'Diaye *et al.,* 1999). Longitudinal surveillance of this family should clarify whether these are early forms of AIMAH. Nonetheless, if these individuals do not have AIMAH, then the linkage analysis is invalid. Moreover, a single generation of affected individuals would not exclude the possibility of a recessive disorder. Incorrect phenotyping also invalidates the exclusion of chromosomal loci derived from linkage analysis.

A confounding factor in the linkage analysis of AIMAH-01 is that according to our phenotype assignment, the disease is not fully penetrant. This is exemplified by individual IV-1 (Figure 2.1) who has been assigned an unaffected phenotype, but who has two children (V-1, V-2; Figure 2.1) with abnormalities suggesting early AIMAH. If the children are affected, then their mother is an obligate carrier of the disease allele. Thus there may be other silent carriers of the disease allele. Also, although there was no history of consanguinity within any of the kindreds; should this have been a remote event, then this could change the possible inheritance

pattern to recessive – invalidating the parametric linkage analysis which has been performed under a dominant disease model.

In AIMAH-04, the assignment of phenotype has been different (Nies *et al.,* 2002). Each individual has been assigned an "affected" or "unaffected" phenotype; isolated (without adrenal morphological abnormalities), subtle biochemical abnormalities were not detected, in contrast with AIMAH-01 and AIMAH-02, and more recent phenotyping data since publication are, to our knowledge, not available (Nies *et al.,* 2002). Also, in AIMAH-04, the presentation of CS has been in the 4th decade – much earlier than in the other families; this could be a hallmark of a distinct genetic cause (Nies *et al.,* 2002). In addition, only females have developed CS; the only known affected male had not developed CS by age 79, although he did have bilateral macronodular adrenal hyperplasia and subtle biochemical abnormalities (Nies *et al.,* 2002). Thus, one could postulate a role for oestrogen as a gene or disease modifier in this family (Nies *et al.,* 2002).

An inherent assumption of the linkage analysis is that within each kindred AIMAH is monogenic, i.e., due to a single disease-causing allele operating within a family, and we have also assumed that the disease allele is rare in the normal population. Whilst this appears to be the most plausible model, we can not exclude that more complex models might be applicable. For example, should there be more than one disease-causing allele operating within any of the families (e.g., rare alleles in some individuals, common alleles in others) or a modifier gene (perhaps introduced by an unrelated spouse) which might alter the function of another gene, then this would have reduced the power of our analyses to identify disease-causing loci, beyond the limitations already discussed. Furthermore, if AIMAH is caused by epigenetic (no change in the DNA sequence) or environmental factors, linkage analysis could not assist in elucidation of the molecular basis of AIMAH.

Copy number variation analysis

This was performed using tumour DNA compared with corresponding germline DNA from the same patient (III-2, III-3; AIMAH-01 – Figure 2.1). For both tumours, regions of deletion were predominant over amplification. The absolute CN increase did not exceed 5.2. CNV analysis using genomic segmentation suggested a CN loss in the region of linkage, 14q32.11-14q32.12 in III-2T (Figure 5.14). The genes in this region were ataxin 3 (*ATXN3*), NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1, 7kDa (*NDUFB1*) and cleavage and polyadenylation specific factor 2, 100kDaA (*CPSF2*) (region average 1.29). This tumour also had a putative CN loss at 2p16 (Figure 5.15), the second locus identified by linkage analysis in kindreds with Carney complex (Stratakis *et al.,* 1996). Other putative CN losses included: 3p21.31 (Figure 5.16), which included *PRKAR2A*, the gene encoding the 2A regulatory subunit of protein kinase A (PKA); 11q13 (Figure 5.17) which did not include *MEN1* and 17q (Figure 5.18), including 17q24, although not the 1A regulatory subunit of PKA (*PRKAR1A*). The importance of *PRKAR1A* in other adrenocortical tumours has been discussed in Chapter 1. At the time of writing this thesis, these remain putative CNVs since the data have not been confirmed. However, review of the pictorial data (Figures 5.14-5.18) is not compelling for any CNV in these regions, nor in other chromosomes (data not shown).

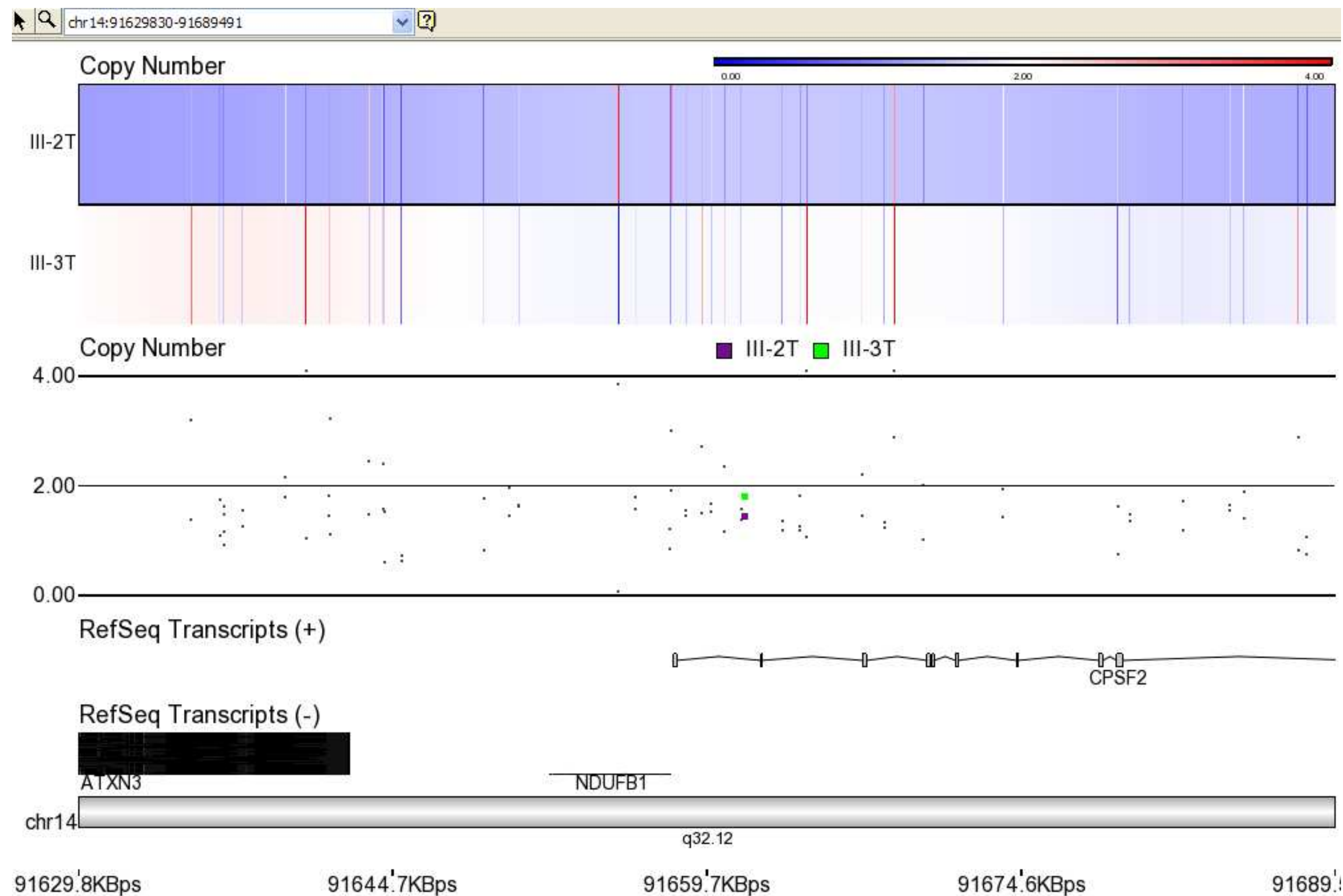


Figure 5.14. Putative somatic copy number loss in the AIMAH-01 locus.
There was a putative copy number loss (calculated region average 1.29) in tumour from III-2 (AIMAH-01; Figure 2.1) in the region of possible linkage. This included the genes *ATXN3, NDUFB1* and *CPSF2*. This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

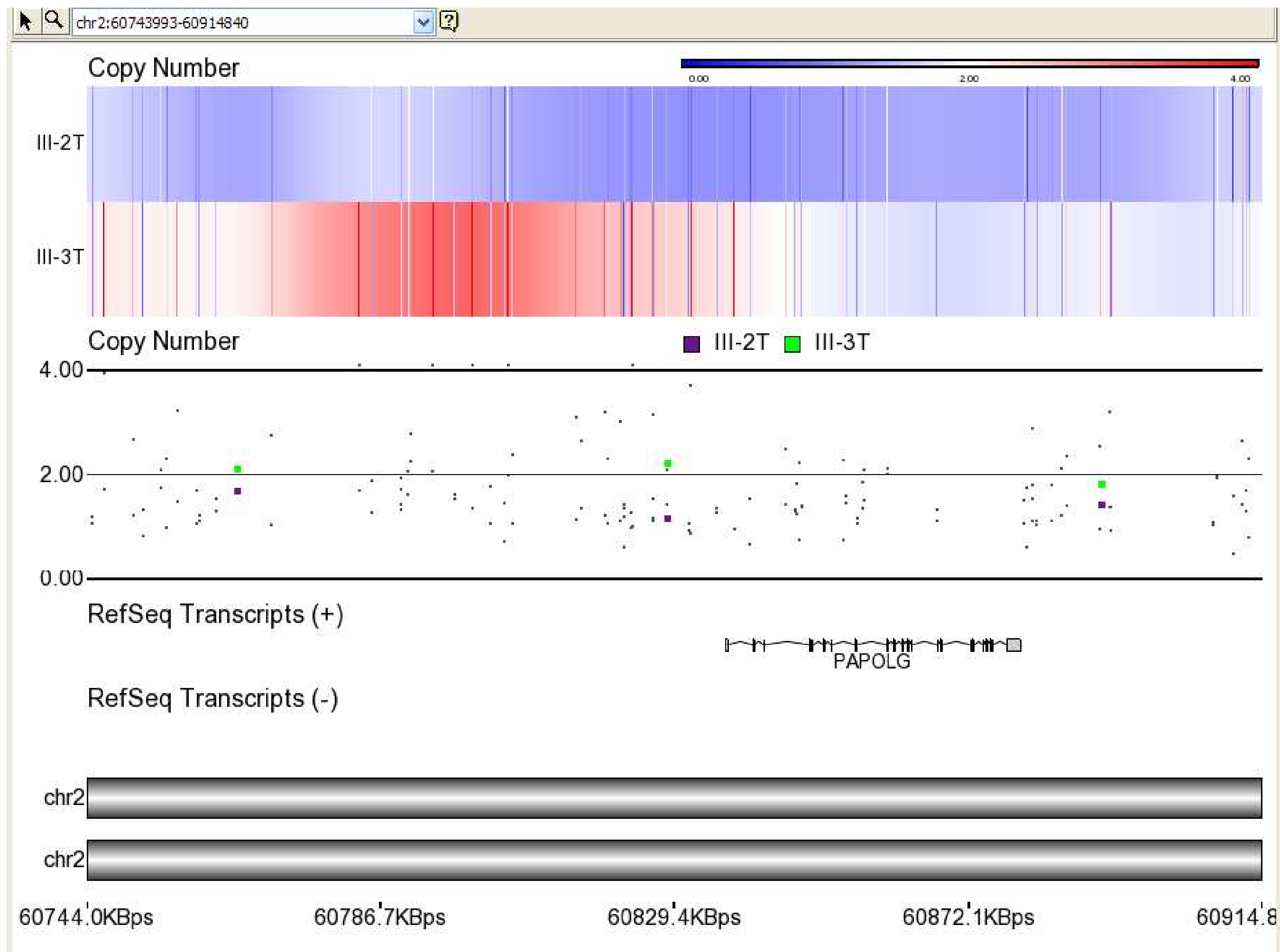


Figure 5.15. Putative somatic copy number loss involving 2p16.
There was a putative copy number loss at 2p16 in tumour from III-2 (AIMAH-01; Figure 2.1). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

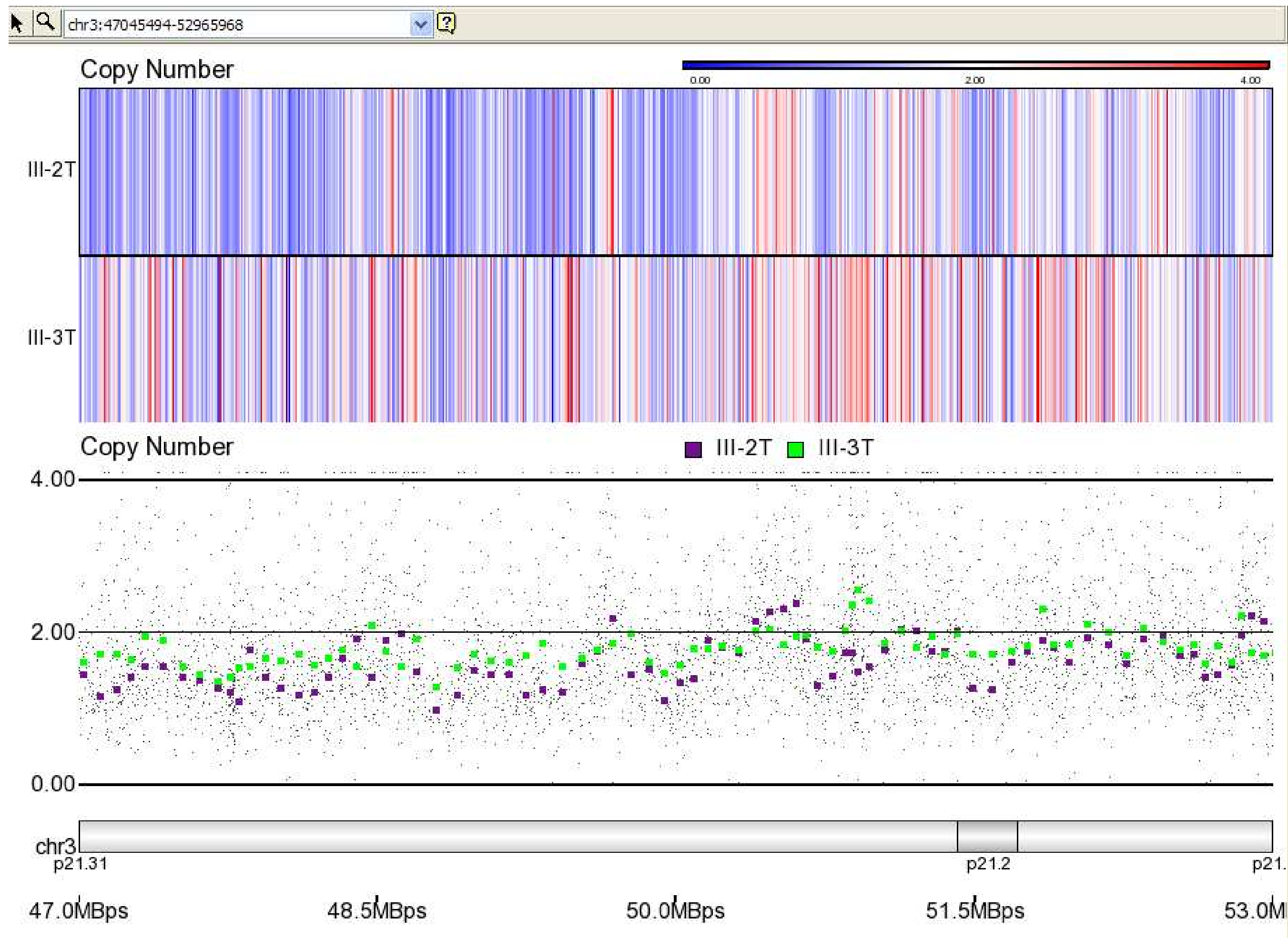


Figure 5.16. Putative somatic copy number loss involving 3p21.
There was a putative copy number loss at 3p21.31 in tumour from III-2 (AIMAH-01; Figure 2.1). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

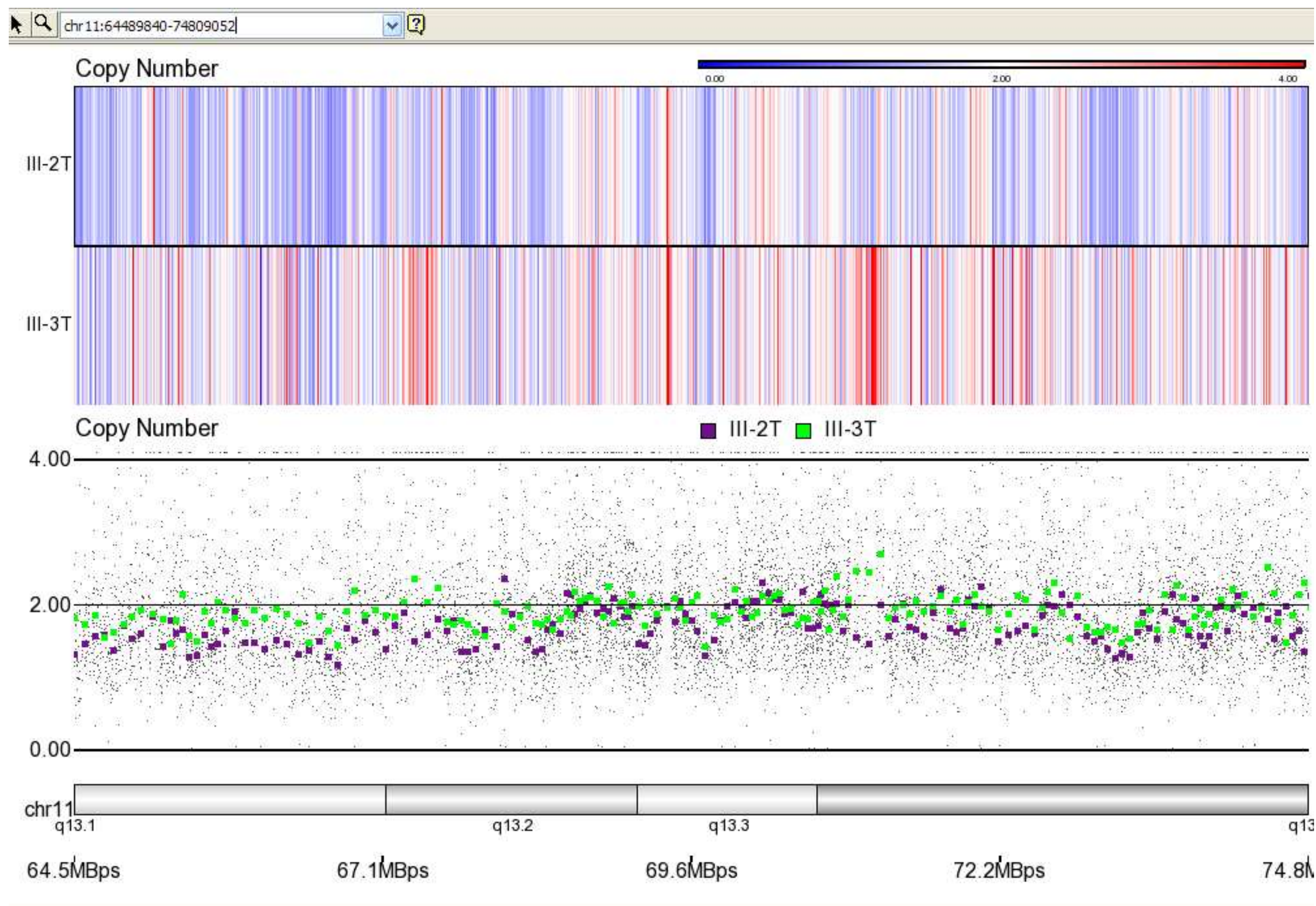


Figure 5.17. Putative somatic copy number loss involving 11q13.
There was a putative copy number loss on 11q13 (excluding the menin locus) in tumour from III-2 (AIMAH-01; Figure 2.1). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

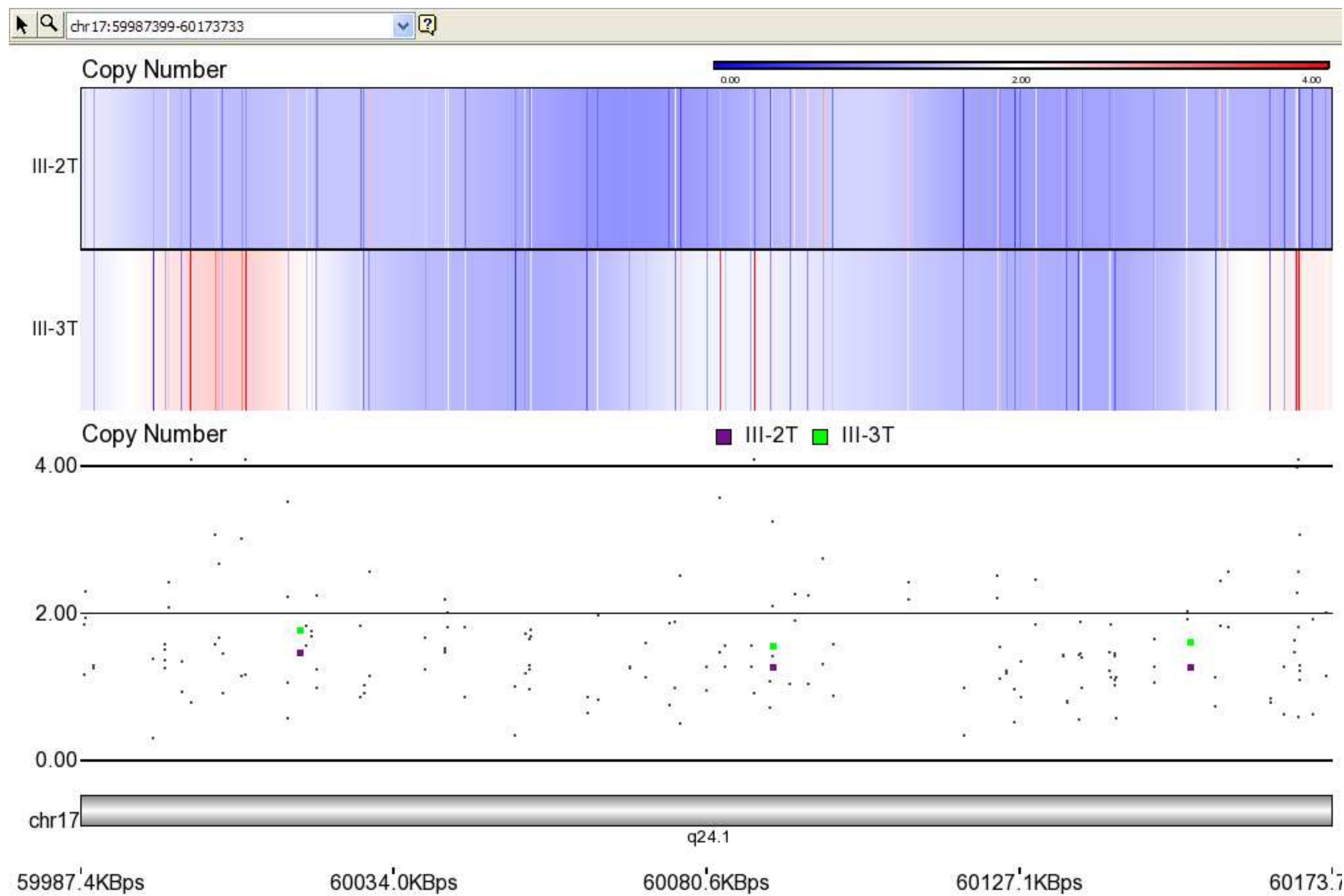


Figure 5.18. Putative somatic copy number loss involving 17q24.
There was a putative copy number loss on 17q24 (excluding *PRKAR1A* locus) in tumour from III-2 (AIMAH-01; Figure 2.1). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

Many more CNVs were present in III-2T compared with III-3T. This likely reflects secondary, rather than causative, genetic changes associated with tumorigenesis. There were few CNVs common to both III-2T and III-3T (Appendix 6). There was a putative common CN loss of 12q24.31 detected using both genomic segmentation (mean 0.985099) and Hidden Markov modelling (region average 1.22382). This region contains exons 2 through 12 of the gene for zinc finger CCHC domain-containing protein 8 (*ZCCHC8*) (Figure 5.19).

ZCCHC8 is a nuclear protein and a member of a family of zinc proteins (Gustafson *et al.,* 2005). It contains a nucleic acid binding domain suggesting a role in transcriptional regulation and another domain that suggests a function in RNA processing and metabolism (Gustafson *et al.,* 2005; Kajimura *et al.,* 2009). In mitotic cells, ZCCHC8 protein is highly phosphorylated (Gustafson *et al.,* 2005). Expression of *ZCCHC8* is increased in neuroblastoma and in mature oocytes and human embryonic stem cells (Wolf *et al.,* 2010; Assou *et al.,* 2009). In neuroblastoma, increased expression is accompanied by CN amplification of 12q24.31 (Wolf *et al.,* 2010). *ZCCHC8* is also upregulated in breast epithelial cells stimulated to proliferate by the overexpression of the transcription factor DEAF-1 (deformed epidermal autoregulatory factor-1); suggesting it may be one of its target genes (Barker *et al.,* 2008). *ZCCHC8* is a frequent component of the gene signature of oestrogen receptor positive breast cancer, suggesting a role in these hormone-dependent tumours (Yu *et al.,* 2007). The significance, if any, of a CN loss affecting *ZCCHC8* in the AIMAH-01 tumours is not yet clear.

Loss of heterozygosity analysis

III-3T had a large region of LOH on 16p13 (Figure 5.20). The tumour samples did not share any regions of LOH.

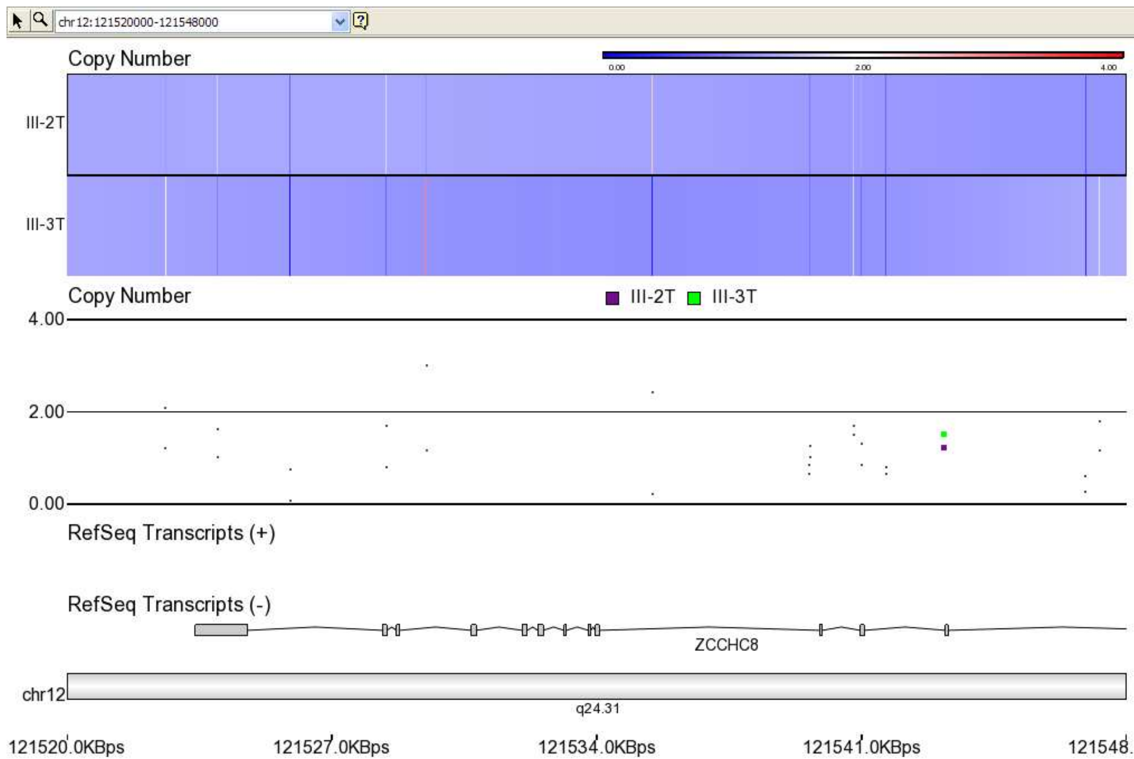


Figure 5.19. Putative somatic copy number loss in region containing *ZCCHC8*. There was a purported copy number loss on 12q24 in AIMAH tumours from III-2 and III-3 (AIMAH-01; Figure 2.1). This region contained the gene zinc finger CCHC domain-containing protein 8 (*ZCCHC8*). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

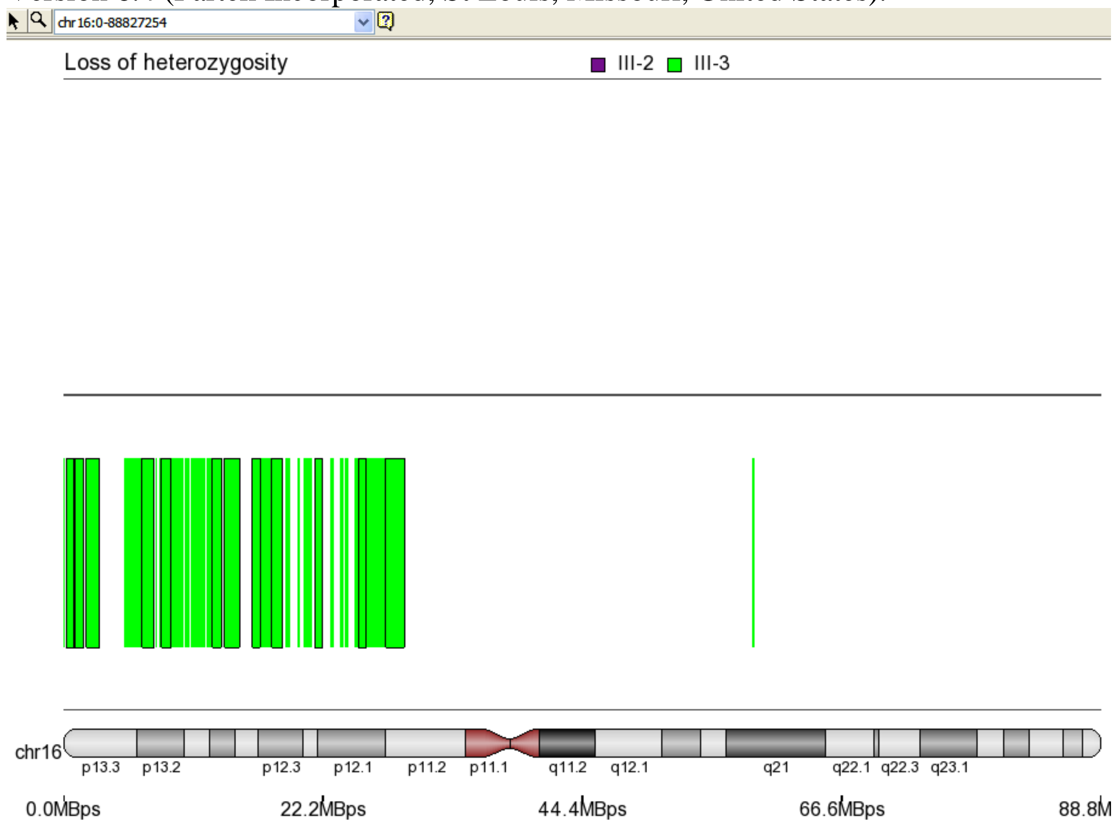


Figure 5.20. Loss of heterozygosity on 16p13 in tumour from III-3 (AIMAH-01). This figure was generated using Partek® Genomics Suite™, Version 6.4 (Partek Incorporated, St Louis, Missouri, United States).

Sequencing of candidate genes in region of possible linkage (AIMAH-01)

Due to limited sample availability, we have not studied the AIMAH-04 locus further at this stage. We undertook sequencing of selected candidate genes in the locus of possible linkage in AIMAH-01. Despite that genome-wide significant evidence for linkage (LOD score > 3) at this locus was not achieved; this was the locus with the strongest, and moreover, only, evidence for linkage in AIMAH-01. It was for these reasons that we studied genes within the locus. Tumour CNV data assisted prioritisation of candidate genes selected for sequencing. There was a CN loss (mean < 1.30) in adrenal tumour DNA from III-2 (AIMAH-01) on 14q32.11-14q32.12 which included the genes *ATXN*3, *CPSF2* and *NDUFB1* (full gene names in Table 5.2) (Figure 5.14). There was a possible CN gain in tumour DNA from III-3, which included *TDP1* – although, the region average of 2.5 did not meet the predefined (though arbitrary) criterion of region average > 2.7. Review of known gene function, Unigene, OMIM and IPA data also assisted prioritisation of candidate genes (Table 5.2).

ATXN3 and *TDP1* were selected on the basis of the CNV data discussed above. *CALM1* was selected because the protein can indirectly activate adenylate cyclase, increasing intracellular cAMP production. Since cAMP activates PKA, this pathway was over-represented in our AIMAH expression data (Chapter 4) and as discussed in Chapter 1, the PKA pathway is involved in adrenocortical tumorigenesis, *CALM1* was considered a good candidate. Aberrant expression of G-protein coupled receptors (GPCR) is a frequent occurrence in AIMAH (Chapter 1); hence *GPR68* which encodes a GPCR was also considered a good candidate. *ITPK1* was selected because it has kinase activity. Finally, *CHGA* which encodes for a protein secreted by a variety of neuroendocrine tumours was also sequenced.

All coding exons (including splice sites) for these genes were directly sequenced in III-2 and III-3 (AIMAH-01; Figure 2.1), II-1 (AIMAH-02; Figure 2.10) and III-3 (AIMAH-03; Figure 2.12). There was no sequence variation noted in *TDP1*, *GPR68* or *CALM1*. The sequence

variants in the other genes are described in Table 5.5, along with the consequence of the nucleotide change and an assessment of the potential for pathogenicity. The potential pathogenicity of a variation was considered to be excluded if the variation was synonymous or had a reported reference population frequency > 10% (dbSNP129). No potentially pathogenic variations were identified. Thus, sequencing of a subset of candidate genes within the AIMAH-01 locus did not identify the causative gene in AIMAH-01. An intronic (splice site) mutation affecting exon splicing in *ATXN3* was excluded by RT-PCR (Figure 5.21). This was performed because preliminary analyses had suggested a CN loss of the region containing this gene in both tumours – however this was not confirmed by later analyses. With the advent and availability of large-scale DNA resequencing methods during the course of this study, evaluation of the potential locus in AIMAH-01 by a candidate gene approach was not pursued further.

Table 5.5. Coding variations of sequenced candidate genes in possible AIMAH-01 locus.

Gene name, Gene symbol	Kindred*	SNP reference	Variant#	Consequence§	Potential pathogenicity
Ataxin 3, ATXN3	AIMAH-01	rs16999141	c.492C>T	p.=; V164V	excl - synonymous
	AIMAH-03	rs16999141	c.492C>T	p.=; V164V	excl - synonymous
	AIMAH-01	rs1048755	c.634G>A	p.V212M	excl - population frequency
	AIMAH-01	rs12895357	c.916G>C	p.G306R	excl – population frequency
Chromogranin A, CHGA	AIMAH-03	rs9658655	c.792G>C	p.E264D	excl - population frequency
	AIMAH-01	rs729940	c.1195C>T	p.R399W	excl - population frequency
	AIMAH-01	rs941581	c.1323A>G	p.=; E441E	excl– synonymous
	AIMAH-02	rs941581	c.1323A>G	p.=; E441E	excl– synonymous
	AIMAH-03	rs941581	c.1323A>G	p.=; E441E	excl– synonymous
Inositol 1,3,4-triphosphate 5/6 kinase, ITPK1	AIMAH-03	rs2295394	c.834C>T	p.=; F278F	excl – synonymous

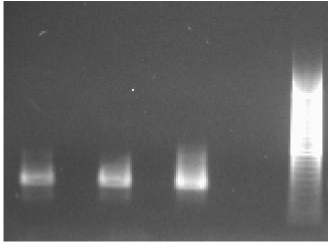
excl – excluded; SNP reference – from National Centre for Biotechnology Information SNP database (dbSNP129)

*Kindreds as described in Chapter 2

#Variant is given in Human Genome Variation Society (HGVS) notation as follows: {nucleotide interval}{changed nucleotide>new nucleotide}
Nucleotide abbreviations are as follows:
A – adenine; C – cytosine; G – guanine; T - thymine

§Consequence given as: (1) p.{code first amino acid changed}(amino acid interval}{code new amino acid} or (2) p.=; indicates a synonymous variant (no change in the protein sequence)
Amino acid abbreviations are as follows:
D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; M: methionine; R: arginine; V: valine; W: tryptophan

1F-6R: Forward primer in exon 1; reverse primer in exon 6
1 2 3 4 5 6 7 8

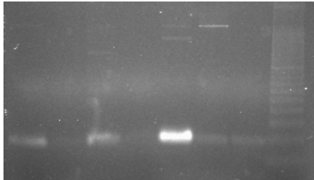


Expected product size: 435bp

6F-10R: Forward primer in exon 6; reverse primer in exon 10
1 2 3 4 5 6 7 8

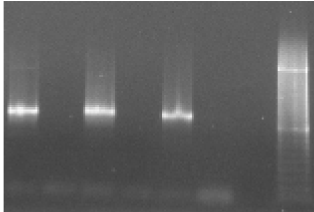
Expected product size: 487bp

10F-11R: Forward primer in exon 10; reverse primer in exon 11
1 2 3 4 5 6 7 8



Expected product size: 198bp

1F-11R: Forward primer in exon 1; reverse primer in exon 11
1 2 3 4 5 6 7 8



Expected product size: 1184bp

Lane 1: III-2
Lane 2: III-2 (RT-)
Lane 3: III-3
Lane 4: III-3 (RT-)
Lane 5: Control
Lane 6: Control (RT-)
Lane 7: No template control
Lane 8: Marker (brightest bands at 1kb and 3kb)

Figure 5.21. RT-PCR of ataxin 3 mRNA from III-2 and III-3 (AIMAH-01).
This was performed to exclude a mutation affecting exon splicing. All products were of the expected size, consistent with the presence of all exons in the mRNA.

5.4 Conclusion and Future Studies

To our knowledge, these studies have been the first performed internationally, attempting to identify the causative genetic basis of familial AIMAH. The linkage studies have identified two distinct loci within two of the families, AIMAH-01 and AIMAH-04, which could potentially harbour the disease-causing allele. Unfortunately, due to limited sample availability, we have not been able to study the AIMAH-04 locus further. Although neither LOD score achieved what has been conventionally considered to be genome-wide significant evidence of linkage in either family, in the future we may be able to reanalyse our data, particularly if and when other individuals develop AIMAH. Also, in the future, we and others may encounter large AIMAH kindreds; analyses of such kindreds could be expected to be more informative for linkage, and our existing data may be in some way contributory.

During the course of this PhD, the approach to novel gene discovery was revolutionised by the ability to "capture" and resequence large compartments of the human genome. Given the inherent limitations of linkage analysis, we proceeded initially with targeted exon capture, and then with whole exome capture and next-generation sequencing studies. These will be discussed in Chapter 6.

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

6.1 Introduction

The linkage analysis studies we have performed with the aim of identifying the locus (or loci) harbouring the disease-causing allele(s) for familial ACTH-independent macronodular adrenal hyperplasia (AIMAH), suggested possible linkage at 14q32.11-12 in AIMAH-01. During the course of this PhD, next-generation sequencing (NGS) methods, described in Chapter 1, became available (Hodges, Xuan *et al.,* 2007; Ng *et al.,* 2009). This technology has enabled relatively cost-effective deep resequencing on a larger (selected exons, whole exome and genome) scale than ever before and has already led to the discovery of novel genes for rare Mendelian disorders of previously unknown genetic basis (Ng *et al.,* 2009; Ng, Bigham *et al.,* 2010; Ng, Buckingham *et al.,* 2010; Gilissen, Arts, Hoischen *et al.,* 2010; Musunuru, Pirruccello, Do *et al.,* 2010; Walsh, Shahin *et al.,* 2010; Bilgüvar, Öztürk *et al.,* 2010).

Having analysed the linkage data, and with acknowledgement of the inherent limitations of the data, as discussed in Chapter 5, we decided the best next step was to proceed with next-generation sequencing (NGS) of germline DNA from affected individuals. We initially performed targeted exon capture (III-2, AIMAH-01; Figure 2.1) of 712 cancer genes, which could provide data on the presence of non-synonymous single nucleotide variants (SNVs) in the exons of selected genes, as well as enable us to familiarise ourselves with this cutting-edge technology, and the issues pertaining to data analysis, albeit on a relatively small-scale.

We subsequently proceeded to whole exome capture (WEC) of two affected siblings (III-2, III-3 – AIMAH-01; Figure 2.1); we chose to study two siblings because we expected that

seeking out only shared variants would substantially reduce the number of potentially pathogenic variants for us to consider. We also anticipated that WEC would enable us to rapidly study the entire AIMAH-01 locus of possible linkage, as well as the whole exome; we believed this was a cost- and time-efficient strategy for novel gene discovery, compared with consecutively sequencing the genes in the locus. At the time we conducted this study, the WEC/NGS technology could provide exome-wide data regarding coding SNVs and insertion/deletions (INDELs; insertions/deletions of 1 to 10,000 bases).

We chose whole exome, rather than whole genome, capture and sequencing, to search for the disease allele underlying familial AIMAH because: (1) positional cloning studies which have focussed on protein-coding regions have successfully identified variants underlying monogenic diseases; (2) the majority of allelic variants known to cause Mendelian disorders disrupt protein-coding sequences; and (3) a large proportion of rare non-synonymous variants are predicted to be deleterious, in contrast to noncoding sequences, where variants are more likely to have weak or no effects on the phenotype, even in well-conserved sequences (Stenson *et al.,* 2009; Kryukov *et al.,* 2007; Chen *et al.,* 2007). Thus, we hypothesized that study of the SNVs shared in both exomes, would place us well to identify the monogenic basis of familial AIMAH in AIMAH-01. Furthermore, WEC/NGS is able to detect coding INDELs. An INDEL may cause disease by altering protein function: if the INDEL length is a multiple of three and coincides with codon boundaries, then it inserts or deletes an amino acid(s); if it does not occur in a multiple of three or does not coincide with codon boundaries, then it alters the reading frame ("frame-shift"), frequently resulting in a premature "STOP" codon: the aberrant mRNA may be subject to nonsense-mediated decay which prevents expression of truncated or erroneous proteins; alternatively, a non-functional protein may be expressed (Mullaney *et al.,* 2010).

We selected this technology, because it would provide the most comprehensive analysis of potentially causative (coding region; exome) genetic variation, which was available at the time that we designed the study, and hence offered the best prospect of leading us to gene discovery. We hypothesised that in addition to prioritising the WEC/NGS data (variants) based on predicted pathogenicity and other assumptions as described in Chapter 1, we would also be able to prioritise the variants based on whether the genes were in the AIMAH-01 locus of possible linkage, as well as diminish the priority of gene variants that were in loci with LOD scores < -2. Ideally, we would have studied the most distantly related affected individuals from AIMAH-01, in order to minimise the number of shared alleles, and thus reduce the number of potentially pathogenic variants for consideration. However, in AIMAH-01, the only definitely affected individuals were (closely related) siblings. It was imperative that there be no uncertainty regarding the phenotypic assignment, and thus we selected these siblings for this study.

6.2 Research Methods

These studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee.

Exon capture, next-generation sequencing and validation

Targeted exon capture of 712 cancer genes was performed using pooled germline DNA from five individuals – III-2 (AIMAH-01; Figure 2.1) and four familial acute myeloid leukaemia (AML) patients from whom DNA was available (the latter supplied by Professor Hamish Scott, Department of Molecular Pathology, SA Pathology). The exon capture was performed using a Roche NimbleGen custom array (Roche NimbleGen Inc, Madison, Wisconsin, United States) which had been designed to capture the coding regions of the 712 genes (Appendix 7). These genes were selected by Dr Christopher Hahn (Department of Molecular Pathology) and Professor Hamish Scott from the following sources:

1. COSMIC v39 release (Catalogue of Somatic Mutations in Cancer; http://www.sanger.ac.uk/genetics/CGP/cosmic/)
2. AML genome (Ley *et al.,* 2008)
3. Lung cancer genes (Campbell *et al.,* 2008)
4. Breast cancer genes (Leary *et al.,* 2008; Sjoblom *et al.,* 2006)
5. Colon cancer genes (Leary *et al.,* 2008; Sjoblom *et al.,* 2006)
6. Glioblastoma (Cancer Genome Atlas Research Network, 2008)
7. Kinome (Greenman *et al.,* 2007)
8. Pancreatic cancer (Jones *et al.,* 2008)

The sequencing was performed using the Illumina Genome Analyser II. The sequencing and preliminary sequencing analyses (including mapping and variant calling) were performed by AGRF. Subsequent analyses were performed by Mr Joe Carolan.

The sequence reads, of length 55 nucleotides, from the capture were aligned to the target regions using two genome alignment programs – Short Oligonucleotide Analysis Package 2 (SOAP2) and Bowtie. The percentage of reads mapping to the target region was 19.24% (SOAP2) and 24.53% (Bowtie). The Bowtie alignments consistently showed a higher percentage of reads mapping to the target region across these and other samples, so these alignments were used for subsequent analyses.

SNV detection programs (Varscan and Sequence Alignment Map [SAM] tools) were used to identify variations in the Bowtie alignment. SNVs that were identified were then matched with all previously known single nucleotide polymorphisms (SNPs) in the SNP database, dbSNP130 (National Centre of Biotechnology Information – NCBI; http://www.ncbi.nlm.nih.gov/snp/), in order to identify novel SNVs for further analysis. The novel SNVs were annotated with:

(1) SeattleSeq Annotation (http://gvs.gs.washington.edu/SeattleSeqAnnotation/), an online tool providing annotations for the functional effect of SNVs; and

(2) PolyPhen (http://genetics.bwh.harvard.edu/pph/), an online tool which predicts the functional effect of amino acid substitutions (Ramensky *et al.,* 2002; Sunyaev *et al.,* 2000; Sunyaev *et al.,* 2001).

To derive a list of sequence variants, the non-synonymous SNVs were selected if they fulfilled any of the following:

(1) SNV leading to a nonsense variant;

(2) SNV at exon-intron boundary (splice site); or

(3) predicted by PolyPhen as "probably damaging".

Having fulfilled any of these, the variants also had to fulfil all of the following:

(1) have an allele frequency of 2-35%;

(2) occur at a depth of sequencing of at least seven;

(3) be detected by both SAMtools and Varscan; and

(4) have more than one sequencing start site.

Validation of all possible pathogenic SNVs was attempted using Sanger sequencing. The methods are presented in Appendix 1. Since the DNA was pooled from five individuals (III-2, AIMAH-01 and four familial AML patients), DNA from each individual was directly sequenced.

Whole exome capture (WEC) and next-generation sequencing (NGS)

WEC and NGS of germline DNA from III-2 and III-3, AIMAH-01 (Figure 2.1) was performed by Beijing Genomics Institute (BGI; Beijing, China). The capture was performed using the SureSelect Target Enrichment System (Agilent Technologies) (Figure 6.1).

NOTE:
This figure is included on page 269 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6.1. The SureSelect Target Enrichment System Capture Process.

The SureSelect Target Enrichment system uses a liquid phase capture process to amplify the exome. Briefly, germline DNA was randomly fragmented and adapters were ligated to both ends of the fragment. The adapter-ligated DNA templates were purified by the Agencourt AMPure SPRI beads and fragments with insert size of 250 base pairs (bp) were excised. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified and hybridized to the SureSelect Biotinylated RNA Library (BAITS) for enrichment. Hybridised fragments were bound to the Streptavidin beads. The magnitude of enrichment of captured LM-PCR products was estimated using the Agilent 2100 Bioanalyser. Sequencing of each captured library was performed using Illumina HiSeq 2000. Raw image files were processed by

Illumina Pipeline v1.6 for base-calling with default parameters and the sequences were generated as 90bp paired-end reads.

The BGI pipeline of bioinformatics analyses is shown in Figure 6.2. Filtering removed low quality sequence (defined as reads that contain more than six N – no base call or forty continuous identical bases - and those polluted by linker or adapter sequences which were introduced when capturing and sequencing; Step (i), Figure 6.2). The clean reads were aligned to the reference Human Genome Assembly 18 (NCBI36/hg18; March 2006; University of California, Santa Cruz (UCSC) Genome Browser - http://genome.ucsc.edu/) using SOAPaligner. SOAPsnp (http://soap.genomics.org.cn/) was used to call genotypes in target regions (Li *et al.,* 2009). SOAPsnp data were filtered based on the following criteria: (1) base quality more than 20; (2) sequencing depth of 4-200; (3) copy number estimate ≤ 2; and (4) more than four bases between two SNVs. The SNP database, dbSNP129, was used to filter out all known variants. This database was used despite more recent versions, because it is based on hg18 which was the reference genome in this study and the data are well-validated. The insertion/deletions (INDELs) were called by the BGI automated pipeline, based on Burrows-Wheeler Aligner. INDEL data not fulfilling the following criteria were excluded from further analyses: (1) root mean square mapping quality more than 20; (2) a minimum of three reads.

NOTE:
This figure is included on page 271 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6.2. Pipeline of bioinformatics analyses. (Beijing Genomics Institute; BGI). (i) The raw Solexa reads were filtered for low quality sequence (described in text). (ii) The "clean reads" were aligned to the reference genome (hg18). (iii) Unique mapped reads – defined as those that have only one location in the reference genome and those which might derive from low complex exome regions and have multiple hits but are randomly aligned onto the exome region. (iv) The data were filtered for prespecified (by BGI) criteria pertaining to integrity of the read (described in text). Advanced analysis – refers to filtering against known sequence variation in the genome (dbSNP129). Subsequent analyses are described in the text.

Bioinformatics analyses of whole exome capture data

The SNVs were annotated using PolyPhen to predict pathogenicity. This was performed by Mr Joe Carolan, Department of Molecular Pathology. Potentially pathogenic SNVs were those that were either: "nonsense", "splice site" or predicted by PolyPhen to be "probably" or "possibly" damaging. The INDEL data were analysed in collaboration with Mr Joe Carolan, using SIFT (http://sift.jcvi.org/) – an online tool which predicts the effect of coding non-synonymous variants on protein function (Kumar *et al.,* 2009). We initially sought to study further the SNVs and INDELs that were shared between the exomes of the two siblings (III-2, III-3; AIMAH-01; Figure 2.1).

We further annotated the shared SNVs and INDELs based on gene information from public databases: known disease associations (OMIM; http://www.ncbi.nlm.nih.gov/omim), tissue

expression (UniGene; http://www.ncbi.nlm.nih.gov/unigene) and pathway involvement (Ingenuity® Pathways Analysis - IPA; http://www.ingenuity.com/) in order to prioritise the candidates for confirmation by direct sequencing. We also interrogated dbSNP131 (http://www.ncbi.nlm.nih.gov/projects/SNP/) – the most recent SNP database available at the time of these analyses - to determine whether any of the novel SNVs had subsequently been reported in the normal population.

Whole exome capture SNV validation

Confirmation of selected potentially pathogenic SNVs and INDELs was attempted using germline DNA from III-2 and III-3 (AIMAH-01) by Sanger sequencing. We sequenced the germline DNA of other affected (AIMAH-01, AIMAH-02, AIMAH-03) and unaffected (AIMAH-01) individuals for the variants. Higher priority was given to SNVs or INDELs if the genes were: (1) in a region of linkage or tumour loss of heterozygosity; or (2) involved in pathways over-represented in the AIMAH-01 expression data (Chapter 4); or (3) expressed in the adrenal gland; and (4) not in dbSNP131 (SNVs only). Although disease associations were noted, this was not an absolute exclusion criterion – since different variants within a gene (and we were considering only novel variants) may have different disease associations. One such example is the lamin A/C gene (*LMNA*) (MIM ID *150330). Of 41 known, mainly missense mutations, 23 cause Emery-Dreifuss muscular dystrophy, three limb-girdle muscular dystrophy, eight dilated cardiomyopathy and seven familial partial lipodystrophy (Genschel and Schmidt, 2000).

We performed a normal population screen for one confirmed SNV using 530 stored normal human DNA samples (supplied by Professor Hamish Scott). The population screen was performed using high-resolution melt (HRM), a post-PCR method that allows rapid sequence variant analysis. The method is described in Appendix 1. The principle is based on melting curve analysis of PCR-generated amplicons during heating and rapid cooling. Melting curve

profiles vary according to amplicon nucleotide composition – thus allowing SNV detection. We used DNA from III-2 and III-3 (AIMAH-01), as “positive controls” and compared the melting curves with that of the normal population.

The criteria we specified for a SNV to be considered potentially causative in AIMAH were: (1) it segregated with the assigned phenotype in AIMAH-01; and (2) it was not found in the normal population (AIMAH is rare, thus so must be the causative mutation).

6.3 Results and Discussion

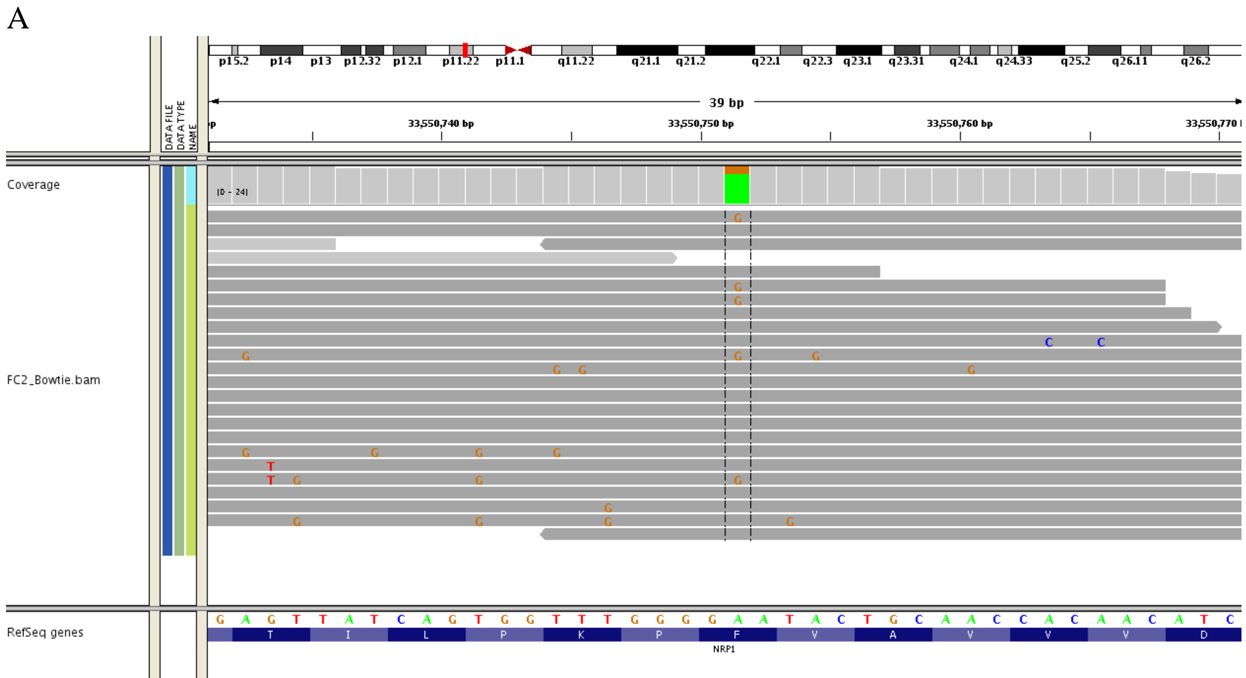
Targeted exon capture and sequencing

Eighty per cent of the target region was mapped to with a minimum depth of 1 and an average depth of 56; on average there was a 45-fold coverage of the total target region. As stated earlier, non-synonymous SNVs were selected if they fulfilled the following criteria: (1) SNV leading to a nonsense variant; or (2) SNV at exon-intron boundary (splice site); or (3) predicted by PolyPhen as “probably damaging”; and (4) had an allele frequency of 2-35%; and (5) occurred at a depth of sequencing of at least seven; and (6) were detected by both SAMtools and Varscan; and (7) had more than one sequencing start site. There were 11 genes that had a non-synonymous SNV that fulfilled these criteria. Examples of the alignment data are shown in figure 6.3. The genes, genomic location (based on hg18) and base and amino acid changes are listed in Table 6.1.

Targeted exon capture variant validation

Direct sequencing across the purported region harbouring the SNV was performed for the AIMAH-01 (III-2) and four familial AML samples. None of the 11 putative SNVs were confirmed. Examples of the direct sequencing result of the genes *NRP1* and *PKHD1* in germline DNA from one patient are shown in figures 6.4 and 6.5.

A



B

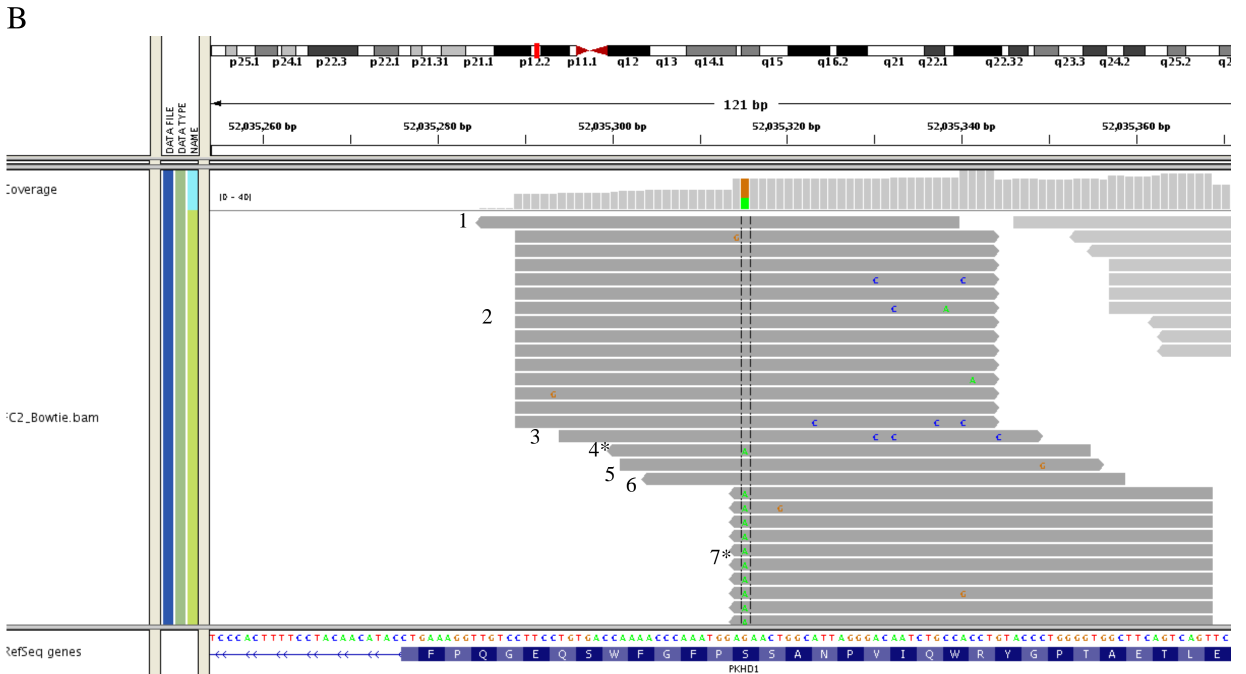


Figure 6.3. Exon capture alignment data for *NRP1* and *PKHD1*. These were two candidates arising from the Roche NimbleGen exon capture and Illumina sequencing. Each grey bar represents a 55 nucleotide "read". Panel A shows the sequence read alignments for *NRP1*. The reference allele is "A", the putative variant, "G". Although there are other variants, these did not fulfill other criteria for selection, e.g., only found in reads with the same sequencing start site. Panel B shows the alignments for *PKHD1*. The reference allele is "G", the putative variant, "A". For *PKHD1* there are seven different "read start sites" – but in only two of these is there a possible variant (indicated by *). These putative variants were not confirmed by direct sequencing (Figures 6.4 and 6.5).

Table 6.1. Summary of single nucleotide variants in a DNA* sample using targeted exon capture.

Gene name, Gene symbol	**Variant#**	**Consequence§**
Breast cancer 2, early onset, BRCA2	c.8021A>G	p.K2674R
Catalase, CAT	c.1384A>G	p.N462D
Endothelial PAS domain protein 1, EPAS1	c.896A>G	p.K299R
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian), ERBB2	c.2798A>G	p.D933G
Polycystic kidney and hepatic disease 1 (autosomal recessive), PKHD1	c.1079G>A	p.S360F
Versican, VCAN	c.2161C>T	p.F721L
Kinectin 1 (kinesin receptor), KTN1	c.1043C>G	p.A348G
mutS homolog 6 (E. coli), MSH6	c.2750A>G	p.D917G
RAN binding protein 17, RANBP17	c.2914A>G	p.M972V
Platelet-activating factor acetylhydrolase 1b, catalytic subunit 2 (30kDa), PAFAH1B2	c.470A>G	p.N157S
Neuropilin 1, NRP1	c.1184A>G	p.F395S

* the DNA was pooled from an AIMAH patient (III-2, AIMAH-01) and four familial acute myeloid leukaemia patients. The exon capture was performed using Roche NimbleGen capture, followed by Illumina sequencing.
#Variant is given in Human Genome Variation Society (HGVS) notation as follows: {nucleotide interval}{changed nucleotide>new nucleotide}
Nucleotide abbreviations are as follows:
A – adenine; C – cytosine; G – guanine; T - thymine

§Consequence given in HGVS notation as follows: (1) p.{code first amino acid changed}(amino acid interval}{code new amino acid}
Amino acid abbreviations are as follows:
A – alanine; D – aspartic acid; F – phenylalanine; G – glycine; K – lysine; L – leucine; N – asparagine; R – arginine; S – serine; V - valine

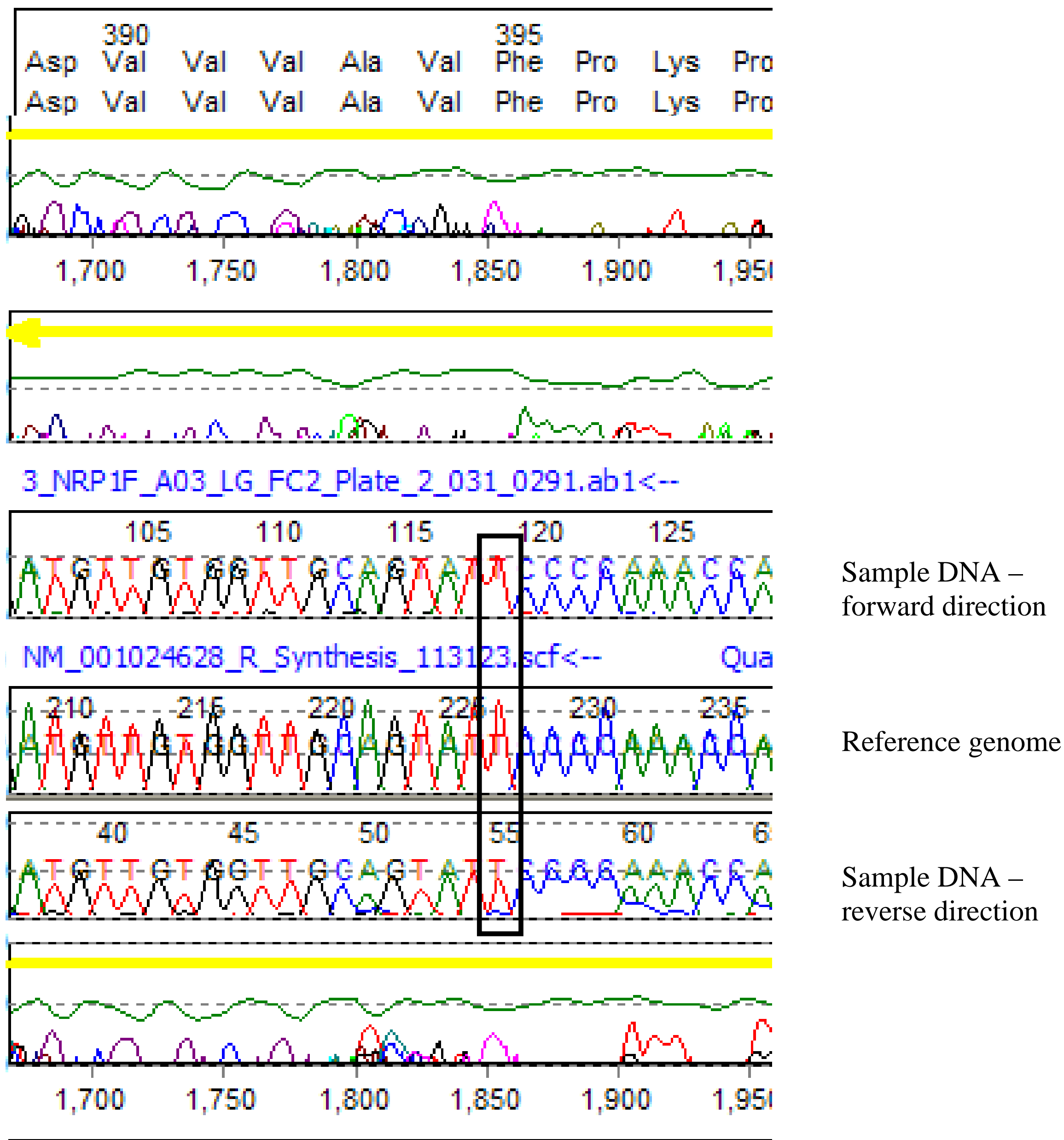


Figure 6.4. The putative SNV ("T to C") in neuropilin 1 (*NRP1*) in one patient.
The top and bottom sequences are the sequences obtained after sequencing in both the forward and reverse directions. The middle sequence is the reference sequence. The putative variant is indicated by the box (□). The "T" in the reference sequence is present in the patient DNA in both the forward and reverse directions. Thus the putative variant was not confirmed.
SNV = single nucleotide variation

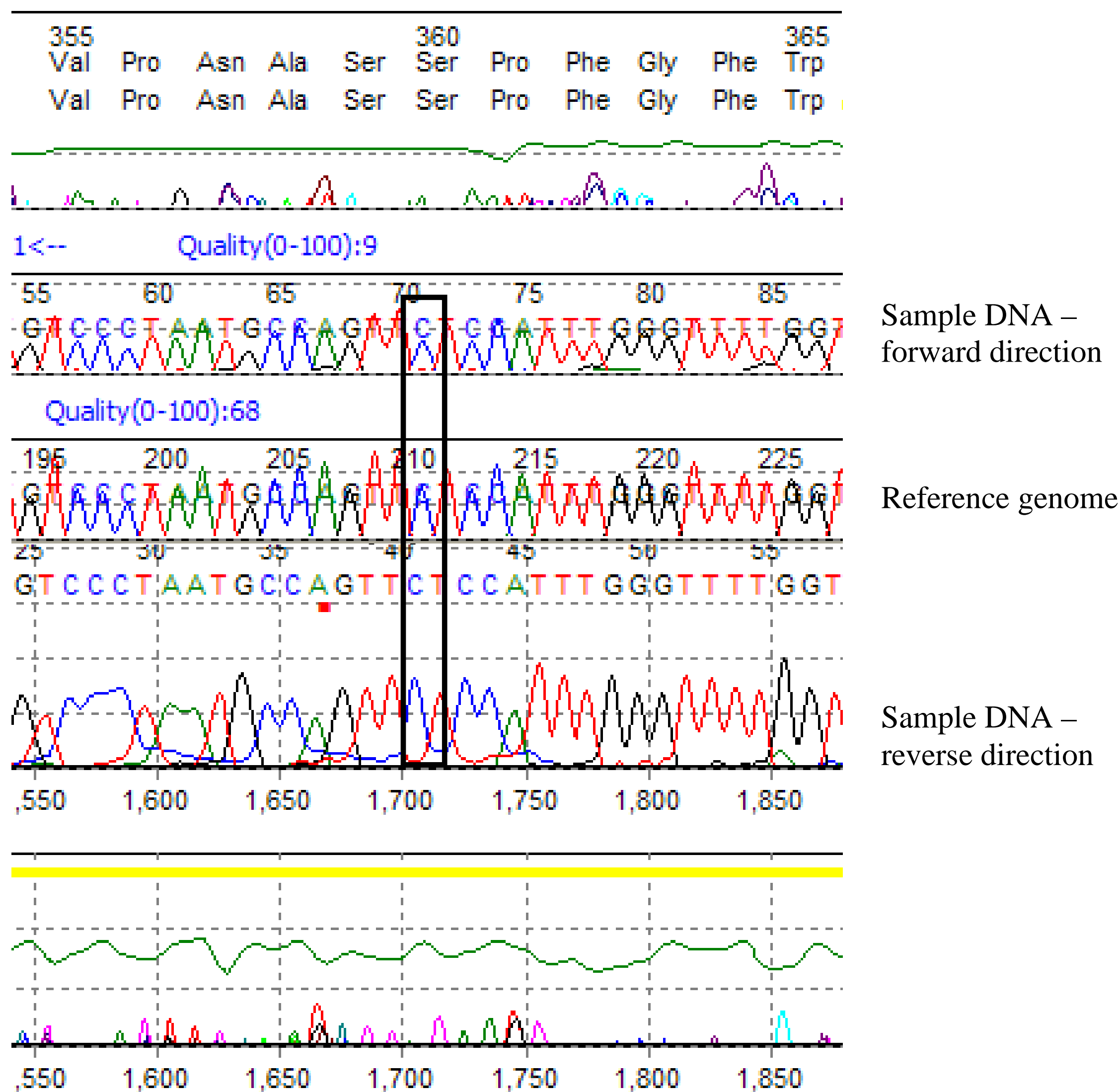


Figure 6.5. The putative SNV ("C to T") in Polycystic kidney and hepatic disease 1 (autosomal recessive) (*PKHD1*) in one patient.
The top and bottom sequences are the sequences obtained after sequencing in both the forward and reverse directions. The middle sequence is the reference sequence. The putative variant is indicated by the box (□). The "C" in the reference sequence is present in the patient DNA in both the forward and reverse directions. Thus the putative variant was not confirmed.
SNV = single nucleotide variation

We attribute the discrepancy between the NGS and direct sequencing data to: (1) PCR errors - errors in base incorporation during the two PCR steps involved in this process; and (2) sequencing errors. In addition, the complexity of the sheared library prepared for sequencing was less with these five samples than for other libraries performed in the Molecular Pathology Research laboratory (personal communication, Dr Christopher Hahn and Professor Hamish Scott). Low complexity is depicted in Figure 6.3, Panel B. Sequencing start sites 2 and 7 appear to contribute greatly to the depth of sequencing. However, each of these start sites appears to come from a single PCR product, since they have the same sequencing start and end sites. Also, because the sequence alignment was to the target region, and not the whole genome, we can not exclude the alignment having been of paralogous or pseudogene sequences to the target region.

Whole exome capture (WEC) and next-generation sequencing (NGS)

A summary of the quality of the sequencing data generated from our two WEC samples (III-2 and III-3, AIMAH-01) is presented in Table 6.2. More than 95% of each exome was captured. Over 85% of each exome was resequenced to a depth of at least four-fold; however resequencing to a depth of at least twenty-fold was achieved in only 40-45% of each exome. As will be discussed, this depth of coverage meant that we had to consider, as candidates, potentially pathogenic variants in either exome.

Forty-two exons within the possible region of linkage in AIMAH-01, 14q32.11-14q32.12, were not resequenced to a depth of at least four-fold in either exome (Table 6.3). Thus, we have not completely excluded the region of possible linkage as harbouring the disease causing gene, and we are currently planning to proceed with targeted exon capture to resequence these 42 exons. In particular, we have not fully sequenced *KCNK13* – due to the recent discovery of potassium channel mutations in familial adrenal hyperplasia and hyperaldosteronism, complete sequencing of this gene is a priority (Choi *et al.,* 2011).

Table 6.2. Summary of the quality of sequencing data generated from whole exome capture and next-generation sequencing.

Exon capture	III-2	III-3
Initial bases on target	37,806,033	37,806,033
Total effective reads*	16,030,644	16,258,134
Average read length (bp)	90.00	90.00
Effective sequence on target (Mb)	792.08	880.65
Average sequencing depth on target	20.95	23.29
Coverage of target region	**96.60%**	**96.70%**
Fraction of target covered with at least 20X	**42.30%**	**47.00%**
Fraction of target covered with at least 10X	**68.70%**	**71.70%**
Fraction of target covered with at least 4X	**87.20%**	**88.30%**

*For a read to be "effective" it needed to fulfill either of the following criteria: (1) the read only aligned to a unique region of the reference genome or (2) the read mapped to multiple regions on the genome (number of hits between 1 and 20) and were randomly aligned onto the target regions. These reads arise mostly from low complex genomic regions, e.g., repetitive sequences, and account for about 2% of total effective reads.

Table 6.3. Coding exons within the AIMAH-01 possible region linkage which were not resequenced to an average four-fold depth by whole exome capture.

Table 5.2 lists the full gene name. The exon and average depth of resequencing is shown.

Gene symbol	Exon number	Average Depth
KCNK13	1	0.4
PSMC1	1	1
TTC7B	1	1.6
TTC7B	20	1.4
RPS6KA5	17	1.4
CCDC88C	1	0.3
CCDC88C	2	0.1
CCDC88C	3	0
CCDC88C	4	0.1
CCDC88C	5	0.2
CCDC88C	6	0
CCDC88C	7	0
CCDC88C	8	0.1
CCDC88C	9	0
CCDC88C	10	0.3
CCDC88C	11	0
CCDC88C	12	0.2
CCDC88C	13	0.2
CCDC88C	14	0
CCDC88C	15	0.1
CCDC88C	16	0.3
CCDC88C	17-21	0
CCDC88C	22	0.2
CCDC88C	23	0.1
CCDC88C	24	0
CCDC88C	25	0
CCDC88C	26	0.2
CCDC88C	27	0.3
CCDC88C	28	0
CCDC88C	29	0.4
CCDC88C	30	0
SMEK1	1	0
SMEK1	11, 15	3.8
PP8961	1	0
SLC24A4	1	1.7
RIN3	1	0
RIN3	10	0.9

The SNVs and INDELs for the two WEC samples are summarised in Table 6.4. III-2 and III-3 (AIMAH-01; Figure 2.1) shared 103 SNVs that were nonsense, at splice sites or predicted by PolyPhen to be "probably" or "possibly" damaging (potentially pathogenic variants). None of the shared SNVs were in genes in the region of possible linkage (14q32.11-14q32.12) in AIMAH-01.

Table 6.4. Summary of SNVs and INDELs for whole exome capture samples.

	III-2	III-3
Single nucleotide variants		
Nō of genomic positions for calling SNVs	80,392,691	80,392,691
Nō of high-confidence genotypes	67,882,458	68,354,850
Nō of high-confidence genotypes in target region	35,573,253	35,651,928
Number of known SNP site in target region	155,132	155,132
Coverage of population SNPs in target region	154,595 (99.65%)	154,595 (99.65%)
3' untranslated region	1,074	1,177
5' untranslated region	831	864
Intergenic	375	380
Intron	18,438	19,444
Missense	5,911	6,088
Nonsense	40	41
Readthrough	10	10
Splice site	13	10
Synonymous-coding	6,957	7,009
Total number of SNVs	33,649	35,023
INDELs		
Number of genomic positions for calling INDEL	59,829	43,553
Number of high-confidence INDEL	4,741	4,638
5' untranslated region	48	56
3' untranslated region	147	164
CDS	169	171
Intron	3,241	3,251
Non-gene	1,122	980
Total number of INDELs	4,726	4,621

Abbreviations: CDS – coding sequence; INDEL – insertion/deletion; SNP – single nucleotide polymorphism; SNV – single nucleotide variation; 5' untranslated region – refers to 200 base pairs upstream of the initiation codon; 3' untranslated region – refers to 200 base pairs downstream of the termination codon

Of the shared SNVs, forty-seven have since been reported in dbSNP131, suggesting that these were more recently identified variants occurring in the normal population and were therefore unlikely to be pathogenic. III-2 and III-3 each had a further 145 and 161 SNVs, respectively, which in addition to the aforementioned functions, included one readthrough (STOP codon to an amino acid) in each individual. Due to the moderate depth (4-fold) of resequencing for the majority (> 85%) of the exome, we considered as candidates not only those potentially pathogenic variants in both exomes, but also those potentially pathogenic variants in either exome. Thus, there were 409 potentially pathogenic variants. None were found on 14q32.11-32.12, the region of possible linkage. The rationale for including variants that occurred in either exome was that the moderate depth of resequencing may have failed to detect the same variant in the other exome.

There were 219 coding INDELs (in-frame amino acid deletion - 87; in-frame amino acid insertion - 50; frameshift - 82) in at least one exome from III-2 or III-3; 105 of these INDELs were found in both exomes. Due to the moderate depth of resequencing, INDELs occurring in either exome are candidate causative variants. Thus, WEC has yielded a large number of novel, potentially pathogenic variants. The number of variants in each exome (≈ 200) is comparable to that found by others in single exomes (Ng, Buckingham *et al.,* 2010).

Whole exome capture validation

We selected and confirmed six (out of 103) shared SNVs by direct Sanger sequencing (Figures 6.6–6.11). As discussed below, none of these SNVs could be established to be the causative pathogenic variant in AIMAH-01. The variants, amino acid change, PolyPhen prediction and the rationale for selecting these variants for validation are summarised in Table 6.5. We also screened other family members from AIMAH-01 (affected and unaffected) and affected individuals from AIMAH-02 and AIMAH-03 for the variants. Segregation of the variant with the phenotype in AIMAH-01, i.e., the presence and absence of the variant in

affected and unaffected individuals, respectively, would provide evidence implicating that variant as causative in AIMAH-01. Detection of the variant in the other AIMAH families, could have implicated the same variant in the other families. The absence of the variant in other AIMAH families, however, would not exclude the gene as potentially being causative in the other AIMAH families because there could be a pathogenic variant elsewhere in the gene (allelic heterogeneity).

The *COL7A1* nonsense variant was one of the first to be confirmed (Figure 6.6A). It did not segregate with the phenotype although, it did segregate with the phenotype in generations III and IV, in whom, because of the late age-of-onset that is typical of AIMAH (discussed in Chapter 2), we could be more confident of the assigned phenotype. The variant was not present in the two affected individuals in generation V (Figure 6.6B) and nor was it present in IV-1 (Figure 6.6B), who although herself unaffected, is an obligate carrier of the disease allele (children affected). Of all the variants we have studied, this variant segregated best, although imperfectly, with the phenotype. It was not found in affected individuals from AIMAH-02 or -03.

Table 6.5. The basis for selection for validation of six SNVs detected by whole exome capture.

Gene symbol#	Variant§; Consequence¶; PolyPhen prediction	Rationale for selection
COL7A1	c.8299C>A p.E2767X N/A	Variant encoded a STOP codon
CCDC88A	c.1016A>G p.I339T possibly damaging	Interacts with GNAS proteins – includes Gsα subunit of G protein Gene is within the 2p16 locus – the second Carney complex locus identified by linkage analysis, although gene not identified
IQGAP1	c.240A>T p.K80N probably damaging	Late-onset gastric hyperplasia develops in a null murine model (MIM ID *603379) Sequence similarity to RAS-related GTPase activating protein family – which are involved in the control of cellular proliferation and differentiation Overexpression of IQGAP1 in colon carcinoma cells enhanced β-catenin transcriptional coactivation (Wnt signalling)
AKAP13	c.3413A>G p.D1138G probably damaging	Involved in cAMP and protein kinase A pathway signalling Directs the activity of protein kinase A by tethering the enzyme near its physiologic substrates (MIM ID *604686)
RASAL1	c.299-4T>C Splice site variant N/A	GTPase activating protein; Splice site variant; Highest expression in adult brain, thyroid and adrenal
MYH8	c.1209G>T p.C403X N/A	Variant encoded a STOP codon In a family proposed to have Carney complex variant, affected individuals had a missense mutation of MYH8 (MIM ID *160741)

Table legend is on page 285.

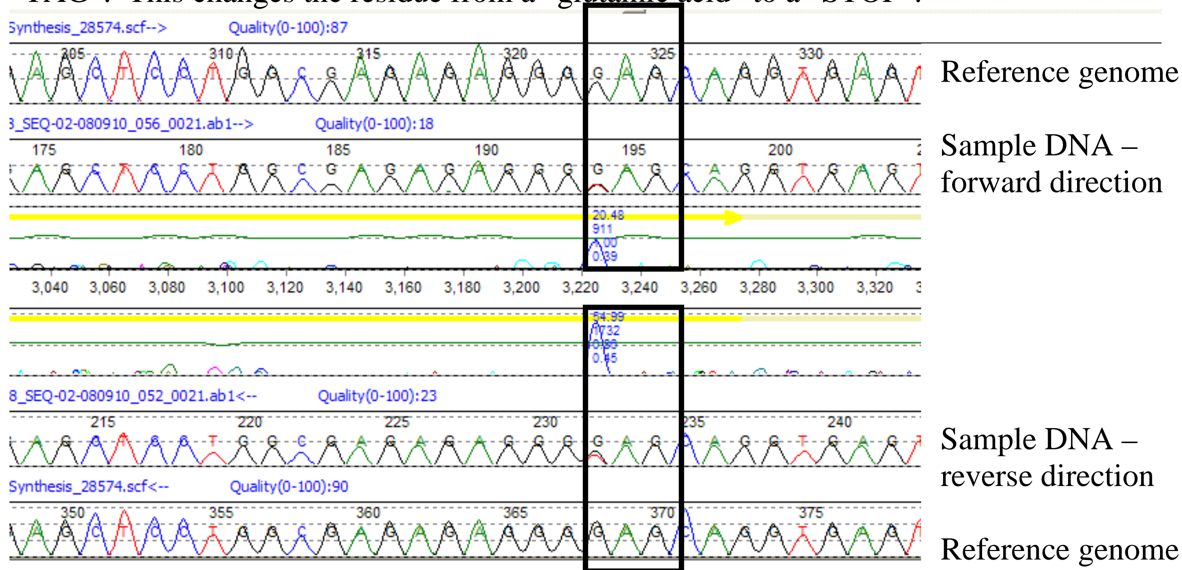
N/A – not applicable; PolyPhen can only predict the effect of amino acid substitutions; it can not predict the effect of nonsense or splice site variants.

#Gene symbol: Full gene names are as follows – AKAP13 – A kinase (PRKA) anchor protein 13; CCDC88A – coiled-coil domain containing 88A; COL7A1: collagen, type VII, alpha 1; IQGAP1 – IQ motif containing GTPase activating protein 1; MYH8 – myosin, heavy chain 8, skeletal muscle, perinatal; RASAL1 – RAS protein activator like 1 (GAP1 like)
§#Variant is given in Human Genome Variation Society (HGVS) notation as follows: {nucleotide interval}{changed nucleotide>new nucleotide}
Nucleotide abbreviations are as follows:
A – adenine; C – cytosine; G – guanine; T - thymine
¶Consequence given in HGVS notation as follows: (1) p.{code first amino acid changed}(amino acid interval}{code new amino acid}
Amino acid abbreviations are as follows:
C – cysteine; D – aspartic acid; E – glutamic acid; G – glycine; I – isoleucine; K – lysine; N – asparagine; T – threonine; X – STOP

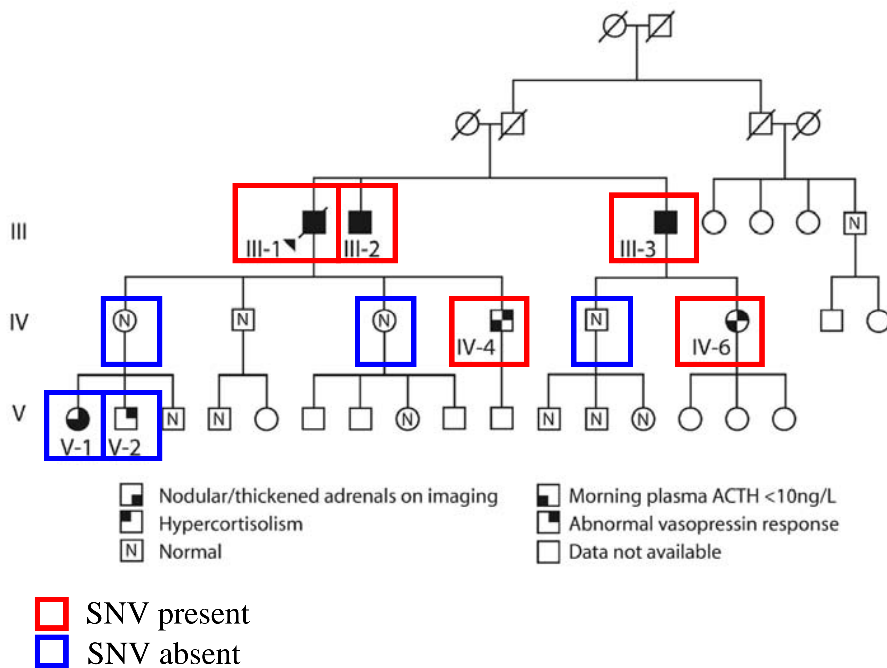
MIM ID = Online Mendelian Inheritance in Man (OMIM) reference (http://www.ncbi.nlm.nih.gov/omim)

Figure 6.6. Collagen, type VII, alpha 1 (*COL7A1*)
A. Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a nonsense variant – "T" instead of "G" changing the codon from "GAG" to "TAG". This changes the residue from a "glutamic acid" to a "STOP".



B. Distribution of the Collagen, type VII, alpha 1 (*COL7A1*) SNV in AIMAH-01.
The variant did not segregate with the assigned phenotype.

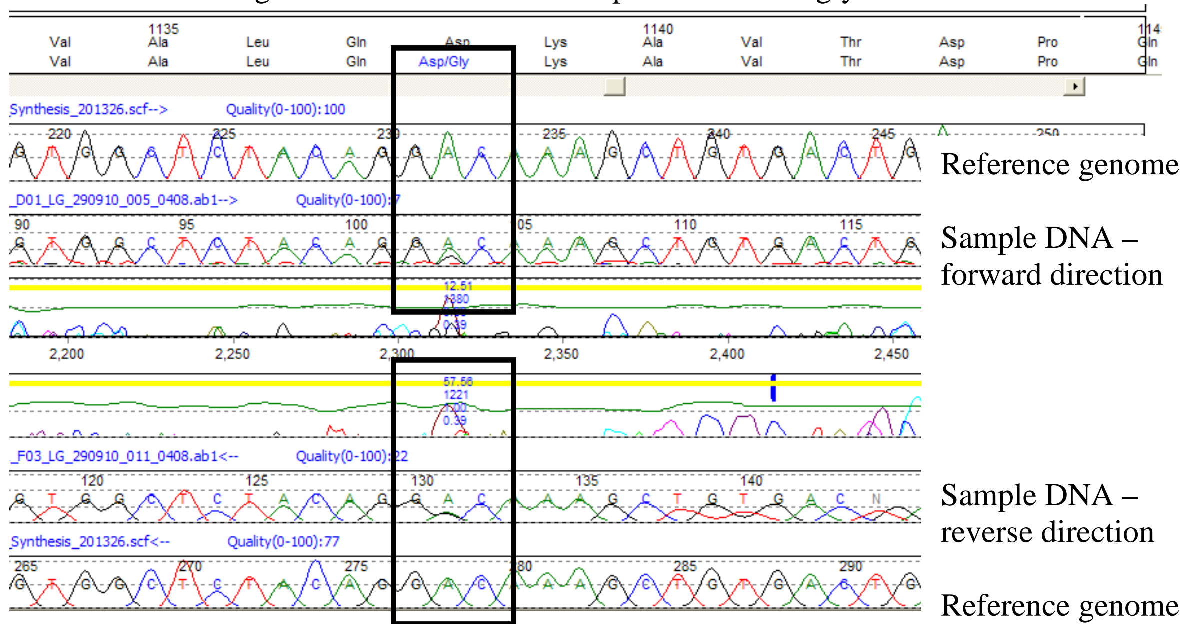


The SNVs we have validated in *AKAP13* (Figure 6.7A) and *RASAL1* (Figure 6.8) have since been reported in the SNP database, dbSNP131. The *AKAP13* SNP has a population frequency of 5% (population: pilot.1.CEU (72 chromosomes) - dbSNP131; reference SNP ID rs75504327). The *RASAL1* SNP (rs73207027) has a population frequency of 20% (population: pilot.1.CEU (72 chromosomes); dbSNP131). That these variants occur in the normal population is sufficient basis for us to exclude these variants as pathogenic in AIMAH-01. Furthermore, the *AKAP13* variant did not segregate with the phenotype (Figure 6.7B).

The SNVs we have validated in *CCDC88A* (Figure 6.9A) and *IQGAP1* (Figure 6.10A) did not segregate with the assigned phenotype in AIMAH-01 (Figures 6.9B and 6.10B, respectively) since several affected individuals did not have either variant. These variants were also not present in the affected individuals of AIMAH-02 or AIMAH-03. Nevertheless we are unable to definitely exclude either of these variants as potentially causative in AIMAH-01, since, for all but the three affected siblings, the assigned phenotypes may change in the future. For similar reasons, we can not definitely exclude the *COL7A1* variant, discussed earlier, either. Furthermore, the absence of these variants in the other AIMAH families does not exclude them as being pathogenic in AIMAH-01 (genetic heterogeneity). In addition, we have only evaluated *a specific SNV* for each gene in the other AIMAH families – thus we can not exclude another SNV elsewhere in the gene in the other families (allelic heterogeneity). This latter point is applicable to all the SNVs we have evaluated.

Figure 6.7. A kinase (PRKA) anchor protein 13 (*AKAP13*)
A. Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a missense variant – "G" instead of "A" changing the codon from "GAC" to "GGC". This changes the amino acid from "aspartic acid" to "glycine".



B. Distribution of the A kinase (PRKA) anchor protein 13 (*AKAP13*) SNV in AIMAH-01.
The variant did not segregate with the assigned phenotype. This variant is now reported as a SNP in dbSNP131.

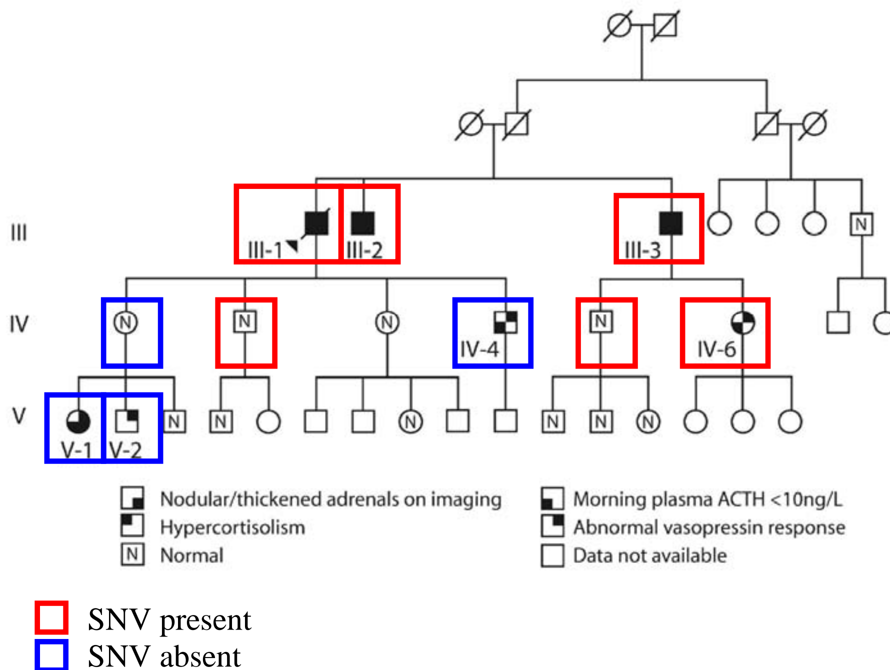


Figure 6.8. RAS protein activator like 1 (GAP1 like) (*RASAL1*)
Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a splice site variant – “T” to a “C”.

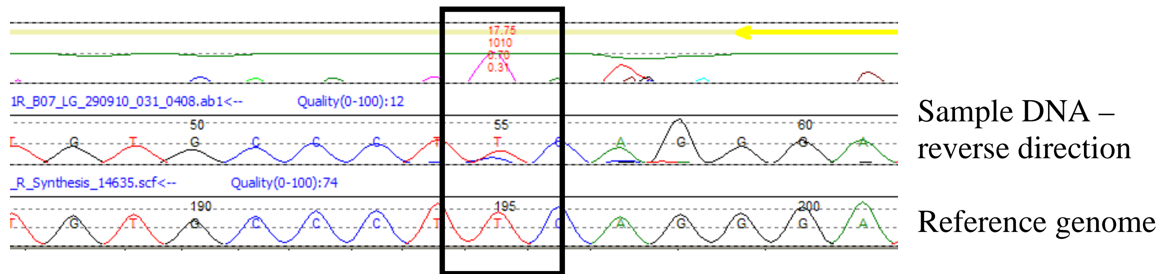
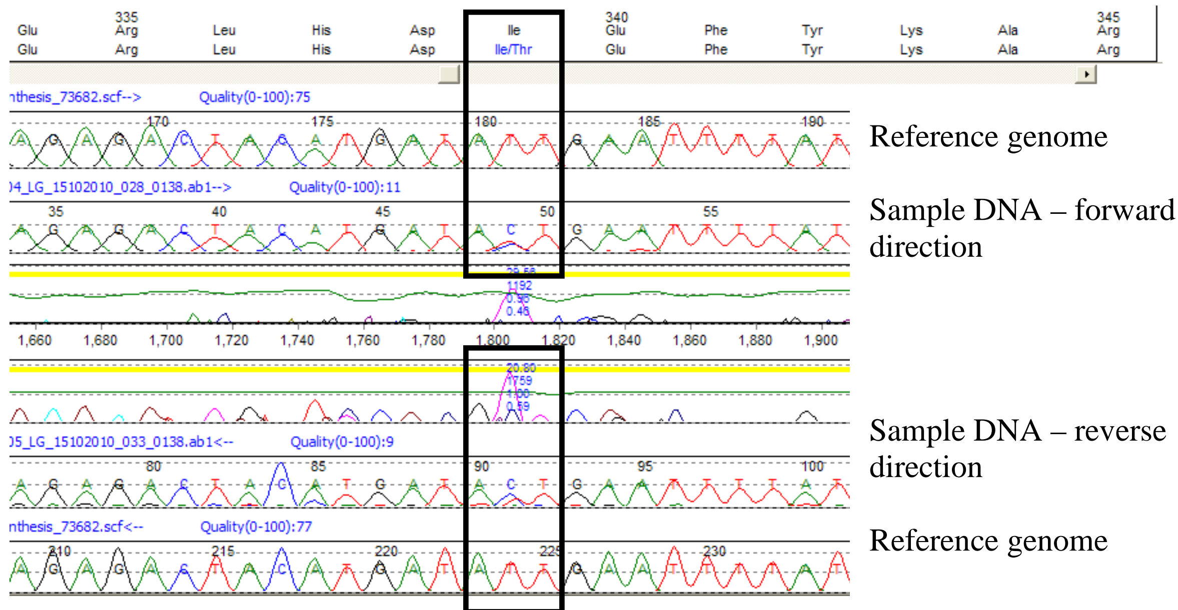


Figure 6.9. Coiled-coil domain containing 88A (*CCDC88A*)

A. Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a missense variant – "C" instead of "T" changing the codon from "ATT" to "ACT". This changes the amino acid from "isoleucine" to "threonine".



B. Distribution of the Coiled-coil domain containing 88A (*CCDC88A*) SNV in AIMAH-01.

The variant did not segregate with the assigned phenotype.

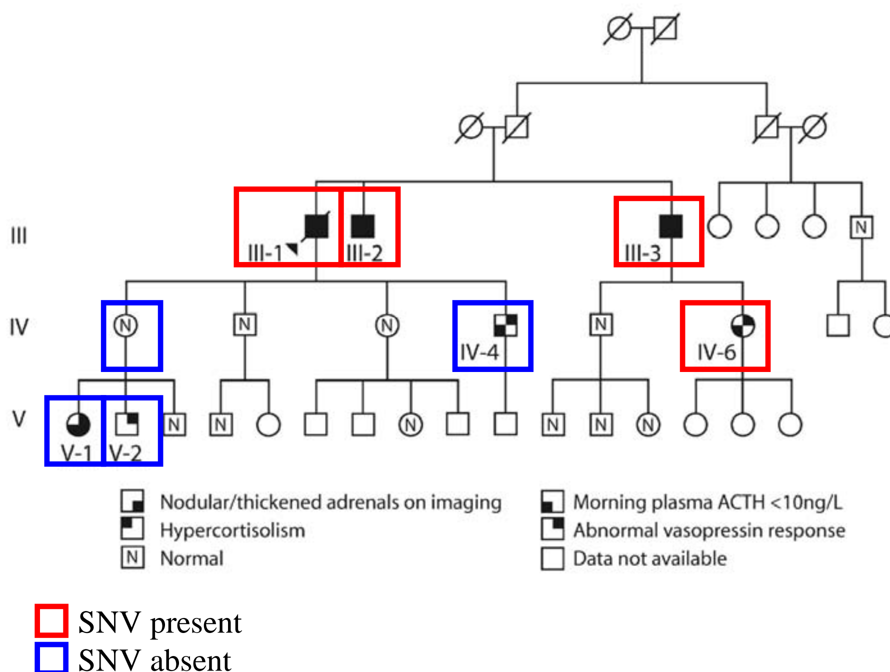
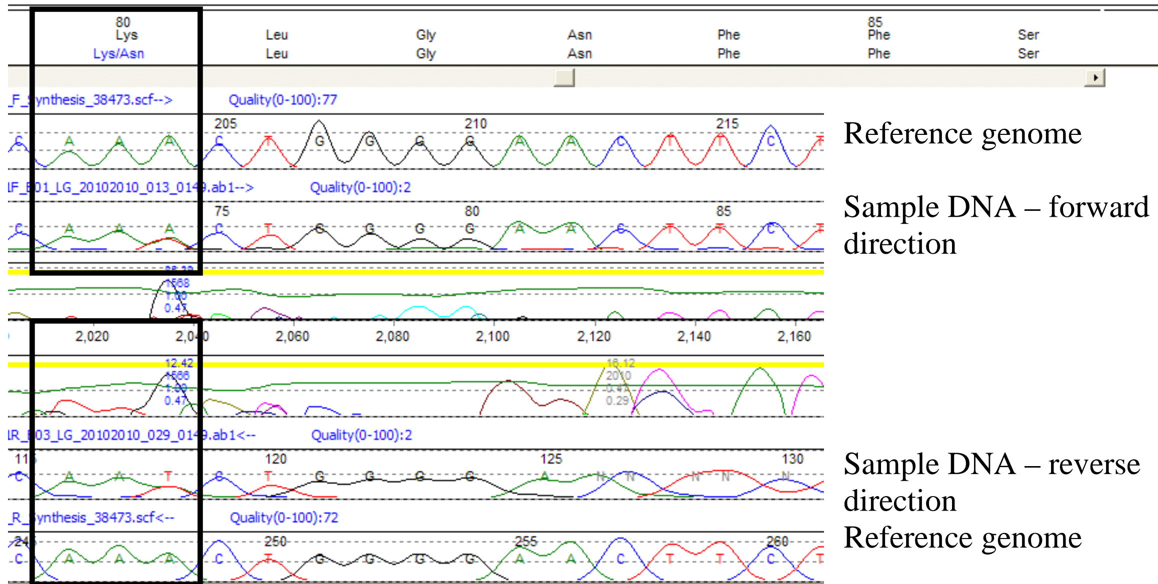
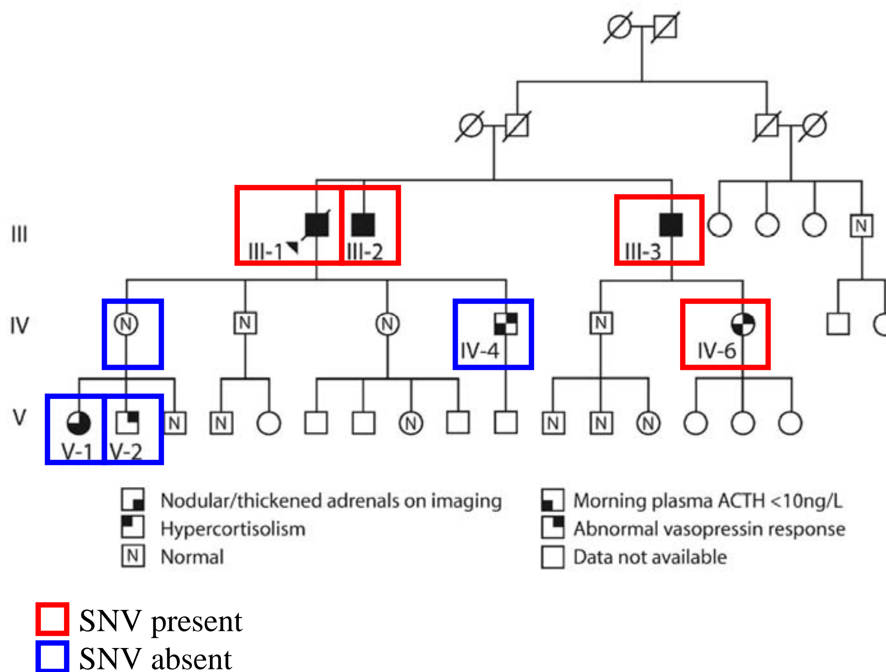


Figure 6.10. IQ motif containing GTPase activating protein 1 (*IQGAP1*)
A. Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a missense variant – "T" instead of "A" changing the codon from "AAA" to "AAT". This changes the amino acid from "lysine" to "asparagine".



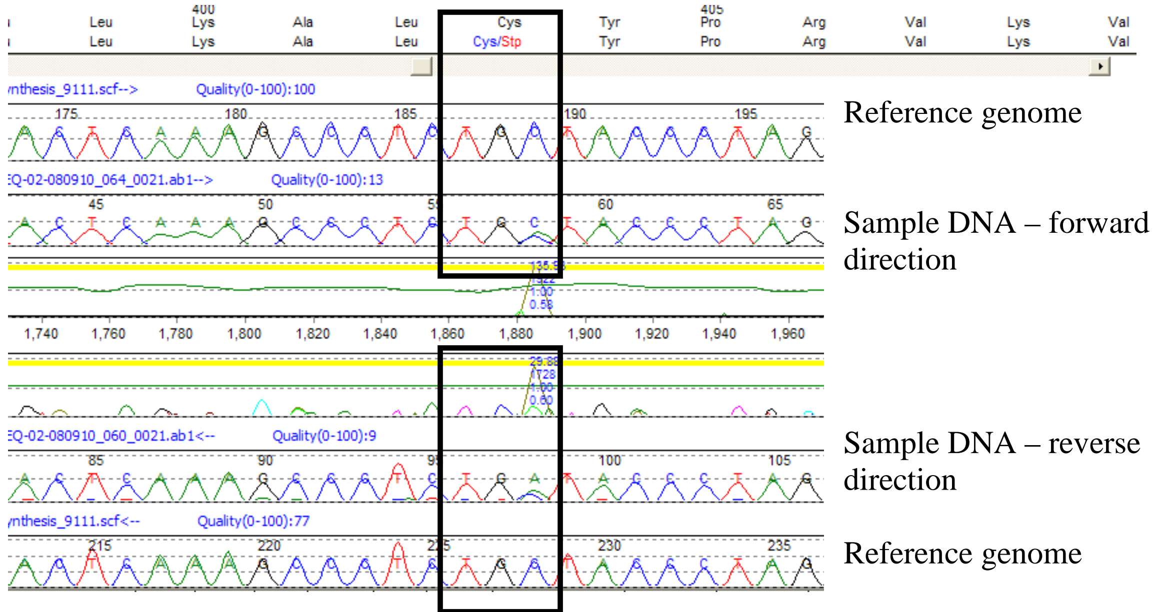
B. Distribution of the IQ motif containing GTPase activating protein 1 (*IQGAP1*) SNV in AIMAH-01.
The variant did not segregate with the assigned phenotype.



The *MYH8* nonsense variant was validated in germline DNA from III-2 and III-3 (AIMAH-01) (Figure 6.11A). The SNV in *MYH8* did not segregate with the assigned phenotype in AIMAH-01 (variant absent in some individuals with possible early manifestations; variant present in unaffected individuals) (Figure 6.11B) and was not present in the affected individuals of AIMAH-02 or AIMAH-03. Notwithstanding this, we pursued a normal population screen for this variant for the following reasons: (1) it was a nonsense variant which is generally considered to be highly pathogenic because it prematurely terminates protein translation; (2) the presence of the variant in unaffected individuals is consistent with incomplete penetrance, as suggested by our phenotyping data (Chapter 2); and (3) there are some, albeit controversial data, suggesting that mutations in *MYH8* may be associated with a variant of Carney complex, which, as discussed in Chapter 1, has amongst its manifestations, micronodular adrenocortical hyperplasia (MIM ID *160741; Veugelers *et al.,* 2004; Stratakis *et al.,* 2004). We found this SNV in 1 of 530 normals screened using HRM (Figure 6.12) and subsequently confirmed this using Sanger sequencing (Figure 6.13). Thus, we suspect this is a previously unreported rare variant in the normal population and therefore do not believe this nonsense variant is the causative genetic mutation in familial AIMAH.

Figure 6.11. Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*)
A. Whole exome capture SNV validation (in germline DNA from III-2 and III-3; AIMAH-01) using direct sequencing.

The variant is a nonsense variant – "A" instead of "C" changing the codon from "TGC" to "TGA". This changes the residue from a "cysteine" to a "STOP".



B. Distribution of the Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV in AIMAH-01.
The variant did not segregate with the assigned phenotype.

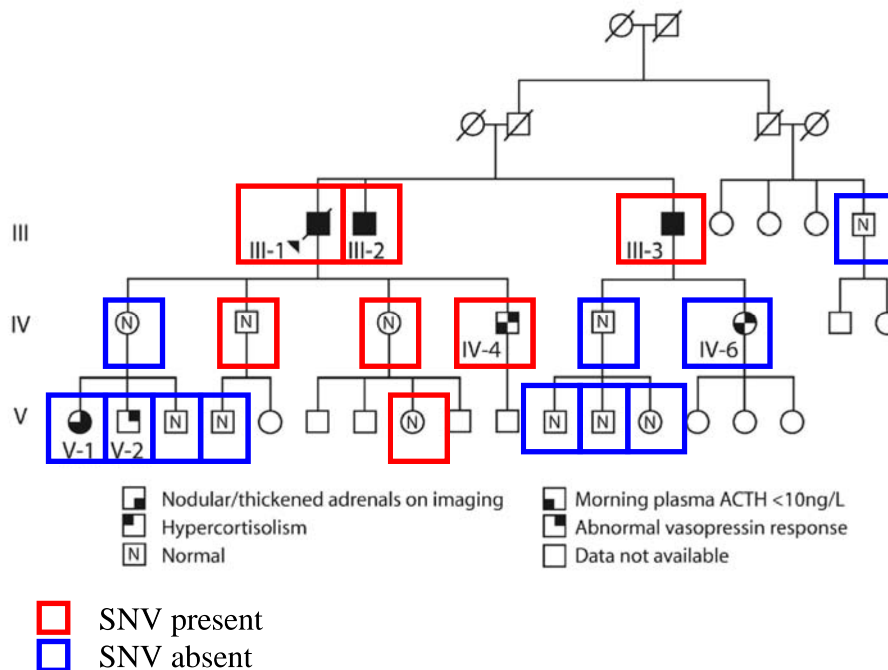


Figure 6.12. High resolution melt analysis of Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV.

The profile of positive controls is shown in red; the normal profile is shown in blue. In one of these analyses, one normal individual had the same profile as the positive controls.

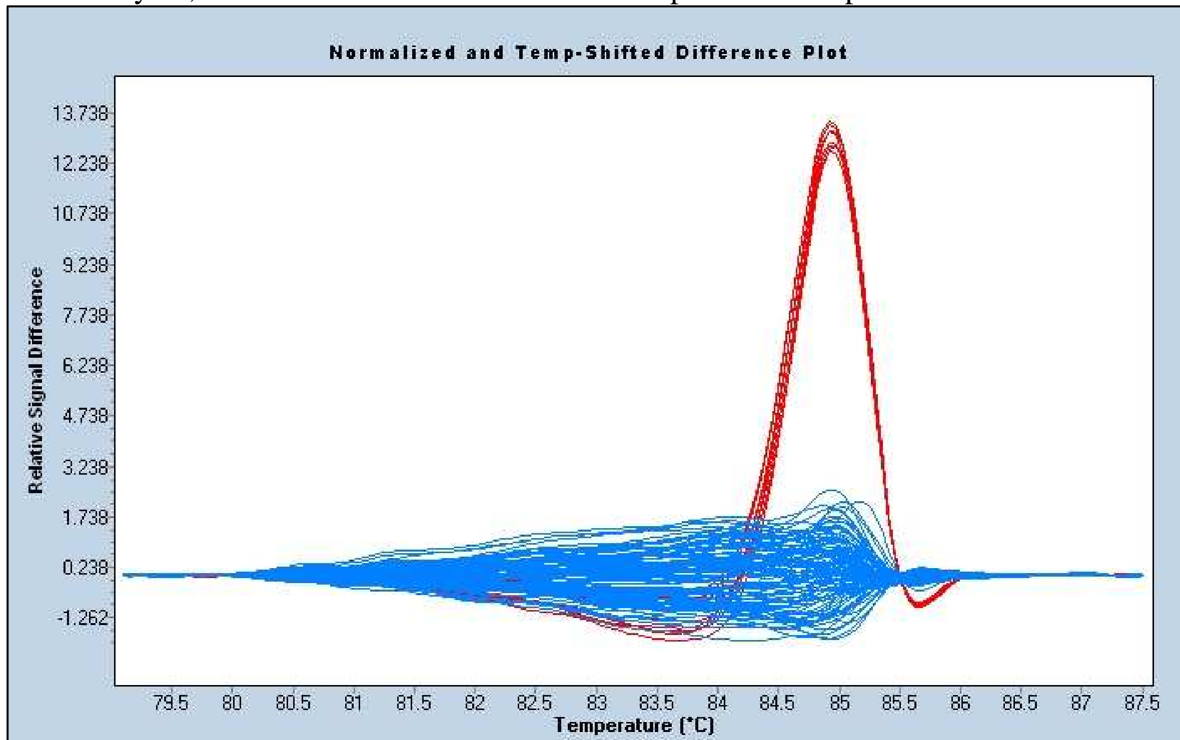
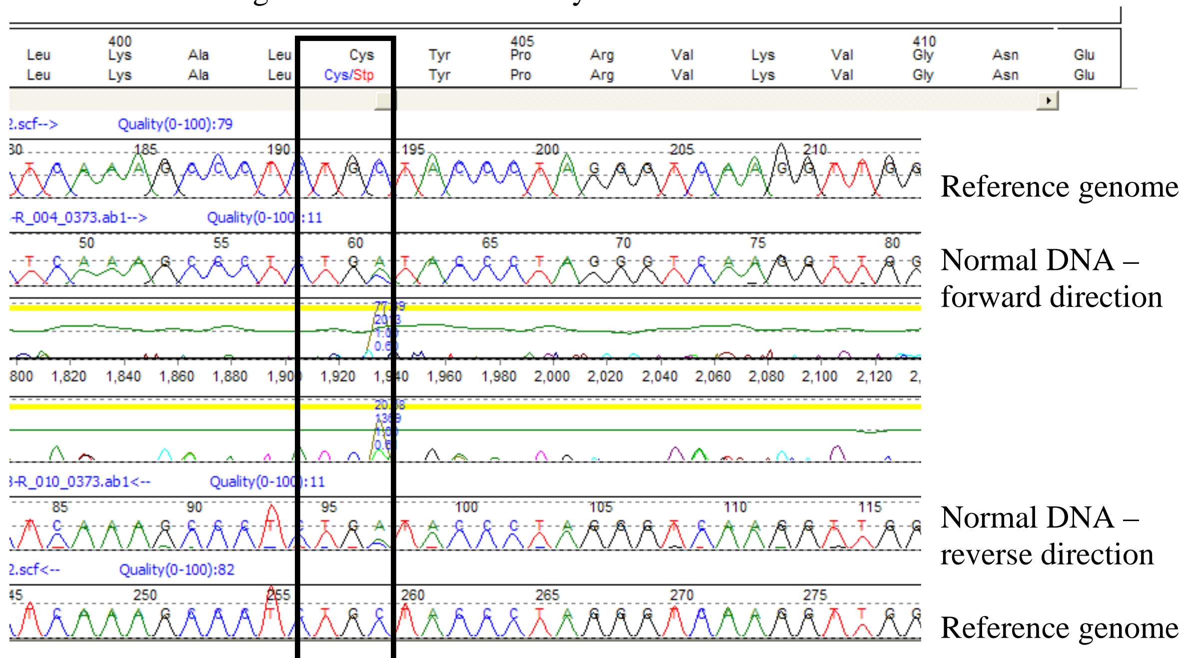


Figure 6.13. Myosin, heavy chain 8, skeletal muscle, perinatal (*MYH8*) SNV in a normal individual.

The variant is a nonsense variant – “A” instead of “C” changing the codon from “TGC” to “TGA”. This changes the residue from a “cysteine” to a “STOP”.



Nonsense variants have traditionally been considered to be associated with disease susceptibility because they introduce premature termination (STOP) codons into genes. Whilst this could result in a shorter protein, truncated proteins are frequently deleterious and may be eliminated by nonsense-mediated mRNA decay (NMD) (Maquat, 2004). Activation of the NMD pathway eliminates production of the protein, with complete loss of the gene product. However, if the premature termination codon is located either in the last exon or within 50 to 55 nucleotides upstream of the last exon-exon boundary, NMD can be evaded, resulting in the production of a truncated protein (Maquat, 2004; Mort *et al.,* 2008).

Although nonsense variants are frequent causes of genetic disease, in some cases they may prove advantageous. For example, a STOP allele in caspase-12 (*CASP12*) is associated with a reduced chance of developing severe sepsis and increased survival in modern-day hospitals (Saleh *et al.,* 2004; Xue *et al.,* 2006). Moreover, in recent times, whole exome and whole genome deep resequencing of DNA from normal individuals, have identified that nonsense variants are not infrequent, may occur in the homozygous state and, even in homozygosity, have no apparent adverse functional consequence (Yngvadottir *et al.,* 2009; MacArthur and Tyler-Smith, 2010).

In one study of 1151 healthy individuals (from 56 worldwide populations) who were SNP genotyped for 805 nonsense SNPs, 20% (167) of genes were found to contain a nonsense SNP (total 169 SNPS – two genes had two nonsense SNPs each) (Yngvadottir *et al.,* 2009). For 99 nonsense SNPs (59%), at least one homozygous sample was found (Yngvadottir *et al.,* 2009). Of the 169 nonsense SNPs, only eight were found in the Human Gene Mutation Database of mutations associated with human inherited disease; suggesting that very few of the nonsense SNPs were low-frequency disease-causing alleles (Yngvadottir *et al.,* 2009; Stenson *et al.,* 2003). On average, each person had 14 homozygous nonsense SNPs and 18 heterozygous nonsense SNPs in their genome (Yngvadottir *et al.,* 2009). The disease allele frequency

spectrum of nonsense SNPs suggested that over an evolutionary timescale, there was negative and positive selection for potentially deleterious and advantageous SNPs respectively (Yngvadottir *et al.,* 2009). Thus, the emerging data provided by NGS of normal human DNA suggest that, in contrast to previous concepts regarding the clinical significance of nonsense variants, many are not overtly associated with disease. The *MYH8* nonsense variant detected in our exome capture data and in one of 530 normal people screened may be one such variant.

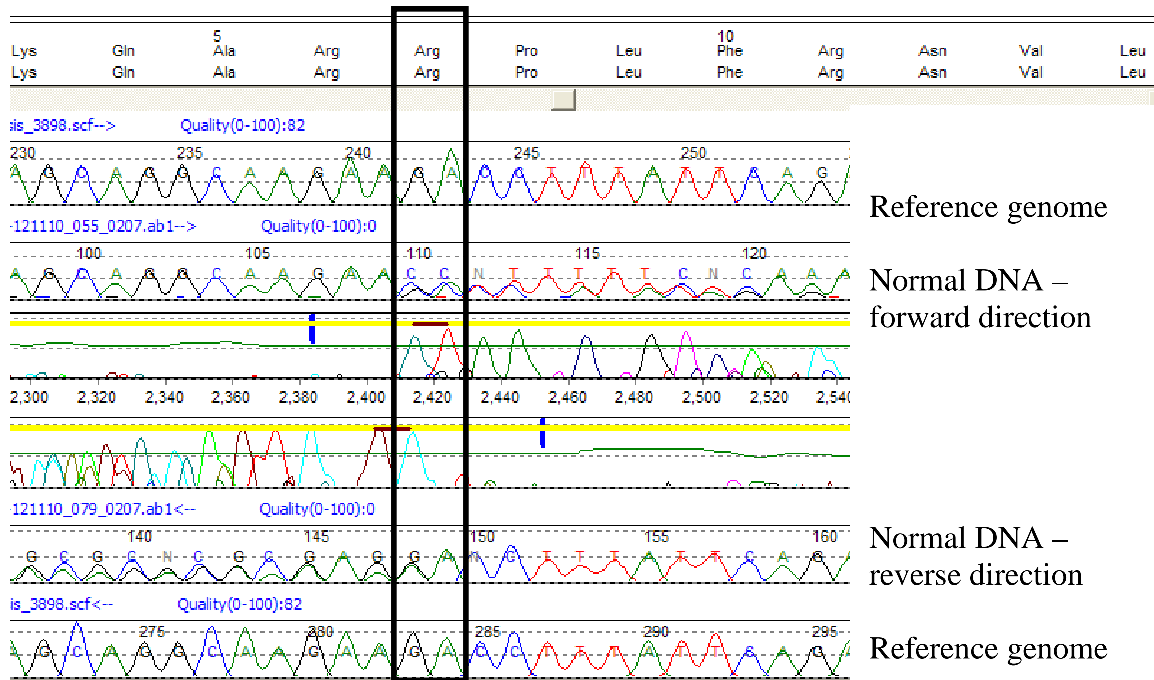
It could be debated however, that the presence of a variant in the normal population should not be used as an exclusion criterion. In patients with familial micronodular adrenocortical hyperplasia, Horvath *et al.,* found various inactivating mutations in the phosphodiesterase 11A (*PDE11A*) gene, isoform 4 (Horvath *et al.,* 2006a). Studies of the adrenal tumours demonstrated decreased PDE11A-4 protein, increased cAMP (phosphodiesterases hydrolyse cAMP) and increased phosphorylated cAMP-response-element binding protein (CREB) suggesting that these mutations had functional implications (Horvath *et al.,* 2006a). These mutations were detected, albeit at a lower frequency, in the normal population (0.1-4%; compared with 6-17.7% in patients with adrenal tumours) (Horvath *et al.,* 2006b). The data suggest that there are disease-causing alleles which occur in the normal population; the development of disease may be modified by other factors (e.g., other genes, hormones).

We annotated the INDELs using OMIM, Unigene and the AIMAH-01 expression data. At the time of writing this thesis, we have validated one shared INDEL (Figure 6.14A). We selected a variant in the *PDE11A* gene because, as discussed, *de novo* and inherited loss-of-function (frame-shift and nonsense) variants have been previously reported in some patients with micronodular adrenocortical hyperplasia (Horvath *et al.,* 2006a). Furthermore, germline *PDE11A* missense variants were found in patients with other adrenal tumours (18.8% of all cases studied *vs* 5.7% in healthy controls): AIMAH (24%), adenoma (19%) and carcinoma (16%) (Libé *et al.,* 2008). The INDEL in our data set was a 2bp deletion in exon 1

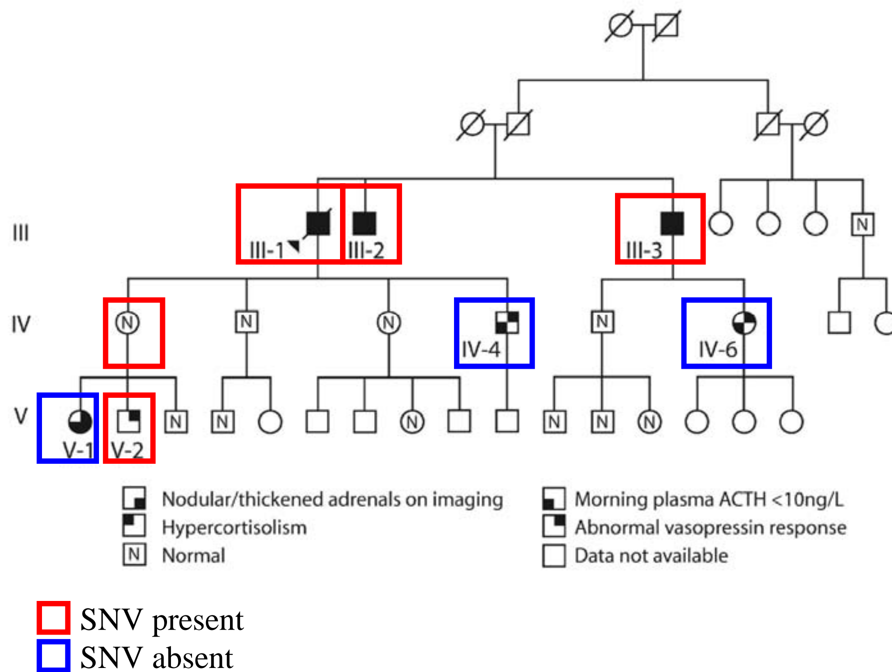
(c.19_20delTC), which resulted in a frame-shift and a truncated (35 amino acid) peptide (Figure 6.14A). This variant did not segregate with the phenotype (Figure 6.14B) and was not present in the affected individuals from AIMAH-02 and -03. Furthermore, exon 1 is a coding exon only for isoform 3 of *PDE11A*, which is not expressed in the adrenal. In addition, inactivating mutations of *PDE11A* would result in increased intracellular cAMP - our gene expression studies (Chapter 4) did not suggest activation of the cAMP pathway; so overall, we believe this candidate is unlikely to be the causative variant in familial AIMAH.

Thus far, we have been able to confirm several SNVs/INDELs which were detected by NGS and which we prioritised for validation (Table 6.5). We have not yet elucidated the genetic basis of familial AIMAH. One of the scientific challenges of NGS is to determine which of the many variant(s) detected is/are pathogenic. This is in no way a trivial task, since variable penetrance of a (pathogenic) variant and the presence and effect of modifier genes (and <u>their</u> penetrance) may confound the relationship between the (pathogenic) variant and the phenotype. It is unlikely that each of the many hundreds of variants in individual exomes is pathogenic or associated with a phenotype: this is likely to be confirmed as our knowledge of recent human genetic variation evolves with the sequencing of exomes and genomes of normal individuals (The 1000 Genomes Project Consortium, 2010). Conversely, the large number of variants detected by NGS may be diminished by applying various (logical) "data filters", described in Chapter 1. Notwithstanding the logical processes on which the data filtering is based, it is possible that the causative variant could be mistakenly eliminated from further consideration.

Figure 6.14A. Phosphodiesterase 11A (*PDE11A*) INDEL in AIMAH-01.
There is a deletion of "GA" on one strand.



B. Distribution of the Phosphodiesterase 11A (*PDE11A*) INDEL in AIMAH-01.



We attempted to reduce the number of variants for initial consideration by NGS of whole exomes of two siblings, intending to only study those variants that were shared. However, the moderate depth of resequencing has subsequently led us to believe that we should consider variants occurring in either exome as possible candidates – and thus we have many more candidate variants than we had anticipated. The power of our study to identify the genetic cause could have been increased by achieving a greater sequencing depth of coverage and by either studying: (1) additional affected related individuals; or (2) two more distantly related individuals. Studying two distant relatives, in combination with screening other related affected individuals for the variants and using linkage data to further prioritise variants, has been a successful strategy for gene discovery (Johnson, Mandrioli, Benatar, Abramzon *et al.,* 2010). Alternatively, the power of our study could have been increased by studying several unrelated affected individuals, in conjunction with the AIMAH-01 siblings; by studying all possible subsets and permutations of exomes, we might have been able to identify shared genes/variants. This strategy has led to novel gene discovery of other rare Mendelian disorders (Ng, Bigham *et al.,* 2010). Even for AIMAH, in which genetic heterogeneity is assumed, studying "sufficient" numbers of (in practical terms, as many as possible) exomes could adequately compensate for genetic heterogeneity. Another way to compare exomes from unrelated individuals might be to search for variants in, albeit different, genes relating to one cellular signalling pathway, whether that pathway is known to be involved in the disease process or not. The possibility of allelic heterogeneity could be accounted for by accepting as candidates, common genes but with different variants, between exomes.

6.4 Conclusion and Future studies

These studies directed at elucidating the genetic basis of familial AIMAH have not yet identified the causative genetic mutation. Our most recent attempt has involved WEC and NGS of two siblings with AIMAH. We found 409 potentially pathogenic SNVs in either exome that were either nonsense, at splice sites or were non-synonymous and predicted to be

damaging variants, and 219 coding INDELs. Although the genetic basis should be identical in these siblings, for reasons already discussed regarding the depth of sequencing achieved, we believe that potentially pathogenic variants in either exome should be considered as candidates. Thus, there is a large number of potentially pathogenic variants for us still to consider. Moreover, these individuals will share a large number of variants (pathogenic or not) because they are closely related genetically (siblings). Analysis of more distantly related individuals (who would therefore share fewer alleles) could reduce the number of variants; however, in AIMAH-01 there are no other individuals in subsequent generations who are definitely affected. Since we did not obtain adequate coverage of the possible locus in AIMAH-01, we have not completely excluded that it harbours the disease-causing gene; and thus our next step will be to perform targeted exon capture of the exons missed by WEC, since the linkage studies, although not without limitations, remain our strongest lead to date towards discovery of the genetic basis of familial AIMAH.

If we subsequently exclude the locus, then our next step could be continuing to validate all of the potentially pathogenic variants from our current exome data. This could be done sequentially, as described above, based on prioritization of the candidates, or could be done relatively cost- and time- efficiently using a multiplex assay (Gabriel *et al.,* 2009). However, we are likely to continue to encounter the problem of variants, even potentially the disease-causing variant, not segregating with the assigned phenotype, and perhaps even detecting it in the normal population. Incomplete penetrance and that the assigned phenotype is provisional, since many unaffected individuals may be too young to manifest the disease, serve as cautions to us, that we may not be able to rely on the disease-causing variant segregating perfectly with the phenotype. This may result in us inadvertently discarding the disease-causing variant. We are mindful of this issue for the variants we have already validated. Thus, we believe that the next best step, if the linkage region is excluded, will be to perform WEC on additional, unrelated individuals with AIMAH.

Several recent studies of WEC of multiple unrelated individuals have shown that this strategy may successfully reduce the number of candidate genes or variants to consider and have led to the discovery of novel genes underlying rare Mendelian disorders (Ng, Bigham *et al.,* 2010). The proof-of-concept was illustrated by exome capture of four unrelated individuals with Freeman-Sheldon syndrome, a rare autosomal dominant disorder known to be caused by mutations in the myosin heavy chain 3, skeletal muscle, embryonic (*MYH3*), gene (Ng *et al.,* 2009). The consecutive addition of unrelated exomes reduced the number of potentially pathogenic genes with rare coding variants (and neither in dbSNP nor in eight HapMap exomes and predicted to be damaging) as follows: 160 (one exome), 10 (two exomes), 2 (three exomes) and 1 (*MYH3*) (four exomes) (Ng *et al.,* 2009). There were three potentially pathogenic variants if any three of the four exomes were considered (Ng *et al.,* 2009).

The genetic basis of Kabuki syndrome (KS), a rare, multiple malformation disorder, has recently been discovered, after the WEC and NGS of ten unrelated, affected individuals (Ng, Bigham *et al.,* 2010). Several parallels can be drawn between KS and AIMAH. Both disorders have been considered to be predominantly sporadic, although familial forms have been described which suggest that they may be autosomal dominantly inherited (Ng, Bigham *et al.,* 2010; Findlay *et al.,* 1993; Minami *et al.,* 1996; Miyamura *et al.,* 2002; Nies *et al.,* 2002; Lee *et al.,* 2005; Vezzosi *et al.,* 2007; Gagliardi *et al.,* 2009). The relatively low number of cases, the lack of multiplex families and phenotypic variability have impeded the discovery of the genetic basis of KS, and, in our experience, AIMAH. In their study, Ng, Bigham *et al.,* ranked ten individuals with KS according to their physical characteristics and studied their exomes in all possible subsets of permutations containing at least two exomes (Ng, Bigham *et al.,* 2010). The four highest ranked individuals had distinct nonsense variants in one potentially pathogenic gene (myeloid/lymphoid or mixed-lineage leukemia 2 – *MLL2*) (Ng, Bigham *et al.,* 2010). Three others had either an INDEL or a nonsense variant in *MLL2* (Ng, Bigham *et al.,* 2010). Thus, WEC and NGS of ten unrelated, affected individuals

successfully led to the identification of novel genetic mutations in seven individuals with a rare Mendelian disease that demonstrates allelic heterogeneity. Sequencing multiple individuals attempted to compensate for the possibility of genetic heterogeneity – which is evidenced by the three individuals in whom the genetic basis of KS remains unknown. It is notable, however, that two exomes was sufficient to restrict the candidates to a single gene. Hence we believe that exome analysis of other individuals with familial AIMAH (AIMAH-02, AIMAH-03) may be required. Variant analysis of additional affected individuals may direct us towards discovery of the genetic mutation underlying AIMAH; sufficient numbers of exomes from unrelated individuals will be needed to compensate for the anticipated genetic heterogeneity.

After having performed multiple exome analyses, it remains possible that we will not discover the genetic basis of familial AIMAH. There are several reasons why this may be. Firstly, in most of the published exome studies, the "WEC" only captured about 75% of the 20,000 target genes (Kaiser, 2010). In addition, exome capture does not detect structural changes (gene deletions or duplications) and does not examine the non-coding DNA (Kaiser, 2010). Non-coding DNA contains regulatory regions – regions of DNA that influence gene expression. If the genetic basis of familial AIMAH is due to a mutation in a non-coding region of DNA or due to gene amplification or deletion, this will not be detected by WEC. Furthermore, genetic heterogeneity may not be sufficiently compensated for by multiple exomes. Finally, should familial AIMAH be caused by epigenetic (no sequence change in DNA – e.g., methylation), a chromosomal deletion or amplification, or environmental factors, these will not be found by exome sequencing. Overall, however, the international reports of familial AIMAH strongly suggest that AIMAH may be inherited, and the reports of affected parent-child dyads, as discussed in Chapter 2 are compelling evidence for the autosomal dominant nature of disease inheritance.

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

7.1 Introduction

The first reports of the regulation of cortisol production by aberrant, adrenocorticotropic hormone (ACTH)-independent mechanisms were in patients with adrenocortical adenomas or ACTH-independent macronodular adrenal hyperplasia (AIMAH) and gastric inhibitory polypeptide (GIP)-sensitive Cushing's syndrome (CS) (Lacroix *et al.,* 1992; Reznik *et al.,* 1992). Subsequently, aberrant sensitivity to other hormones (including vasopressin [VP], luteinizing hormone / human chorionic gonadotropin, catecholamines, angiotensin II and glucagon) has been identified in adrenocortical adenomas and AIMAH (Lacroix *et al.,* 2001).

The aberrant adrenocortical sensitivity is generally correlated with the expression of illegitimate receptors on tumour cells (Lacroix *et al.,* 2001). These receptors are illegitimate because they are: (1) either not normally expressed by adrenocortical cells (ectopic expression) or their expression on tumour cells is increased (eutopic overexpression); and (2) coupled to steroidogenesis, such that administration of a specific receptor ligand, or physiological modulation of its endogenous levels, results in an ACTH-independent increase in cortisol (Lacroix *et al.,* 2001). In AIMAH, the aberrant responses have been (arbitrarily) classified according to the percentage increase in cortisol from baseline as follows: < 25% - no response; 25-49% - partial response; ≥ 50% - positive response (Lacroix *et al.,* 1999b).

As discussed in Chapter 2, we had three families with VP-sensitive AIMAH, in which an aberrant, ACTH-independent increase in cortisol could be demonstrated. In this study, we

wanted to determine the cortisol response of secretory and non-secretory adrenocortical adenomas to VP, using the VP stimulation testing protocol described in Chapter 2.

As discussed in Chapter 1, in the healthy human VP acts independently and synergistically with corticotrophin-releasing hormone (CRH) to stimulate ACTH, and hence, cortisol secretion, in order to effect a stress response (Salata *et al.,* 1988). An ACTH-cortisol increase after exogenous VP (10 I.U. intramuscularly [IM]) therefore reflects the presence of an intact hypothalamic-pituitary-adrenal (HPA) axis (McDonald and Weise, 1956). However, 1 I.U./70kg body weight of intravenous (IV) VP (physiologic dose VP [PD-VP]) is sufficient to stimulate the intact HPA axis; this dose is at the top of the dose-response curve for ACTH, and represents a more physiological stimulus (Torpy *et al.,* 1994). The mean peak VP level achieved after PD-VP is 229-267pmol/L, well above the 46-60pmol/L threshold for ACTH release in humans and basal circulating VP levels (usually less than 3pmol/L) (Torpy *et al.,* 1994; Hensen *et al.,* 1988; Robinson and Verbalis, 2003). The threshold in humans is comparable to circulating VP levels in non-human primates; in whom VP concentrations in the hypophyseal-portal circulation are 300-fold higher than in the periphery (Zimmerman *et al.,* 1973).

Following injection of PD-VP, the *normal* response is a sequential increase in ACTH and cortisol, peaking at 15 and 30 minutes, respectively (Torpy *et al.,* 1994). We define an *aberrant* response as one where there is an increase in cortisol with either no change or a fall in plasma ACTH, indicative of adrenal hypersensitivity to VP. This was seen in our patients with VP-sensitive AIMAH, discussed in Chapter 2. Although in Chapter 2 we did not stipulate a minimum percentage increase in cortisol from baseline to define an aberrant response; for the purposes of our study in adrenal adenomas, we predefined a minimum, percentage increase in cortisol of 20% from baseline, in the absence of an ACTH increase, as indicative of an aberrant response. Hence a lack of ACTH and cortisol response, referred to

as an *absent* response, may signify pituitary corticotroph suppression from cortisol excess and, in the absence of overt hypercortisolism, may be a marker of subclinical CS (SCS).

We chose a 20% threshold increase in cortisol from baseline, in contrast to the minimum 25% increase that has been the threshold for determining a VP response in other studies. We chose a lower threshold because we were administering a substantially lower dose of VP (1 I.U./70kg IV), than has been conventionally used (10 I.U. IM), but we also needed a sufficient threshold that would not be confounded by the intra-assay variation in cortisol (Lacroix *et al.,* 1999b). Our rationale for choosing a lower threshold was that, as discussed below, the aberrant cortisol response to VP is dose-dependent; hence we hypothesised that in cases where we could demonstrate an aberrant response, we should expect a smaller cortisol increment from baseline because of the lower dose of VP administered.

An aberrant and dose-dependent cortisol increase following VP administration has been observed in functioning adrenal adenomas and AIMAH, suggesting heightened adrenocortical tumour sensitivity and responsiveness to VP (Perraudin *et al.,* 1995; Horiba *et al.,* 1995; Lacroix *et al.,* 1997b; Iida *et al.,* 1997; Daidoh *et al.,* 1998; Arnaldi *et al.,* 1998; Mune *et al.,* 2002; Tatsuno *et al.,* 2004; Reznik *et al.,* 2004; Lee *et al.,* 2005; Gagliardi *et al.,* 2009). Corresponding *in vitro* studies have established that VP directly stimulates adrenocortical production of cortisol, operating principally through eutopic overexpression of AVPR1A and ectopic expression of AVPR1B (Perraudin *et al.,* 1995; Lacroix *et al.,* 1997b; Arnaldi *et al.,* 1998; Mune *et al.,* 2002; Tatsuno *et al.,* 2004). Whilst AVPR1A is expressed on the normal adrenal and may be involved in the paracrine regulation of cortisol secretion, as discussed in Chapter 1; AVPR1B is expressed on pituitary corticotrophs and not by the normal adrenal (Perraudin *et al.,* 1993; Zingg, 1996).

The abolishment of the *in vitro* cortisol response to VP by the selective AVPR1 antagonist, $[d(CH_2)_5,Tyr(Ome)^2]$AVP or OPC-21268 provided further evidence to the regulation of cortisol secretion by VP in some adrenal tumours (Arnaldi *et al.,* 1998; Daidoh *et al.,* 1998). *In situ* hybridisation studies confirmed adrenocortical VP receptor expression (Arnaldi *et al.,* 1998). Ectopic V2 receptor (AVPR2) expression has also been detected (Lee *et al.,* 2005). Aberrant VP receptor expression has not been universally found in VP-sensitive adrenal adenomas: intra-tumoral VP (immunohistochemistry) detected in some cases suggests that VP may operate *via* autocrine or paracrine mechanisms to stimulate steroidogenesis; alternatively, increased coupling efficiency of the VP receptor to the steroidogenic pathway, or aberrant expression of a novel receptor have been postulated (Joubert *et al.,* 2008).

Thus, the aberrant cortisol response to VP of adrenocortical adenomas may be due to eutopic AVPR1A overexpression, ectopic expression of other VP receptors (AVPR1B, AVPR2, or a novel receptor), increased coupling efficiency of the VP receptor to the steroidogenic pathway or by an, albeit unknown, autocrine or paracrine mechanism that may involve the intra-adrenal production of VP.

Although supraphysiological doses (5-10 I.U. IM) of VP have generally been administered to evaluate VP sensitivity, with ACTH suppression induced by pre-treatment with dexamethasone, PD-VP without dexamethasone has the capacity to detect an aberrant HPA axis response (fall in or unchanged ACTH and rise in cortisol) in AIMAH (Gagliardi *et al.,* 2009). High doses of VP may induce hypertension, tachycardia and abdominal cramping due to the vasoconstrictor properties of VP; cardiovascular comorbidities may therefore preclude evaluation of the VP response using such doses (Lacroix *et al.,* 1997b; Mune *et al.,* 2002; Lefebvre *et al.,* 2003). The ACTH-cortisol response of non-AIMAH adrenal tumours to PD-VP has not been previously evaluated.

The principal aims of this study were to determine: (1) if the aberrant ACTH-cortisol response to PD-VP was seen commonly in adrenal tumours, or represented a specific phenomenon for VPs-AIMAH; and (2) if the absent responses correlated with other biochemical tests suggesting SCS. Only with the use of PD-VP would our study have been able to address both aims. Hence, we recruited patients referred with adrenal tumours discovered either incidentally on radiological testing or after clinical suspicion of hormone excess. Another aim was to study gene expression profiles of VP-sensitive non-AIMAH adrenocortical tumours and compare with normal adrenal cortex and with the AIMAH expression data (Chapter 4). We hypothesised that: (1) the aberrant ACTH-cortisol response to PD-VP seen in AIMAH would not be seen more generally in adrenal tumours; and (2) an absent response to VP is indicative of pituitary corticotroph suppression due to cortisol excess from an adenoma, and would be more likely in patients with other tests suggestive of SCS.

7.2 Research Methods

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee. Sixteen participants with adrenal adenomas were recruited from the endocrine outpatient clinic. Evaluation for a functioning adenoma was performed by the treating clinician. A history of ischaemic heart disease was the only exclusion criterion, due to the vasoconstrictive properties of VP.

The VP stimulation test was performed in the morning (commencing between 0800 and 0900h); the protocol, blood sampling for ACTH and cortisol, and assays used have been described in Chapter 2. We used our published historical control data to define a "normal" response to VP as an increase in ACTH and cortisol from baseline (peaking at 15 and 30 minutes, respectively) (Torpy *et al.,* 1994) (Figure 7.1). The baseline ACTH and cortisol levels were taken as the average of the -30, -15 and 0 min measures prior to VP administration. The ACTH and cortisol increments were calculated using the peak measured

respective hormone level after VP administration. Thus, the percentage change in ACTH was calculated as follows:

$$\%\ \text{change in ACTH} = \frac{\text{(peak measured ACTH level after VP – baseline ACTH)}}{\text{baseline ACTH}} \times 100\%;$$

where baseline ACTH was the average of the ACTH levels measured at -30, -15 and 0 mins prior to VP administration. Where ACTH was < 10ng/L; we substituted an ACTH of 9ng/L so as to obtain an integer appropriate for the calculation of an average concentration. A similar calculation was performed for the percentage change in cortisol.

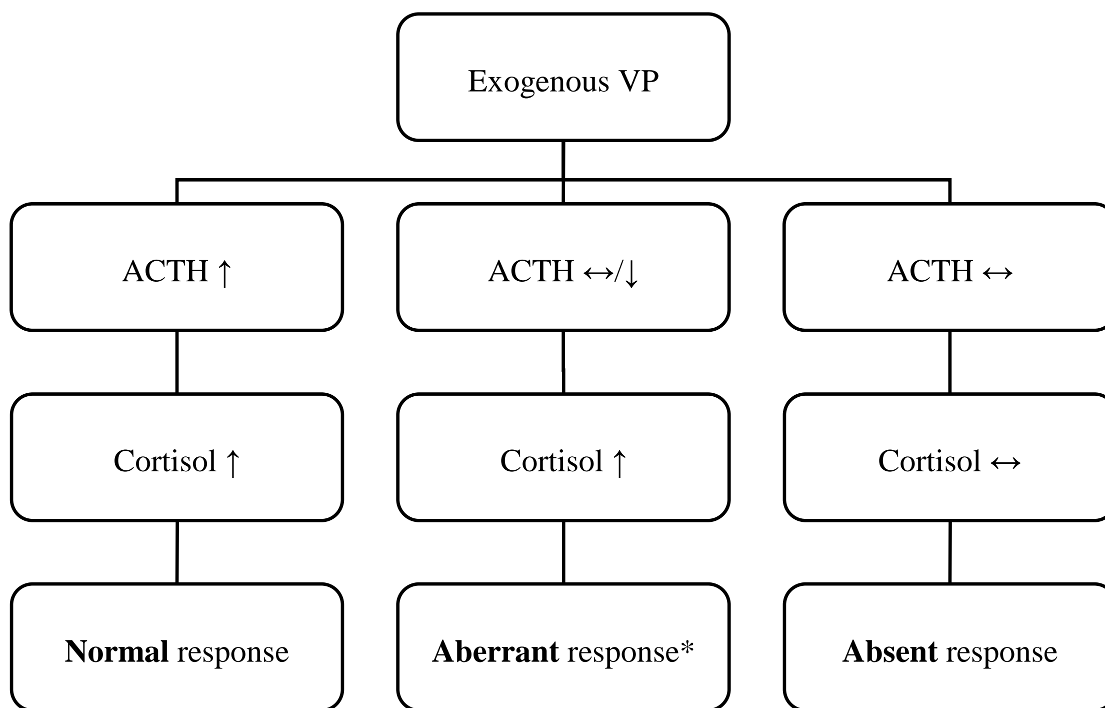


Figure 7.1. Classification of ACTH-cortisol responses to physiologic-dose vasopressin.
ACTH – adrenocorticotropic hormone; VP – vasopressin; hormone level after VP:
↔ unchanged, ↓ decreased, ↑ increased
*An aberrant response was defined as an unchanged or fall in ACTH and a minimum 20% increase in cortisol from baseline#
#Baseline cortisol was taken as the average of the serum cortisol levels measured at -30, -15 and 0 mins before VP administration; the % increase was calculated as

$$\frac{\text{(peak measured cortisol level after VP – baseline cortisol\#)}}{\text{baseline cortisol\#}} \times 100\%;$$

The response to VP was “aberrant” if cortisol increased from baseline by at least 20% and ACTH decreased or was unchanged (Figure 7.1). An “absent” response to VP was defined as one where neither ACTH nor cortisol increased (Figure 7.1).

7.3 Results and Discussion

Participants

The participants’ characteristics are shown in Table 7.1. Twelve had a non-functioning adrenal incidentaloma (NFAI), two had overt CS and two had primary aldosteronism (PA); those with PA had presented with hypertension and hypokalaemia. Of the 12 with NFAI, three had SCS according to current biochemical criteria (Arnaldi *et al.,* 2003).

VP stimulation test

This was well tolerated. Eleven participants (2 PA, 9 NFAI) had a normal ACTH and cortisol response (Table 7.1). Both participants with CS and two participants with NFAI had an absent response (Table 7.1). One participant with NFAI had an aberrant response (24% increase in cortisol; ACTH unchanged) (Table 7.1).

The ACTH-cortisol responses to PD-VP were absent in both cases of CS, consistent with suppression of pituitary ACTH release by ACTH-independent hypercortisolism. Both patients had definite HPA axis suppression as evidenced by a subsequent prolonged requirement (> 1 year) for exogenous glucocorticoid replacement following adrenalectomy. We did not detect an aberrant response to PD-VP in either case of CS (4 and 7% cortisol increase). This may be either because: (1) the tumours were not VP sensitive or (2) the dose of VP was insufficient to detect an aberrant response. In this study of adrenal adenomas, we detected only one person with an aberrant cortisol response to VP; furthermore she had a subtle (24%) cortisol increase, compared with the cortisol increments (45-340%) we have demonstrated using this protocol in VPs-AIMAH (Chapter 2; Gagliardi *et al.,* 2009).

Table 7.1 (part 1 of 2). Characteristics of participants and their ACTH and cortisol responses to physiologic-dose vasopressin.

Age, Gender	Adrenal adenoma, Diagnosis	24h UFC (50-350mmol/d)	ACTH (10-60ng/L)	1mg DST	NSC (<13nmol/L)	VP response‡ ACTH (ng/L): Basal→Peak (Δ%);* Cortisol (nmol/L): Basal→Peak (Δ%)*
60, F	R, 2cm AI	120	<10	<25	ND	Normal 9→26 (189); 305→564 (85)
39, F	L, 4cm, Cushing's	3266	<10	892	42	Absent 9→9 (0); 435→466 (7)
59, M	R, 2.5cm AI	ND	11	44	<8	Absent 11→12 (9); 299→265(-11)
64, M	L, 3.4cm AI	265	<10	ND	ND	Normal 9→22 (144); 193→669 (246)
71, F	**L, 2cm AI**	**93**	**<10**	**62**	**<8**	**Normal** **9→21 (133); 199→620 (211)**
47, F	L, 3cm, Cushing's	1233	<10	848	ND	Absent 9→9 (0); 759→789 (4)
53, M	L, 1.2cm AI	299	12	44	12	Normal 9→11 (22); 180→226 (25)
59, F	L, 2.8cm AI	209	10	22	12	Normal 12→19 (58); 584→622 (6)
63, F	L, 2cm AI	114	<10	29	10	Normal 9→35 (289); 303→742 (145)

The data for the participants with subclinical Cushing's syndrome are shown in bold.
ACTH – adrenocorticotropic hormone; AI – adrenal incidentaloma; 1mg DST – 1mg overnight dexamethasone suppression test – result is 0900h serum cortisol after 1mg oral dexamethasone at 2300h the night prior (reference: cortisol <50nmol/L); F – female; L – left; M – male; ND – not done; NSC – nocturnal salivary cortisol; PA – primary aldosteronism; R – right; UFC – 24 hour urinary free cortisol; VP – vasopressin; ‡VP response - "normal" if ACTH increased, cortisol increased; "absent" if ACTH and cortisol unchanged after VP; "aberrant" if ACTH unchanged or decreased, cortisol increased
basal – the average of -30, -15 and 0 min levels prior to VP administration; peak – maximum hormone level after VP administration; Δ% = (peak-basal)/basal* X 100%

Table 7.1 (part 2 of 2). Characteristics of participants and their ACTH and cortisol responses to Vasopressin.

Age, Gender	Adrenal adenoma, Diagnosis	24h UFC (50-350mmol/d)	ACTH (10-60ng/L)	1mg DST	NSC (<13nmol/L)	VP response‡ ACTH (ng/L): Basal→Peak (Δ%);* Cortisol (nmol/L): Basal→Peak (Δ%)*
62, M	L, 2.2cm AI	134	30	72	<8	Normal 23→61 (165); 303→490 (62)
78, F	**Bilateral, bulky, AI**	**ND**	**<10**	**62**	**<8**	**Absent 9→11 (22); 570→542 (-5)**
65, F	**R, 2.5cm, AI**	**76**	**<10**	**96**	**11**	**Normal 9→13 (44); 340→593 (74)**
69, M	L, 1.6cm AI	266	12	44	<8	Normal 12→22 (83); 501→741 (48)
75, F	L, 2.6cm AI	76	<10	ND	<8	Aberrant 9→9 (0); 414→515 (24)
75, M	Bilateral, PA	ND	ND	ND	<8	Normal 15→22 (47); 325→452 (39)
57, M	L, bulky, PA	ND	ND	ND	ND	Normal 11→17 (55); 207→504 (143)

The data for the participants with subclinical Cushing's syndrome are shown in bold.
ACTH – adrenocorticotropic hormone; AI – adrenal incidentaloma; 1mg DST – 1mg overnight dexamethasone suppression test – result is 0900h serum cortisol after 1mg oral dexamethasone at 2300h the night prior (reference: serum cortisol <50nmol/L); F – female; L – left; M – male; ND – not done; NSC – nocturnal salivary cortisol; PA – primary aldosteronism; R – right; UFC – 24 hour urinary free cortisol; VP – vasopressin; ‡VP response - "normal" if ACTH increased, cortisol increased; "absent" if ACTH and cortisol unchanged after VP; "aberrant" if ACTH unchanged or decreased, cortisol increased
Basal – the average of -30, -15 and 0 min levels prior to VP administration; Peak – maximum hormone level after VP administration; Δ% = (peak-basal)/basal* X 100%

We have previously shown that our protocol for VP stimulation testing is able to detect an aberrant cortisol response in VPs-AIMAH and we confirmed aberrant VP receptor expression on the AIMAH tumours (Chapter 2; Gagliardi *et al.,* 2009). The magnitude of the cortisol increment to PD-VP from baseline varied from 45-340% in our patients with VPs-AIMAH (Chapter 2; Gagliardi *et al.,* 2009). However, compared with single adrenal adenomas, the tumour cell burden in AIMAH is much higher, since in AIMAH the adrenal glands are bilaterally and diffusely diseased and normal adrenal cortex is not found. Conversely in single adrenal adenomas, normal adrenal is usually found attached to the adenoma. Therefore the number of cells aberrantly expressing VP receptors is potentially much larger in AIMAH, resulting in relatively exquisite tumour sensitivity to VP, and hence a more robust cortisol response to PD-VP, than one might expect could be mounted by a solitary adrenal adenoma. In part this is a reasonable justification for the lower percentage increment in cortisol in our definition of an aberrant cortisol response for this study. We postulate that whilst PD-VP is able to detect an aberrant response in VPs-AIMAH, a disease characterised by bilateral and diffuse adrenocortical hyperplasia, in the case of a single adrenal adenoma such a dose may either: (1) detect relatively milder aberrant cortisol increases; or (2) be insufficient to detect an aberrant cortisol response.

Although speculative, one could envisage therefore that an aberrant (> 20% increase) cortisol response may have been detected in the two participants with CS and a more robust cortisol increment may also have been detected in the participant with the aberrant response, if we had administered a higher dose of VP. This was not a practical possibility in those with CS since one person had moderate hypertension (a relative contraindication to higher dose VP) and the other person had had an adrenalectomy when the results of the VP stimulation test were available. We have not invited the participant with the aberrant cortisol response to return for evaluation using a higher dose of VP.

Horiba *et al.,* observed a two-fold increase in cortisol after VP (10 I.U. IM) in two patients with AIMAH compared with a less than 25% increase from baseline, in those with cortisol-secreting solitary adenomas (Horiba *et al.,* 1995). These data provide further credence to our postulate that the dose of VP we administered, although sufficient to detect VP sensitivity in AIMAH, may have been insufficient to detect an aberrant response in cortisol-secreting solitary adenomas, and was able to detect only a small cortisol increment in NFAI.

To resolve this issue, future studies could compare the ACTH-cortisol response to PD-VP with the conventional doses used (5-10 I.U. IM). Alternatively, in our small series, we could determine VP receptor expression (RT-PCR) on the resected adrenal tumours from the patients with CS. However, merely establishing aberrant receptor expression would not prove that the receptors were coupled to cortisol synthesis. This has been demonstrated in AIMAH where tumours aberrantly expressed receptors, but an aberrant *in vivo* cortisol response to administration of the specific ligand was absent (Miyamura *et al.,* 2002; Miyamura *et al.,* 2003). In other AIMAH tumours, aberrant receptors were expressed and coupled to steroidogenesis *in vitro* in the absence of an *in vivo* cortisol response (Louiset *et al.,* 2008). The reasons for this are not clear. Nevertheless we have not established adrenocortical tumour cell lines from either patient, and thus can not examine the cortisol response to VP *in vitro*.

The ACTH-cortisol responses to PD-VP were normal in both cases of PA. This is consistent with normal HPA axis function, an expected finding. Our findings do not suggest a role for VP in the regulation of aldosterone secretion in PA. This is in contrast to the study by Perraudin *et al.,* in which a role for VP in the autocrine/paracrine regulation of aldosterone secretion by aldosteronomas was suggested (Perraudin *et al.,* 2006). The evidence comprised: (1) detection of VP protein (immunohistochemistry) in aldosteronoma cells; (2) *in vitro* demonstration of the regulation of aldosterone secretion using perifusion of adrenocortical tumour explants with VP (10^{-7}M); (3) abrogation of the *in vitro* aldosterone response to VP

perifusion by administration of the specific AVPR1A antagonist $[d(CH_2)_5,Tyr(OMe)^2]AVP$ and; (4) the detection of VP receptor mRNA on tumour cells (RT-PCR) (Perraudin *et al.,* 2006). The discrepancy between that study and our findings may be due to the small number of patients we studied, truly VP-insensitive aldosteronomas in our study, or that the dose of VP we administered may not have been sufficient to detect an aldosterone response. However, higher doses of VP may stimulate aldosterone secretion via an ACTH-dependent effect on zona glomerulosa cells; pre-treatment with dexamethasone to suppress ACTH may, therefore, be necessary (Quinn and Williams, 1988).

The ACTH-cortisol responses to PD-VP were normal in 75% (n=9) of participants with NFAI, consistent with normal HPA axis function. During the endocrine evaluation for hormone hypersecretion in four of these participants, baseline ACTH was < 10ng/L, with no other biochemical or clinical features of hypercortisolism; perhaps suggesting pituitary corticotroph suppression due to subtle (subclinical) hypersecretion of cortisol (Table 7.1). However, our findings of normal ACTH-cortisol responses to PD-VP contradict the notion of pituitary corticotroph suppression in these individuals. One participant with NFAI had an aberrant adrenal cortisol response to PD-VP. She had an ACTH-independent 24% increase in cortisol. This finding is in keeping with the aberrant VP sensitivity of adrenal tumours that we and many other groups have reported (Perraudin *et al.,* 1995; Lacroix *et al.,* 1997b; Iida *et al.,* 1997; Horiba *et al.,* 1995; Daidoh *et al.,* 1998; Mune *et al.,* 2002; Arnaldi *et al.,* 1998; Tatsuno *et al.,* 2004; Reznik *et al.,* 2004; Lee *et al.,* 2005; Gagliardi *et al.,* 2009).

Two participants with NFAI had absent responses (Table 7.1). Both had marginal increases in ACTH and a fall in cortisol (Table 7.1). One person had an ACTH increment from 11ng/L to 12ng/L and the other 9ng/L to 11ng/L (Table 7.1). In both cases, cortisol decreased; 11% and 5% respectively (Table 7.1). These were unexpected findings because an increase in ACTH should stimulate an increase in cortisol. However, the increases in ACTH were quite small;

such that they could be considered negligible increases. In such a case, the fall in measured cortisol could be explained by the normal circadian variation in cortisol secretion (Horrocks *et al.,* 1990). The lack of a physiological ACTH response in these individuals may be an indicator of subtle HPA axis suppression; consistent with this was the suppressed ACTH and non-suppression of cortisol to dexamethasone (cortisol 62nmol/L) in one participant. In the other participant, ACTH was low-normal, but other measures of cortisol were normal; this may reflect the limitations of the available diagnostic tests for subtle cortisol hypersecretion.

Amongst participants with NFAI, three fulfilled biochemical criteria for SCS (Arnaldi *et al.,* 2003). Amongst these three participants, two had a normal VP response, and one had an absent response; the latter has been discussed above. The normal ACTH-cortisol response to VP in the other two participants suggests normal HPA axis function and is not consistent with HPA axis suppression due to subtle hypercortisolism.

An international consensus on the definition and diagnosis of SCS is lacking (Stewart, 2010). Although previously thought to be clinically silent, it is now increasingly recognised that a metabolic syndrome equivalent (manifesting as central obesity, hypertension, glucose intolerance, dyslipidaemia) and subtle catabolic tissue effects of cortisol excess (osteoporosis) do occur (Rossi *et al.,* 2000; Chiodini *et al.,* 2009). There are also data now that treatment of SCS may be beneficial in reversing components of the metabolic syndrome, although this finding has not been universal (Chiodini *et al.,* 2010; Toniato *et al.,* 2009; Sereg *et al.,* 2009; Mitchell *et al.,* 2007). However the data are prone to bias, since ethical considerations prohibit the conduct of a double-blinded, placebo-controlled clinical trial of adrenalectomy *vs* “sham” surgery. Moreover, it has been contended that patients with the aforementioned clinical features of metabolic syndrome, which are also found in patients with overt CS, should hardly qualify as having a subclinical endocrinopathy (Stewart, 2010). These issues

are discussed further in Chapter 8. Nevertheless, increasing efforts have been made to detect SCS, although the optimal diagnostic test currently eludes us.

The current diagnostic criteria for SCS require any two abnormal tests of the HPA axis consistent with hypercortisolism, in the absence of the classic Cushingoid phenotype. Diagnosis of SCS is largely based on dexamethasone suppression testing, despite its inherently high false positive rate (Gagliardi and Torpy, 2010; Arnaldi *et al.,* 2003). There are major limitations in evaluating for SCS since there is no gold standard test for diagnosis, or consensus on its definition and diagnosis (Stewart, 2010). Moreover there is a reliance on tests that perform imperfectly in the diagnosis of overt hypercortisolism. In a large study (*n*=369) of overweight or obese patients with an average of five to six additional features suggestive of CS, the specificity of screening tests was as follows: urinary free cortisol – 96%; dexamethasone suppression test – 90%; nocturnal salivary cortisol – 84% (radioimmunoassay), 92% (liquid chromatography-tandem mass spectrometry) (Baid *et al.,* 2009). These tests could perform more poorly in the diagnosis of SCS.

The PD-VP test, which evaluates the presence of corticotroph suppression, may be more accurate in predicting progressive deleterious tissue effects of hypercortisolaemia, although further studies are required. In two of three participants with apparently NFAI, but possible SCS based on other biochemical criteria, the VP response was normal, which questions the sensitivity of VP testing for diagnosis of SCS. In this pilot study, sensitivity of PD-VP testing for SCS can not be determined. Moreover, mere comparison of PD-VP testing with other biochemical tests with their own inherent false-positive and false-negative rates could confound the potential clinical utility of PD-VP testing (Gagliardi and Torpy, 2010). Less equivocal measures of corticotroph suppression, such as contralateral adrenal suppression on scintigraphy or a blunted ACTH response to CRH injection, may be more appropriate comparisons that could be considered in future studies (Gagliardi and Torpy, 2010). Our data,

although requiring verification by a much larger study, suggest that PD-VP stimulation testing may have a clinical application in the diagnosis of SCS.

Most studies have utilised VP stimulation to detect aberrant receptor expression in adrenal tumours, since this could have a role in their pathogenesis. In contrast to our evaluation protocol, these studies have universally used dexamethasone suppression and frequently VP doses of 5-10 I.U. IM (Perraudin *et al.,* 1995; Arnaldi *et al.,* 1998; Joubert *et al.,* 2008). In our, albeit small study of the ACTH-cortisol response to PD-VP in adrenal adenomas, we detected only one aberrant response. As already discussed, this could be a reflection of differences in the testing protocol. Consistent with this is the dose-response relationship between VP and cortisol secretion observed in some adrenal tumours (Daidoh *et al.,* 1998). However, we suggest that PD-VP may have an application in HPA axis evaluation in adrenal incidentaloma – which can not be performed when administering supraphysiological doses of VP, nor in the presence of dexamethasone suppression.

A secondary aim of our study was to compare gene expression of resected VP-sensitive adrenocortical tumours with VP-sensitive AIMAH and normal adrenal cortex. We did not detect VP-sensitivity in any of the functioning adrenal tumours (CS, PA) in this study, and thus have not been able to achieve this aim at this time. There has been no clinical indication to resect the adrenal adenoma in the only patient with VP-sensitive cortisol secretion.

7.4 Conclusion and Future Studies

On the basis of this study, aberrant responses to PD-VP in adrenal adenomas appear to be less frequent, in contrast to AIMAH. In patients with adrenal adenomas, an absent ACTH-cortisol response to PD-VP may reflect cortisol secretory autonomy. Whilst these data are preliminary, and require confirmation in a larger study, PD-VP testing could represent another method of evaluation for SCS in adrenal adenomas.

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

8.1 Introduction

Cushing's syndrome (CS) refers to the constellation of clinical signs associated with prolonged tissue glucocorticoid excess. These include many features of the metabolic syndrome, e.g., central obesity, hypertension and type 2 diabetes mellitus (T2DM) (Newell-Price *et al.,* 2006). In CS, T2DM may be of new-onset, or worsening hyperglycaemia may occur in those previously well-controlled who are continuing, or despite escalating, therapy. Treatment of hypercortisolism *per se* is required to improve or ameliorate hyperglycaemia (Munir and Newell-Price, 2010).

Recently, the entity of subclinical hypercortisolism (subclinical Cushing's syndrome, SCS) in adrenal incidentaloma patients has been recognised, and may also be associated with subtle clinical features of the metabolic syndrome (Reincke *et al.,* 1992; Rossi *et al.,* 2000). These observations, together with anecdotal reports of occult CS in patients with poorly-controlled T2DM, and the difficulty in the clinical distinction of CS from "simple" metabolic syndrome, have led to the notion that SCS/CS may be highly prevalent, although undetected, amongst patients with T2DM (Leibowitz *et al.,* 1996). Accordingly, screening studies for SCS/CS have been conducted in order to determine their prevalence in patients with T2DM; those studies which were published prior to the initiation of our study are summarised in Table 8.1.

Table 8.1. Prevalence studies of Cushing's syndrome or subclinical Cushing's syndrome in patients with diabetes mellitus.

Study; Number of subjects	Participant characteristics; Screening test, threshold	Results of screening	Subsequent work-up	Prevalence and nature of CS
Leibowitz *et al.*, 1996; *n*=90	BMI > 25 and HbA1c > 9%; Overnight 1mg DST; cortisol > 140nmol/L	4 positive DST (i.e., cortisol did not suppress)	Liddle's 2mg and 8mg DST and imaging studies	3.3% (2 CD; 1 adrenal CS)
Catargi *et al.*, 2003; *n*=200	BMI >25 and HbA1c > 8%; Overnight 1mg DST; cortisol > 60nmol/L	52 positive DST (26%)	0800h and 2400h ACTH and cortisol, 24h UFC <u>and</u> 4mg IV DST Any one test positive – imaging	2% (3 CD; 1 adrenal CS)
Chiodini *et al.*, 2005; Case-control study: 294 cases, 184 controls	Consecutive T2DM inpatients referred for poor metabolic control, BMI 19-50; Overnight 1mg DST; cortisol > 50nmol/L	Cases: 51 positive DST Controls: 10 positive DST	24h UFC, plasma ACTH and midnight plasma cortisol and/or dex/CRH; imaging guided by biochemical investigations	9.4% (21 adrenal; 4 CD; 2 ectopic)*
Reimondo *et al.*, 2007 *n*=99	Consecutive DM inpatients#; newly-diagnosed; Overnight 1mg DST; cortisol > 110nmol/L; All positives had repeat 1mg DST and a 2-day, 2mg DST; cortisol > 50nmol/L	First 1mg DST: 5 positive (5%) Second 1mg DST: 1 positive (1%)	24h UFC, midnight plasma cortisol, baseline ACTH and after CRH; imaging guided by biochemical investigations	1% (1 CD)

Abbreviations: ACTH – adrenocorticotropic hormone; BMI – body mass index (kg/m^2); CD – Cushing's disease; CRH – corticotrophin releasing hormone; CS – Cushing's syndrome; dex/CRH – dexamethasone/CRH stimulation test; DM – diabetes mellitus; DST – dexamethasone suppression test; HbA1c – glycosylated haemoglobin; T2DM – type 2 diabetes mellitus; UFC – urinary free cortisol

*Causes of "ascertained subclinical hypercortisolism" – diagnosed if positive screening DST and at least 2 other positive biochemical investigations (subsequent work-up) and source of glucocorticoid excess identified by imaging

7.1% of study subjects had type 1 diabetes mellitus

Table 8.1 (continued). Prevalence studies of Cushing's syndrome or subclinical Cushing's syndrome in patients with diabetes mellitus.

Study; Number of subjects	Participant characteristics; Screening test, threshold	Results of screening	Subsequent work-up	Prevalence and nature of CS
Caetano *et al.*, 2007; *n*=103	BMI > 25; NSC > 7nmol/L; Overnight 1mg DST; serum cortisol > 50nmol/L; salivary cortisol > 1.3nmol/L; Any two positive results were followed with a confirmatory 2-day low dose DST^	29 positive 1mg DST (saliva or serum); 1 positive 2-day low dose DST; 7 normal 2-day low dose DST but positive NSC and 1mg DST	Plasma ACTH, DHEAS, 24h UFC, DDAVP stimulation; CT scan adrenal Pituitary MRI	No case confirmed
Newsome *et al.*, 2007; *n*=171	Consecutive BMI > 25; T2DM > 12 months; Overnight 1mg DST; cortisol > 50nmol/L	31 positive	24h UFC	0%

Abbreviations: ACTH – adrenocorticotropic hormone; BMI – body mass index (kg/m^2); CD – Cushing's disease; CRH – corticotrophin releasing hormone; CS – Cushing's syndrome; CT – computed tomography; dex/CRH – dexamethasone/CRH stimulation test; DDAVP – desmopressin stimulation test; DHEAS – dehydroepiandrosterone sulphate; DM – diabetes mellitus; DST – dexamethasone suppression test; HbA1c – glycosylated haemoglobin; MRI – magnetic resonance imaging; NSC – nocturnal salivary cortisol; T2DM – type 2 diabetes mellitus; UFC – urinary free cortisol

^Positive results were either (i) positive 1mg DST (serum or saliva) or elevated baseline salivary cortisol; had 2-day low dose DST: 0.5mg 6 hourly for 2 days

The rationale for these studies has been that the diagnosis and treatment of SCS in T2DM might ameliorate hyperglycaemia; an extrapolation from the improvement in glycaemia observed after clinical cure of CS (Munir and Newell-Price, 2010). Most prevalence studies of SCS/CS in T2DM have utilised the 1mg overnight dexamethasone suppression test (DST) as the initial screening test (Leibowitz *et al.,* 1996; Catargi *et al.,* 2003; Chiodini *et al.,* 2005; Caetano *et al.,* 2007; Newsome *et al.,* 2007). In several of these studies there were many positive DST results which required further, often extensive, investigation of study participants, although relatively few cases of SCS/CS were ultimately confirmed (Table 8.1). We believe this is an important limitation of using the DST in this clinical setting; although in retrospect now, the high-false positive rate may reflect the overall low pretest probability of SCS/CS in this patient cohort.

The DST cortisol threshold used in many of the SCS screening studies in T2DM was 50nmol/L - the currently recommended threshold to enhance the sensitivity of the test for the diagnosis of CS (Arnaldi *et al.,* 2003). The optimal threshold for the DST to determine adrenal autonomy is debated, but 138nmol/L has been recommended because this level is considered to be a reasonable criterion for clinically significant glucocorticoid autonomy (Young, Jr, 2007; Nugent *et al.,* 1965). Thus, the cortisol threshold of 50nmol/L is likely to be too low for the diagnosis of SCS; and is likely also contributory to the substantial false-positive rate in the SCS screening studies. Evidence to this is the approximately 30% positive DST when the cortisol threshold was 50-60nmol/L, which fell to 5% when the threshold was 110nmol/L (Reimondo *et al.,* 2007). Ultimately, however, no single cortisol threshold for the DST reliably separates patients with CS or SCS from normal individuals (Findling *et al.,* 2004).

The nocturnal salivary cortisol (NSC) is a sensitive and specific screening test for CS; and is now recommended as a first-line screening test (Nieman *et al.,* 2008). In this study, we

wanted to evaluate the clinical utility of NSC testing in the evaluation for SCS in selected ambulatory patients with T2DM – those who were overweight or obese and who were not achieving glycaemic targets (defined as a glycosylated haemoglobin [HbA_{1c}] >8%). Specifically, the aim of this study was to evaluate the performance of NSC in a patient group purportedly at increased risk of SCS, to determine its diagnostic usefulness. We hypothesized that NSC would have a better specificity (lower false positive rate) than that reported for the DST in similar patient cohorts. Our study results, presented in this chapter, confirmed our hypothesis (Gagliardi *et al.,* 2010). We also anticipated that by screening for SCS, we might detect additional cases of adrenal incidentaloma, in which we could study VP sensitivity (Chapter 7).

8.2 Research Methods

Recruitment for this study was conducted between January 2008 and March 2009 from two tertiary referral centres in South Australia: the Royal Adelaide Hospital (RAH) and The Queen Elizabeth Hospital (TQEH) diabetes outpatient clinics and T2DM diabetes database (TQEH). The T2DM database contained all patients referred to the diabetes unit for assessment of diabetes-related complications and/or cardiovascular risk. This study was approved by the RAH and TQEH Human Research Ethics Committees and all participants gave written, informed consent.

Participant selection

Participants aged 40-75 years, with a body mass index (BMI) > 25kg/m^2, waist circumference > 80cm (females) or > 94cm (males) and recent (< 3 months) HbA_{1c} > 8%, were eligible. The BMI and HbA_{1c} criteria matched those of another study (Catargi *et al.,* 2003). The waist circumference criteria were based on the International Diabetes Federation recommendations for the diagnosis of metabolic syndrome (Alberti *et al.,* 2005). The exclusion criteria were clinical features of CS; type 1, late-onset autoimmune or secondary diabetes mellitus; history

of pituitary or adrenal disease; comorbidities including New York Heart Association Class III/IV heart failure, renal impairment (eGFR < 60ml/min), chronic liver disease, malignancy, alcohol dependence disorder or major psychiatric disorder; glucocorticoid therapy; acute illness; shift-work.

Anthropometric Data

Height (nearest 0.5cm; Seca 202 mechanical telescopic measuring rod, Seca, Germany) and weight (nearest 0.1kg; Seca 763, Seca) were recorded. Waist circumference (nearest 0.5cm) was measured as the part of the trunk midway between the lower costal margin and the iliac crest. Blood pressure was taken as the mean of two readings, obtained with a mercury sphygmomanometer and an appropriately-sized cuff, after five minutes seated.

Screening protocol

The screening protocol is shown in Figure 8.1.

Nocturnal salivary cortisol (NSC) (reference range, RR: < 13nmol/L) was collected using a Salivette® swab (Sarstedt, Germany), according to the manufacturer's instructions. The cotton salivette was placed in-between the gum and buccal mucosa for approximately three minutes. The sample was collected at home and at bedtime, but no later than midnight, having refrained from smoking, eating, drinking and brushing teeth for at least 30 minutes prior. The next day, between 0800h and 0900h, blood was drawn for plasma ACTH (RR: < 10ng/L). The details of the assays used have been described in Chapter 2.

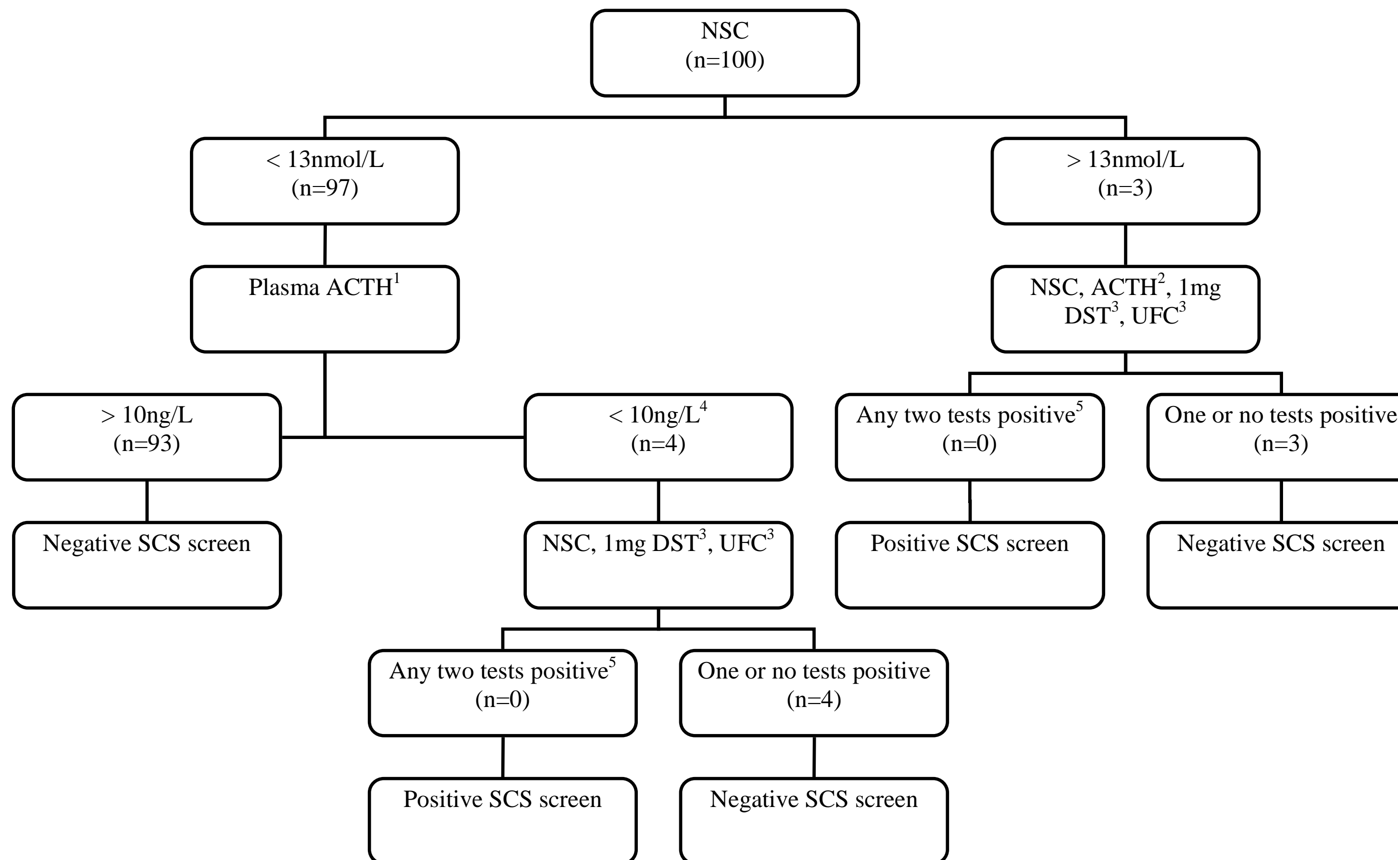


Figure 8.1. Subclinical Cushing's syndrome screening protocol.
[1]ACTH performed to exclude false negative NSC; [2]ACTH measured to assist in the differential diagnosis if SCS confirmed; [3]Confirmatory tests for SCS; [4]ACTH result repeated for confirmation; [5]Two positive tests - any two of the following: NSC > 13nmol/L, positive 1mg DST – 0900h serum cortisol > 50nmol/L after 1mg oral dexamethasone at 2300h the night prior, UFC > 350nmol/24h. Abbreviations: ACTH – adrenocorticotropic hormone; DST – dexamethasone suppression test; NSC – nocturnal salivary cortisol; SCS – subclinical Cushing's syndrome; UFC – urinary free cortisol

We previously measured NSC in healthy individuals and in suspected and overt CS (unpublished). Amongst 19 healthy adults (laboratory staff members) aged 44.6 ± 13.7 (mean ± standard deviation) years; the range of NSC levels was 1.7-15nmol/L (8.33 ± 3.6nmol/L; 5th and 95th percentiles, 1.66nmol/L and 12.5nmol/L) (Figure 8.2). Amongst 24 patients with CS, NSC ranged between 15-74.5nmol/L (27.4 ± 16.8nmol/L) (Figure 8.2). Amongst 13 patients with suspected CS, and in all of whom this was subsequently excluded, NSC ranged between 3-13nmol/L (8.0±2.8nmol/L) (Figure 8.2). We derived a normal threshold of < 13nmol/L; comparable to the threshold of 14.2nmol/L proposed by Yaneva *et al.,* for the same assay (Yaneva *et al.,* 2009).

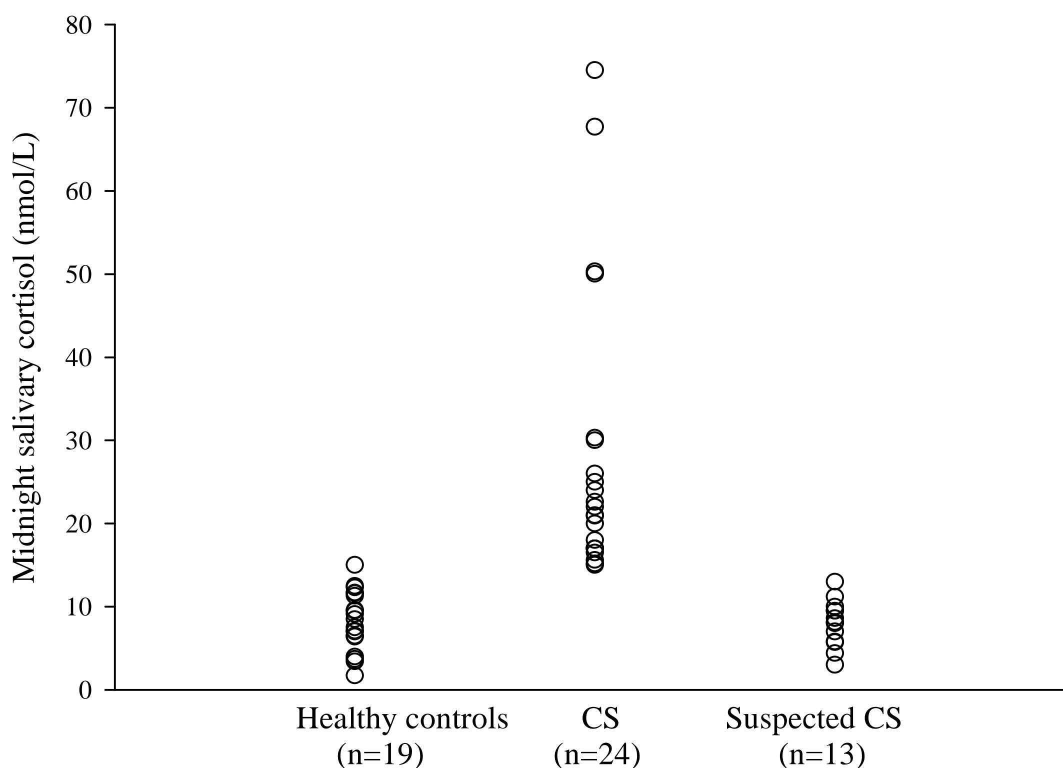


Figure 8.2. Midnight salivary cortisol concentrations using the Roche Elecsys Cortisol Electrochemiluminescence Immunoassay in healthy controls, Cushing's syndrome (CS) and suspected CS. The highest and lowest midnight salivary cortisol in healthy controls and in confirmed CS, respectively, was 15nmol/L. The highest midnight salivary cortisol in suspected CS was 13nmol/L. Suspected CS included patients screened because of clinical features including obesity, hypertension, but none was proven to have CS.

In the case of either an elevated NSC or low plasma ACTH, further assessment for SCS was undertaken with 24h urinary free cortisol (UFC) (RR: 50-350nmol/24h) and 1mg DST (Figure 8.1).

Definition of SCS

SCS is conceptually defined as the presence of hypercortisolism in the absence of clinically apparent CS (Mantero *et al.,* 1997). Operationally, it is defined as the presence of at least two abnormal basal or dynamic tests of the hypothalamic-pituitary-adrenal (HPA) axis (Mantero *et al.,* 1997). For the purposes of this study, we considered SCS screening to be positive if the following conditions were fulfilled:

(1) NSC ≥ 13nmol/L <u>AND EITHER</u>

(2) 24h UFC > 350nmol/24h <u>OR</u>

(3) 0900h cortisol > 50nmol/L after 1mg dexamethasone at 2300h the night prior (1mg DST).

The concept of SCS arose from studies of adrenal function in adrenal incidentalomas, leading to the notion that mild, clinically inapparent CS may be frequent based on reports of subclinical cortisol hypersecretion in ≈ 10% of adrenal incidentaloma, and a frequency of adrenal incidentaloma of ≈ 5% in the age groups screened (NIH consensus statement, 2002; Kloos *et al.,* 1995). A potential population frequency of SCS of 0.5%, greatly outweighs that of CS (incidence of 0.7 to 2.4 per million per year) (Lindholm *et al.,* 2001). Hence a measure of ACTH was performed as an adjunct in our protocol, to assist stratification of those with abnormal NSC.

8.3 Results and Discussion

106 participants (64 males; 65 participants RAH) were recruited. The cohort comprised 94% Caucasian, 4% Asian, 2% other (Hispanic, Aboriginal). Medical history and physical

examination did not reveal anyone with clinically apparent CS. One participant withdrew from the study prior to completing any investigations. One participant performed a salivary cortisol collection only, and another was unable to provide a salivary sample despite repeated attempts. Four participants did not complete any investigations. The characteristics of the 100 participants (60 males) who underwent evaluation for SCS are shown in Table 8.2. Sixty-seven individuals had been previously diagnosed with hypertension (Table 8.2). Of the remaining thirty-three individuals, we ascertained more than half as having "undiagnosed hypertension". "Undiagnosed hypertension" was considered to be present if the average of the two measured blood pressure readings we obtained was > 130/80, the recommended diagnostic cut-off in patients with T2DM (American Diabetes Association, 2008). Thirty-four per cent was on oral hypoglycaemics, 12% insulin and 53% was receiving both. Fifty-five per cent was on one or two antihypertensives, whilst 22% was on at least three.

Table 8.2. Clinical and metabolic parameters of the study participants (*n*=100).

	All participants (*n*=100) mean ± SD
Age (years)	58.6 ± 8.5
Duration of diabetes (years)	11.6 ± 7.3
Glycosylated haemoglobin (HbA_{1c}) (%)	9.5 ± 1.4
Diagnosis of hypertension [no(%) of cohort]	67 (67)
Undiagnosed hypertension* [no(%) of those without a previous diagnosis of hypertension]	17 (51.5%)
Blood pressure (mmHg)	
Systolic	140.7 ± 16.9
Diastolic	80.8 ± 9.8
BMI (kg/m^2)	33 ± 5.0
Waist circumference (cm)	113.2 ± 12.2

*Undiagnosed hypertension was considered to be present if the blood pressure was >130/80, the recommended blood pressure target in patients with type 2 diabetes mellitus, with no previous diagnosis of hypertension (American Diabetes Association, 2008)

NSC results were available for 100 participants. One participant (2, Table 8.3) had an elevated NSC (14nmol/L), two consecutive ACTH < 10ng/L and demonstrated normal cortisol suppression after dexamethasone (cortisol < 25nmol/L). UFC and repeat NSC were normal (Table 8.3). She had set an alarm-clock as a reminder to collect the NSC and this may have resulted in the initial false positive result. An augmented relative awakening cortisol increment with forced (alarm-clock) waking supports our postulate (Garde *et al.,* 2009). Another participant (1, Table 8.3) had NSC 27nmol/L and two consecutive ACTH < 10ng/L. She had been lightly sleeping prior to collection of the NSC sample. The 1mg DST, UFC and repeat NSC were normal (Table 8.3). A third participant (3, Table 8.3) had a single elevated NSC (75nmol/L); repeat NSC was normal, and the spuriously elevated result was attributed to sample contamination with blood (she was taking warfarin). Ninety-seven participants had NSC < 13nmol/L. Thus, the overall false positive rate of NSC in this patient group was 3%, which decreased to 1% when only properly collected samples were considered. No case of SCS was detected.

Four participants (4-7, Table 8.3) had low morning plasma ACTH confirmed, but normal NSC. All underwent further evaluation with repeat NSC and morning plasma ACTH, 24h UFC and 1mg DST. Individual characteristics and results are shown in Table 8.3. Further testing excluded SCS.

Table 8.3. Clinical characteristics and results of study participants requiring further evaluation for possible hypercortisolism.

Nō	Gender, Age, BMI (kg/m^2)	T2DM (yrs); HbA_{1c}; Rx	HT	NSC (nmol/L)	ACTH (ng/L)	UFC (nmol/24h)	1mg DST
1	Female, 49 39.4	0.5; 9.2; OHA	Y	27, < 8	< 10	74	< 25
2	Female, 61 27.6	11; 9.1; OHA	N	14, 8	< 10	169	37
3	Female, 71 30	20; 10.5; OHA, insulin	Y	75, 11, < 8	14	19	ND
4	Female, 50 30	8; 10.1; OHA	Y	< 8, < 8	< 10	91	22
5	Female, 59 29.2	3; 9.5; OHA	Y	< 8, < 8	< 10	63	24
6	Male, 54 36.3	9; 9.3; OHA, insulin	Y	10, < 8	< 10	212	14
7	Female, 67 37.2	26; 9.9; insulin	Y	< 8, < 8	< 10	118	49

Reference ranges: ACTH 10-60ng/L; 1mg DST: result listed is 0900h serum cortisol after 1mg oral dexamethasone at 2300h the night prior, normal < 50nmol/L; NSC < 13nmol/L; UFC 50-350nmol/24h

Abbreviations: ACTH – adrenocorticotropic hormone; BMI – body mass index; DST – dexamethasone suppression test; HbA_{1c} – glycosylated haemoglobin; HT – hypertension; N – no, not hypertensive; ND – not done; NSC – nocturnal salivary cortisol; OHA – oral hypoglycaemic agent; Rx – treatment; T2DM (yrs) – years since diagnosis of type 2 diabetes mellitus; UFC – 24 hour urinary free cortisol; Y – yes, hypertensive

Thus, we did not detect a single case of SCS after screening 100 patients with T2DM. Moreover, this is after having selected participants for characteristics which purportedly increased the pretest probability of SCS – overweight and failing glycaemic therapy. Our data suggest that SCS is less prevalent in an ambulatory cohort of patients with T2DM than reported in other studies. Our data do not support routine screening for SCS in patients with T2DM and features of the metabolic syndrome.

Other prevalence studies of SCS in T2DM have mostly been performed in inpatients and the data may not be applicable to ambulatory patients (Chiodini *et al.,* 2005; Catargi *et al.,* 2003; Reimondo *et al.,* 2007; Leibowitz *et al.,* 1996). Those studies that applied minimal selection criteria, reported a low prevalence of SCS (0-1%), whilst those that selected obese or poorly

controlled patients with T2DM reported a higher prevalence (2-7%) (Chiodini *et al.,* 2005; Catargi *et al.,* 2003; Reimondo *et al.,* 2007; Leibowitz *et al.,* 1996; Newsome *et al.,* 2008; Caetano *et al.,* 2007). In one study, patients with SCS had more severe features of metabolic syndrome - insulin-requiring T2DM, hypertension and dyslipidaemia (Chiodini *et al.,* 2005). Our data conflict with that from a study in which the reported prevalence of occult CS was 2%, utilising the same BMI and HbA_{1c} selection criteria (Catargi *et al.,* 2003). However, in that study, 75% of subjects were female, compared with only 40% in our study, which may account for the discrepancy, since overall, CS is much more frequent in women than in men (Catargi *et al.,* 2003; Lindholm *et al.,* 2001).

Interim results of the largest multi-centre screening study performed for SCS in ambulatory unselected patients with T2DM have been reported recently (Terzolo *et al.,* 2009). In this study, 793 patients were recruited from 23 outpatient clinics (Terzolo *et al.,* 2009). Although they were "unselected", a large percentage had features of the metabolic syndrome: 86.7% had abdominal obesity, 91% had BMI > $25kg/m^2$ and 77% were hypertensive (Terzolo *et al.,* 2009). In this study, five individuals (0.6% of the series) fulfilled diagnostic criteria for SCS (positive 1mg DST – cortisol > 138nmol/L and 2-day 2mg DST – cortisol > 50nmol/L). However, only one patient had surgically confirmed CS (0.1% of the series) (Terzolo *et al.,* 2009). These data support our findings; SCS is rare amongst ambulatory T2DM and there is no justification for routine screening.

When we commenced our study, only one other group had evaluated NSC in screening consecutive, overweight, ambulatory patients with T2DM (*n*=103) for SCS (Caetano *et al.,* 2007). Eleven per cent (*n*=12) had a NSC in the upper quintile of the normal range, and of these, eight were diagnosed with SCS, on the basis of concomitant dexamethasone suppressed serum or salivary cortisol results (Caetano *et al.,* 2007). In three individuals, SCS remained unconfirmed at study completion; however no case was confirmed by surgery or pathology

(Caetano *et al.,* 2007; Table 8.1). These data support our conclusion that NSC has a low false positive rate.

Most recently, another group also used NSC to screen 79 healthy controls and 201 consecutive ambulatory patients with T2DM who fulfilled at least two of the following criteria: $HbA_{1c} \geq 7\%$, BMI $\geq 25 kg/m^2$, history of hypertension or blood pressure $\geq$ 140/90 mmHg (Mullan *et al.,* 2010). In this study, NSC had a high (23%) false positive rate. However, the NSC threshold was intended to be conservative and, due to the absence of NSC data from patients with CS, was arbitrarily determined (Mullan *et al.,* 2010). Thus, a high false positive rate could have been predicted. In contrast, we used a NSC threshold that had been derived from our experience of NSC levels in patients with definite CS and in healthy controls; this likely accounts for the low false positive rate of NSC in our study. Not a single case of SCS amongst T2DM was detected in the study by Mullan *et al.,* suggesting, as do our data, that this is less common than previously thought (Mullan *et al.,* 2010).

The low false positive rate obtained in our study also contrasts with a study of elderly male veterans in whom an overall 20% false positive rate was reported (Liu *et al.,* 2005). Whilst this could suggest a need for age-specific NSC reference ranges, screening was performed in a cohort not suspected of having CS and the assay used had not been validated in patients with CS (Liu *et al.,* 2005). Conversely, we selected a high-risk cohort, and we had validated our assay in CS and in healthy individuals.

In (obese) control subjects, and in those with subclinical, overt, cured or recurrent, CS, salivary cortisol levels were comparable regardless of the sampling conditions (inpatient *vs* outpatient; midnight *vs* bedtime), suggesting that an outpatient bedtime collection may suffice (Nunes *et al.,* 2009). Thus, NSC lends itself favourably to repeated measurements, which may be required for diagnosis of SCS (Kidambi *et al.,* 2007). NSC could be a practical screening

tool in large high-risk cohorts although its sensitivity needs to be established. This requires comparison of NSC to a gold standard test for SCS – which, except for postoperative hypocortisolism, currently eludes us. Other measures of corticotroph suppression, such as contralateral adrenal suppression on scintigraphy, or a blunted ACTH response to corticotrophin-releasing hormone (CRH) injection, could represent appropriate surrogates (Gagliardi and Torpy, 2010). Any screening test must have a low false positive rate and acceptable sensitivity, as subsequent testing is inconvenient, expensive and potentially invasive.

The parotid gland harbours 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) which converts cortisol to cortisone (Smith *et al.,* 1996). Thus some salivary cortisol is metabolised to cortisone, which is not measured by the salivary cortisol assay we used (assay cross-reactivity with cortisone 0.3%; Chapter 2). Hence it is conceivable that we failed to detect a case of SCS, because we used NSC as the only screening test.

The hypercortisolism of SCS is generally milder than overt CS; hence mildly elevated cortisol levels may not be detected by salivary cortisol measurement, due to the metabolism of cortisol to cortisone, together with the use of an assay in which there is negligible cross-reactivity between cortisol and cortisone. Further credence is given to this postulate by two recent studies which have concluded that NSC lacks sensitivity for SCS (Nunes *et al.,* 2009; Masserini *et al.,* 2009). However, only small numbers of patients (total *n*=34) were evaluated, which reduces the ability to ascertain the sensitivity of NSC for SCS (Nunes *et al.,* 2009; Masserini *et al.,* 2009). Moreover, the conclusion relied upon an assumption that is debatable: that an unequivocal diagnosis of SCS had been made (Nunes *et al.,* 2009; Masserini *et al.,* 2009). In these studies, the diagnosis of SCS required a positive DST and at least another HPA axis aberration; although this is in keeping with currently suggested diagnostic criteria,

there is no consensus on the diagnosis of SCS in the form of established evidence-based, peer-reviewed clinical guidelines (Stewart, 2010).

Notwithstanding the limitations of these studies, presently emerging data suggest that the metabolism of cortisol to cortisone by salivary 11βHSD2 may significantly alter the measured salivary cortisol value, such that measurement of salivary cortisone might be a better biomarker of circulating free cortisol (Perogamvros *et al.,* 2010). Salivary cortisone has not yet been evaluated in patients with CS; this certainly needs to be performed before any claim can be made that evaluation of a cortisol metabolite is superior to measurement of salivary cortisol (Raff and Findling, 2010). Whether measurement of salivary cortisone has utility beyond that provided by measurement of salivary cortisol in our study is also not known.

A limitation of our study is that we do not know the sensitivity or the false negative rate of NSC for SCS in T2DM, because we did not detect any cases. In part this is due to the lack of a comparison gold standard diagnostic test for SCS, as well as, by definition, the absence of clinical diagnostic clues. We used morning plasma ACTH as an ancillary measure; our rationale was that in primary adrenocortical hyperfunction, glucocorticoid negative feedback would suppress pituitary corticotrophs, and hence, ACTH. In cases where low plasma ACTH was confirmed, regardless of the NSC result, a complete evaluation was performed, however no case of SCS was detected. Hence, we believe it is unlikely that a case of ACTH-independent SCS was undiagnosed, although it is possible that we overlooked ACTH-dependent disease. The recent data that NSC has poor sensitivity for SCS and that salivary cortisone may be a better biomarker of circulating free cortisol, must call into question the validity of using NSC as the sole screening test for SCS, by definition a much more subtle entity than CS (Nunes *et al.,* 2009; Masserini *et al.,* 2009; Perogamvros *et al.,* 2010). In retrospect, a study of NSC *vs* 1mg DST, traditionally the most frequently utilised screening test for SCS in T2DM, may have been informative, although the study would have then

required extensive HPA testing in more individuals due to the high false positive rate of the 1mg DST demonstrated in similar patient cohorts (Table 8.1). Another limitation of our study is the small sample size compared to other prevalence studies.

The impetus driving all of these screening studies for SCS in T2DM has been the premise that diagnosis and treatment of SCS might ameliorate, if not cure, hyperglycaemia. However, whether treatment of SCS has any clinical benefit remains a controversial area since: (1) there is no consensus on its diagnosis; and (2) the conduct of a randomized, double-blind, placebo-controlled study is unethical (Stewart, 2010). Moreover, there is a nomenclature issue: for how are individuals with glucose intolerance or obesity considered to have "subclinical" CS when these are established features of CS (Stewart, 2010)? The time is nigh for an international consensus on the definition and diagnosis of SCS.

Adrenal incidentaloma has been the principal clinical context for the study of the diagnosis and efficacy of treatment of SCS; with the observation of post-operative hypocortisolism a clue to prior hyperfunction of otherwise clinically silent adrenal masses (Reincke *et al.,* 1992; Terzolo *et al.,* 1998; Tauchmanovà *et al.,* 2002; Terzolo *et al.,* 2002; Bernini *et al.,* 2003; Mitchell *et al.,* 2007; Toniato *et al.,* 2009). Mitchell *et al.,* purported to show a benefit of adrenalectomy on metabolic perturbations associated with SCS in subjects with adrenal incidentaloma (Mitchell *et al.,* 2007). However, all patients with "SCS" had clinical features suggestive of CS – atrophic skin, a bruising tendency, proximal muscle weakness, supraclavicular fat pads or facial plethora - although no patient was floridly biochemically hypercortisolaemic, according to the UFC and 1mg DST (Mitchell *et al.,* 2007). The discrepancy between clinical and biochemical features reflects the limitations of the currently available biochemical tests for hypercortisolism. Nevertheless the presence of these clinical signs raises the suspicion that the subjects may have had mild CS. Indeed, some or all of the clinical features, as well as hypertension and glycaemia improved postoperatively, and the

majority of patients required postoperative hydrocortisone – but all of this may have reflected surgical cure of CS, rather than SCS; with implications for the study's conclusions (Mitchell *et al.,* 2007).

The results of the first randomised, prospective trial with a mean of seven years (range 2-17 years) of follow-up of surgically *vs* conservatively managed SCS in adrenal incidentaloma have now been reported (Toniato *et al.,* 2009). The two groups were matched at randomisation for the prevalence of features of the metabolic syndrome. Amongst the surgically treated patients the following improvements were noted: normalisation or improvement of glycaemic control permitting the cessation of or reduction in hypoglycaemic therapy (62.5%); improvement in hypertension permitting reduction in or cessation of antihypertensive therapy (67%); weight reduction (50%) and an improvement in the lipid profile (37.5%) (Toniato *et al.,* 2009). In the conservatively managed group, each of these comorbidities either remained unchanged or deteriorated, necessitating an escalation of therapy (Toniato *et al.,* 2009). However, this was not a double-blind, placebo-controlled study, granted that ethical considerations preclude such a study in humans. It is conceivable that the surgical group experienced metabolic improvements because of factors unrelated to the adrenalectomy (e.g., lifestyle changes) in association with, and influenced by, their own or their endocrinologist's, preoperative expectations. Also, biochemical cure of SCS was not proven postoperatively, since all received prophylactic glucocorticoid replacement.

A recent trial of surgical *vs* conservative management of patients with adrenal incidentaloma and SCS reported an improvement in glycaemia and blood pressure in the surgically treated group (Chiodini *et al.,* 2010). However, this was not a randomised study: all patients with SCS were offered surgery; 25 agreed and 16 declined; amongst those who did not have SCS, 30 fulfilled criteria for surgical resection based on the size of the adrenal mass or an increase in size during follow-up and thus were offered surgery (Chiodini *et al.,* 2010). Improvement

in blood pressure was also observed in the treated group who did not have SCS (Chiodini *et al.,* 2010). This suggests an adrenalectomy-induced effect independent of cortisol; perhaps that adrenal incidentalomas *per se,* and independent of cortisol secretion, may be associated with features of the metabolic syndrome. Bernini *et al.,* also reported a significant decrease in body weight, blood pressure and glucose levels in response to oral glucose tolerance testing, following adrenalectomy in patients whether or not they had fulfilled diagnostic criteria for SCS (Bernini *et al.,* 2003). Alternatively, purported improvement in metabolic features in patients without SCS treated with adrenalectomy may reflect our inability to reliably detect subtle hypercortisolism. A long-term prospective study of patients with adrenal incidentaloma with or without SCS showed no improvement in metabolic features in those treated with adrenalectomy (Sereg *et al.,* 2009).

All the studies conducted in this field have some limitation: first and foremost, the lack of consensus on diagnostic criteria for SCS; other limitations include uncontrolled and non-blinded study designs, short-term follow-up, small case numbers and lack of unequivocal evidence of cure of SCS (Reincke *et al.,* 1992; Rossi *et al.,* 2000; Tauchmanovà *et al.,* 2002; Chiodini *et al.,* 2005; Taniguchi *et al.,* 2008). In addition, the data are conflicting. Thus, whilst there is uncertainty regarding the diagnosis of SCS, and whilst it remains unclear whether there is any objective benefit to be gained from its treatment, we can not recommend routine screening in patients with T2DM and other features of the metabolic syndrome.

8.4 Conclusion and Future Studies

The recent evolution of the field of SCS followed the observation of apparent hypercortisolism in cases of adrenal incidentaloma, with the implication that due to the relatively high prevalence of abnormal HPA axis tests (> 10%) and adrenal incidentaloma (5%), subtle hypercortisolism might be sufficiently frequent to justify screening (NIH consensus statement, 2002; Terzolo *et al.,* 2002; Mantero *et al.,* 1997; Kloos *et al.,* 1995). Of

the three tests (1mg DST, UFC, NSC) used for the diagnosis of CS, the NSC appeared to have lower false positive results; our study aimed to determine the rate of false positive results in a patient group where screening might be expected to detect significant numbers of SCS cases (Raff *et al.,* 1998; Papanicolaou *et al.,* 2002; Yaneva *et al.,* 2004; Viardot *et al.,* 2005). We found a low false positive rate of NSC for SCS, but were unable to evaluate its sensitivity since we did not detect any cases of SCS (Gagliardi *et al.,* 2010).

As the field has now progressed, it appears that SCS is probably not common amongst patients considered to be "at risk" for the disease, when evaluation is performed with the usual investigations used for screening for CS. Perhaps it is not surprising that these tests, which often yield contradictory results in CS, have led to widely varying results in "at risk for SCS" groups. Emerging data, including those from this study, refute the notion of screening for SCS in purportedly "at risk" groups based on metabolic factors.

We are left, however, with data suggesting that abnormal HPA axis testing is common in adrenal incidentaloma, that hypocortisolism may develop post-operatively and that unilateral adrenalectomy may improve metabolic parameters, in the absence of overt CS. This suggests that adrenal adenomas may have detrimental metabolic effects, and, at least occasionally, hypersecrete cortisol. Further progress in this field awaits an HPA axis testing protocol that is capable of detecting subtle dysregulation or a test of tissue glucocorticoid excess.

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

9.1 Introduction

The regulation of cortisol secretion has been discussed in Chapter 1; the salient features are recapitulated here. Cortisol is produced by the adrenal cortex following stimulation of zona fasciculata cells by adrenocorticotropic hormone (ACTH), which is secreted in a pulsatile manner by the pituitary gland (Gallagher *et al.,* 1973; Hellman *et al.,* 1970). Initiation of pulsatile cortisol secretion occurs shortly (≈ 10 minutes) after an ACTH pulse (Gallagher *et al.,* 1973). Periods of increased cortisol concentration are due to more frequent and longer secretory episodes (Weitzman *et al.,* 1971).

There is a light-entrained circadian rhythm of cortisol secretion, in which cortisol levels reach their nadir at midnight, begin to rise at approximately 0200h and continue to rise into the early hours of the morning, peak within thirty minutes of waking (cortisol-awakening response) and then gradually decrease throughout the day (Weitzman *et al.,* 1971; Pruessner *et al.,* 1997). Superimposed on this is the ultradian rhythm of cortisol secretion – the pulsatile secretion of cortisol in response to psychological and physical stressors and other stimuli (e.g., protein-containing mid-day meal) (Kirschbaum *et al.,* 1996; Quigley and Yen, 1979; Ishizuka *et al.,* 1983). The cortisol surge in the early hours of the morning may physiologically prepare the individual for the active period (Scheer and Buijs, 1999). The secretion of cortisol in response to stressors is a mechanism of defence against real or perceived threats to homeostasis. A mid-day, protein-containing meal appears to act as an exogenous synchronizer and amplifier of normal cortisol fluctuation (Quigley and Yen, 1979; Follenius *et al.,* 1982). The cortisol

response to food may also modulate the effects of meal composition on mood and cognitive function (Gibson *et al.,* 1999).

Conventional glucocorticoid replacement (oral, twice or thrice daily) therapy reproduces neither the circadian, nor the ultradian, rhythms of cortisol secretion (Scott *et al.,* 1978; Levin *et al.,* 1981; Mah *et al.,* 2004). In contrast, conventional glucocorticoid replacement therapy produces endogenous cortisol levels that: (1) are unmeasurable during the period of active cortisol secretion (0300h-0800h) in normal humans; and (2) frequently peak at supraphysiological levels shortly after administration and decline rapidly thereafter, reaching very low levels prior to the next dose (Scott *et al.,* 1978; Levin *et al.,* 1981; Mah *et al.,* 2004).

Before the widespread availability of glucocorticoid replacement therapy in the 1950s, the one year survival of patients with Addison's disease was less than 20% (Dunlop, 1963). Although, as discussed in Chapter 1, glucocorticoid replacement has markedly improved, albeit perhaps not normalized, mortality in Addison's disease, subjective health status in this patient group has been shown to be consistently and severely impaired across various ethnic groups compared with matched controls (Løvås *et al.,* 2002; Alonso *et al.,* 2004; Hahner *et al.,* 2007; Gurnell *et al.,* 2008; Bleicken *et al.,* 2008). Patients with Addison's disease reported a health status comparable with a normal population almost twice their age, and with patients with chronic congestive cardiac failure (Løvås *et al.,* 2002). The impairments in health status were associated with a significant work disability (26% *vs* 10% in the normal general population) (Løvås *et al.,* 2002). In a recent study by Bleicken *et al.,* these deficits in health status persisted despite "best practice" thrice daily hydrocortisone dosing (Bleicken *et al.,* 2010).

Gastrointestinal symptoms are also frequently reported (Hahner *et al.,* 2007). Whilst such symptoms (nausea, anorexia, vomiting and abdominal pain) may be prominent prior to the

diagnosis of adrenal insufficiency, or may herald concomitant coeliac disease, anecdotal reports suggest that these symptoms may be due to inadequate glucocorticoid replacement (Tobin *et al.,* 1989).

The explanations proposed to account for the impaired health status in Addison's disease have been discussed in Chapter 1 – they are: (1) DHEA deficiency, since this is not routinely replaced; and (2) non-physiological glucocorticoid replacement, since cortisol has important neurocognitive and arousal effects. However, neither is a proven cause of impaired subjective health status. A meta-analysis of the studies evaluating the effect of DHEA replacement on quality of life in women with adrenal insufficiency suggested a small, possibly clinically trivial effect on quality of life and a small improvement in depression (Alkatib *et al.,* 2009). There was a non-statistically significant trend to improved anxiety and sexual well-being (Alkatib *et al.,* 2009).

The effect of circadian (and ultradian) glucocorticoid replacement on health status has not been studied in a randomised, double-blind, placebo-controlled clinical trial. However, the fervour for the hypothesis that impaired subjective health status in Addison's disease is due to non-physiological glucocorticoid replacement has already inspired the development of modified-release hydrocortisone tablets: Chronocort (Phoqus Pharmaceuticals Limited, West Malling, UK) – a twice daily delayed and sustained-release hydrocortisone preparation and DuoCort™ (DuoCort Pharma AB, Helsingborg, Sweden) - a once-daily dual (immediate and sustained) release hydrocortisone tablet (Newell-Price *et al.,* 2008; Debono *et al.,* 2009; Johannsson *et al.,* 2009). These preparations may produce a circadian cortisol profile comparable to the normal diurnal rhythm, but not the ultradian pulses associated with daily life stress. It is not known if either circadian or ultradian cortisol release is important for normal well-being.

In this study, we wanted to evaluate, in a randomised, double-blind, placebo-controlled clinical trial, the hypothesis that impaired subjective health status (quality of life; well-being) in patients with Addison's disease was due to non-physiological glucocorticoid replacement. A secondary outcome of this study was the effect on gastrointestinal symptoms – since such symptoms are frequent amongst patients with Addison's disease (Hahner *et al.,* 2007).

We attempted to reproduce the normal circadian rhythm of cortisol production using continuous subcutaneous hydrocortisone infusion (CSHI) delivered by a proprietary insulin pump. Others had already performed a proof-of-concept study which had demonstrated that CSHI therapy was safe and could re-establish the circadian variation in cortisol levels (Løvås and Husebye, 2007). We further attempted to reproduce some aspects of the ultradian rhythm of cortisol production by superimposing on the basal infusion, hydrocortisone boluses on waking, with lunch and in response to the experience of a "daily life stress" (defined in Methods section, this chapter). In our study, patients with Addison's disease were assigned to receive each of two treatments, separated by a two-week interval, in a double-blind and random order: (1) one month of oral placebo and CSHI therapy and (2) one month of thrice daily oral hydrocortisone and placebo infusion therapy. We assessed health status in patients receiving "best practice" thrice daily oral hydrocortisone with their health status whilst receiving CSHI.

Although having limited clinical applicability, serum cortisol-day curves have been used to measure endogenous cortisol levels in those on glucocorticoid replacement (Arlt *et al.,* 2006). Since there is a close correlation between serum and salivary cortisol levels, we used serial salivary cortisol sampling to compare endogenous cortisol levels achieved with each treatment (Løvås *et al.,* 2006; Dorn *et al.,* 2007). Apart from the convenience of collection, salivary cortisol measurement is superior to plasma cortisol because it reflects free circulating cortisol, which is available to tissues, rather than total cortisol which includes cortisol bound to

corticosteroid-binding globulin or albumin. At the time of writing this thesis, this study remains in progress.

9.2 Research Methods

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee, and was registered as a clinical trial with the Therapeutic Goods Administration Clinical Trials Notification Scheme (Drugs), Trial Number 2008/217. All participants gave written, informed consent.

Participants

Participants were recruited after either being informed of the study by: (1) their treating endocrinologist and referred to the principal investigators (myself and Associate Professor David Torpy); or (2) the South Australian division of the Australian Addison's Disease Association Incorporated (SA division of AADAI; an Addison's disease support group); and then initiating contact with the investigators. Participants were reimbursed for travel expenses associated with study participation. We aimed to recruit a minimum of six participants.

To facilitate recruitment we:

(1) sent a study information letter to 23 active endocrinologists in SA outlining the study and requesting they inform/contact any potential participants and provide them with the contact details of the study investigators;

(2) obtained ethics approval to recruit from the Queen Elizabeth Hospital;

(3) liaised with the SA division of AADAI as follows:

   a. we attended the annual SA division of AADAI dinner in 2008 and 2009 as guest speakers (myself and Associate Professor David Torpy);

b. the association sent an information letter to all past and present members, inviting interested individuals to contact the study investigators for further information;
c. we placed an advertisement in the AADAI newsletter and on the AADAI website (http://addisons.org.au/core.htm), inviting interested individuals to contact the study investigators for further information.

Participants were eligible for the study if they had Addison's disease, lived in the Adelaide metropolitan area and did not meet any of the exclusion criteria. They were required to wear their Medic-Alert at all times. Any known endocrine comorbidity (eg. hypothyroidism, type 2 diabetes mellitus) needed to be appropriately treated, and considered stable, by the investigators and the participant's treating endocrinologist, based on standard clinical, and where appropriate, biochemical, parameters. Medication doses needed to have been stable for three months prior to study entry. Mineralocorticoid replacement also needed to be adequate; this was guided by blood pressure, serum electrolytes and direct renin concentration.

The exclusion criteria were: age < 18 years, Addison's disease due to bilateral adrenalectomy, secondary adrenal insufficiency, medical or psychiatric illness which, at the discretion of the investigators, was deemed to potentially affect well-being or limit study participation (e.g., type 1 diabetes mellitus, coeliac disease, NYHA Class III or IV heart failure, renal disease [CrCl < 30ml/min], previous stroke, psychiatric illness), shift-work, pregnancy.

The withdrawal criteria were: diagnosis of pregnancy and, at the discretion of the investigators, any problem experienced during the trial which was not expected to be resolved in the short-term, and which was deemed to potentially compromise patient safety or confound quality of life assessments.

Study Protocol

This was a randomized, double-blind, placebo-controlled clinical trial. The study protocol is shown in Figure 9.1.

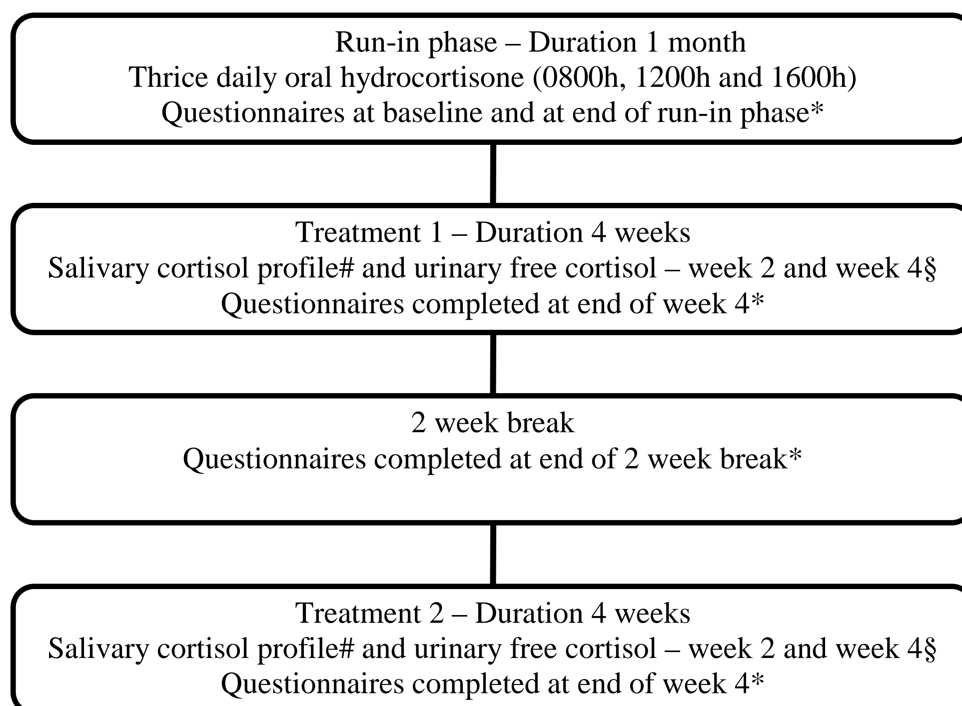


Figure 9.1. Continuous subcutaneous hydrocortisone infusion pump study protocol.
*Questionnaires – Short form 36 health survey (SF-36), Fatigue Questionnaire, General Health Questionnaire, Gastrointestinal Symptom Rating Scale
§Participants were reviewed during week 2 and week 4 of each treatment
#Salivary cortisol profile – salivary cortisol was collected at 0800h, 0830h, 0900h, 1200h, 1230h, 1300h, 1600h and 2100h

Each participant was assigned to treatment 1 and treatment 2 in random order (computer-generated randomisation, performed by the Royal Adelaide Hospital Clinical Trials pharmacist); there was a 2 week interval between treatments 1 and 2. The treatments were:

(i) oral placebo and 24 hour CSHI for one month

(ii) oral hydrocortisone and 24 hour subcutaneous saline infusion for one month.

Hydrocortisone sodium succinate (Solu-Cortef; Pfizer Australia Pty Ltd, North Ryde, NSW, Australia) was diluted in sterile water for injection, to a concentration of 50mg/ml. The placebo infusion was normal saline. The infusions were delivered by a MiniMed insulin pump (model 712; Medtronic Australasia Pty Ltd, North Ryde, NSW), which was loaned to us, by Medtronic, for the duration of the study. The oral placebo was a lactose tablet. The oral placebo and hydrocortisone were encapsulated, so as to be indistinguishable to the participants and study investigators.

Participants underwent a one month run-in phase during which glucocorticoid replacement was changed to a thrice daily oral hydrocortisone dose comparable with (within 10% of) their usual total daily dose of oral glucocorticoid and the calculated subcutaneous dose. A run-in phase was necessary because we wanted: (1) to enable participants to adjust to thrice daily dosing; (2) to prevent any changes to quality of life being confounded due to a change from regular (once or twice-daily) dosing immediately before commencing active study treatments; and (3) to compare CSHI therapy with the current best practice oral intermittent hydrocortisone dosing (thrice daily).

Participants had one education session with a diabetes nurse educator, certified in insulin pump training, to be instructed in the use of the infusion pump and a follow-up session with me that same week. They were provided with written instructions in the use of the pump. Throughout the study, they were able to contact me at any time with any queries or problems, and were reviewed clinically during week 2 of the run-in phase, and weeks 2 and 4 of each treatment (specifically for postural blood pressure measurement, weight, infusion-related complications, e.g., infected cannula sites). The CSHI dosing protocol was based on the dose-response evaluation studies which we performed in a single patient with Addison's disease (discussed later), each participant's pre-study hydrocortisone total daily dose, as well as a modification of previously published CSHI basal rates (Løvås and Husebye, 2007). For

participants who were on a glucocorticoid other than hydrocortisone before the study, the dosing protocol was based on published glucocorticoid equivalency doses (e.g., 5mg oral prednisolone ≈ 20mg oral hydrocortisone) (Stewart, 2003).

In addition to the basal infusion, participants self-administered a hydrocortisone bolus on waking, with lunch and with the experience of a daily life "psychic" stress. To prevent excessive hydrocortisone administration, we advised a maximum of two "stress" boluses per day. For the purposes of the study, we defined "daily life psychic stress" as: "An episode of increased emotional tension, often associated with symptoms such as rapid heart beat, sweating, tummy rumbling. The episode must last longer than two minutes and often follows a socially challenging situation".

Dosing

Overall, we aimed to administer equivalent doses, as far as practically possible, of hydrocortisone during treatments 1 and 2, and wanted the dose to be within 10% of the participant's usual total daily dose of hydrocortisone. We performed dose-response evaluations of bolus doses and basal infusions in a single patient (described below), to assist in determining the distribution of hydrocortisone throughout the 24 hour period. However, for each participant, dosing was individualized and calculated as $mg/m^2/h$. Approximately 30-40% of the total daily dose of hydrocortisone was given in bolus form; the remainder of the total daily dose comprised the basal dose.

Subcutaneous hydrocortisone bolus dose-response evaluation

We performed a subcutaneous hydrocortisone bolus dose-response evaluation in a patient (male, aged 48) with Addison's disease to profile the serum and salivary cortisol increments following a bolus. His maintenance glucocorticoid replacement was hydrocortisone 10mg 0800h and 4mg at 1200h and 1600h. On the day prior to each evaluation, the afternoon

(1600h) dose was replaced with 0.25mg dexamethasone. This was to ensure that he had circulating glucocorticoid during the dose-response study, but which would not interfere with salivary and serum cortisol measurements. He presented for 5 study evaluations, each separated by at least 1 week.

Evaluation 1

Bolus doses: 0.09mg, 0.18mg, 0.36mg and 0.72mg hydrocortisone administered subcutaneously and consecutively, with two hours between each bolus.

Monitoring: Blood pressure and heart rate were measured at 60 minutes after administration of the bolus.

Hormone measurements: Salivary cortisol, serum cortisol and plasma ACTH, sampled at 0, 20, 40, 60 and 120 minutes after bolus. The method for salivary cortisol collection (Chapter 8) and the details of the assays used to measure salivary and serum cortisol and plasma ACTH (Chapter 2) were as previously described.

The bolus doses were selected based on the molecular weight of cortisol (362g/mol), an assumed circulating blood volume of 5L, aiming to reproduce the documented average cortisol increases in response to waking, lunch and a psychic stress, discussed in Chapter 1 (Pruessner *et al.,* 1997; Quigley and Yen, 1979; Kirschbaum *et al.,* 1996).

Evaluation 2

The bolus doses in the previous evaluation did not achieve adequate serum or salivary cortisol levels, necessitating higher bolus dose-response evaluations. The same participant returned and 2.16mg and 2.88mg hydrocortisone were administered subcutaneously with monitoring as for evaluation 1.

Subcutaneous hydrocortisone basal-bolus dose-response evaluation

The purpose of these evaluations was to determine the optimal continuous (basal) infusion rates to mimic the nocturnal cortisol rise that occurs between 0200-0800h, and which is followed by an additional surge in cortisol on awakening (the bolus dose).

Evaluation 3

Hydrocortisone was infused subcutaneously at a rate of 0.6mg/m^2/h for 6 hours and a 3mg bolus was administered at the end of the 6 hour infusion, with a simultaneous reduction in the basal infusion rate to 0.2mg/m^2/h. Salivary cortisol and serum ACTH and cortisol were sampled hourly during the infusion, and at 0, 20, 40, 60 and 120 minutes after the bolus. Blood pressure and heart rate were recorded at two-hourly intervals.

Evaluation 4

Hydrocortisone was infused subcutaneously at a rate of 0.8mg/m^2/h for 6 hours; a 3mg bolus was administered at the end of the infusion, together with a reduction in the basal infusion rate to 0.2mg/m^2/h. Patient monitoring and blood and saliva sampling were as for evaluation 3.

Evaluation 5

Hydrocortisone was infused subcutaneously at a rate of 1mg/m^2/h for 6 hours; a 5mg bolus was administered at the end of the infusion, together with a reduction in the basal infusion rate to 0.2mg/m^2/h. Patient monitoring and blood and saliva sampling were as for evaluation 3.

Biochemical evaluation

Biochemical evaluation using salivary cortisol day profiles (0800h, 0830h, 0900h, 1200h, 1230h, 1300h, 1600h and 2100h) and a 24 hour urinary free cortisol were performed during weeks 2 and 4 of each treatment phase (Figure 9.1). The salivary cortisol day profile was used as an objective measure of differences in endogenous cortisol between the two treatments.

The urinary free cortisol was performed as a biochemical safety measure, to ensure that no-one was receiving excessive or inadequate doses of hydrocortisone.

Assessment of subjective health status

Participants were asked to record all boluses administered, to describe the experience that preceded a "stress" bolus and the perceived efficacy of the bolus on their experience of stress. Subjective health status was assessed using Short Form 36 Health Survey (SF-36), General Health Questionnaire, Fatigue Scale and Gastrointestinal Symptom Rating Scale (Appendix 8), completed at the time points shown in Figure 9.1.

The SF-36 questionnaire is the most widely used generic instrument to assess quality of life (Ware, Jr and Sherbourne, 1992). There are eight multi-item domains representing physical functioning, role functioning physical, bodily pain, general health perception, vitality, social functioning, role functioning emotional and mental health. The domain scores range from 0 to 100; higher values indicate better quality of life (Nemeth, 2006; Garratt *et al.,* 2002).

The General Health Questionnaire is a 28-item survey which screens for psychiatric disorder (Goldberg, 1972). It evaluates for severe depression, anxiety/stress and somatic symptoms (Huppert *et al.,* 1989). The Fatigue Scale is an 11-item questionnaire developed to measure the severity of fatigue (Chalder *et al.,* 1993). Gastrointestinal symptoms were evaluated using the Gastrointestinal Symptom Rating Scale (Svedlund *et al.,* 1988). At the end of the study each participant was asked in a free-response questionnaire (Appendix 8), whether they had a preference for either treatment, and if so, why.

Safety considerations

In the event of an intercurrent illness, participants suspended all study drugs, increased their glucocorticoid dose and sought medical assistance in accordance with their usual practice, and were asked to notify us.

9.3 Results and Discussion

Subcutaneous hydrocortisone bolus and basal-bolus infusion dose-response evaluations

The 0.09mg, 0.18mg, 0.36mg and 0.72mg boluses were administered consecutively on the same day; each bolus succeeded the previous by two hours. Administration of 0.09mg or 0.18mg hydrocortisone did not produce a detectable rise in serum or salivary cortisol (both < 8nmol/L two hours after bolus administration – data not shown).

The serum cortisol profiles for the 0.36mg and 0.72mg boluses are shown in figure 9.2. These boluses resulted in a serum cortisol increment of ≈ 20nmol/L (0.36mg) and 40nmol/L (0.72mg). The basal serum cortisol prior to the 0.72mg bolus was 11nmol/L (this bolus had been preceded two hours earlier by the 0.36mg bolus), and the peak cortisol measured was 41nmol/L (Figure 9.2). Although this suggests a 30nmol/L increment, the serum cortisol due to the previous (0.36mg) bolus would have been falling, so we estimate a 40nmol/L increment resulting from the 0.72mg bolus. These bolus doses did not achieve sufficient cortisol increments to reproduce the pulses associated with waking (9nmol/L increase in salivary cortisol), stress (6nmol/L increase in salivary cortisol) or a mid-day meal (200nmol/L increase in serum cortisol) (Pruessner *et al.,* 1997; Kirschbaum *et al.,* 1996; Quigley and Yen, 1979).

Based on these data, we selected 2.16mg and 2.88mg as the bolus doses for further evaluation. The serum cortisol increments achieved with these doses are shown in figure 9.2. The serum cortisol increment after the 2.16mg bolus was ≈ 100nmol/L (< 8 to 102nmol/L at 40 mins) and after the 2.88mg bolus was also ≈ 100nmol/L (64 to 167nmol/L at 40 mins). Again, however,

if we consider that the baseline serum cortisol for this bolus (64nmol/L) was a consequence of the 2.16mg bolus administered two hours prior, and that serum cortisol due to this bolus would be falling, we estimate that the actual serum cortisol increment would be ≈ 150nmol/L.

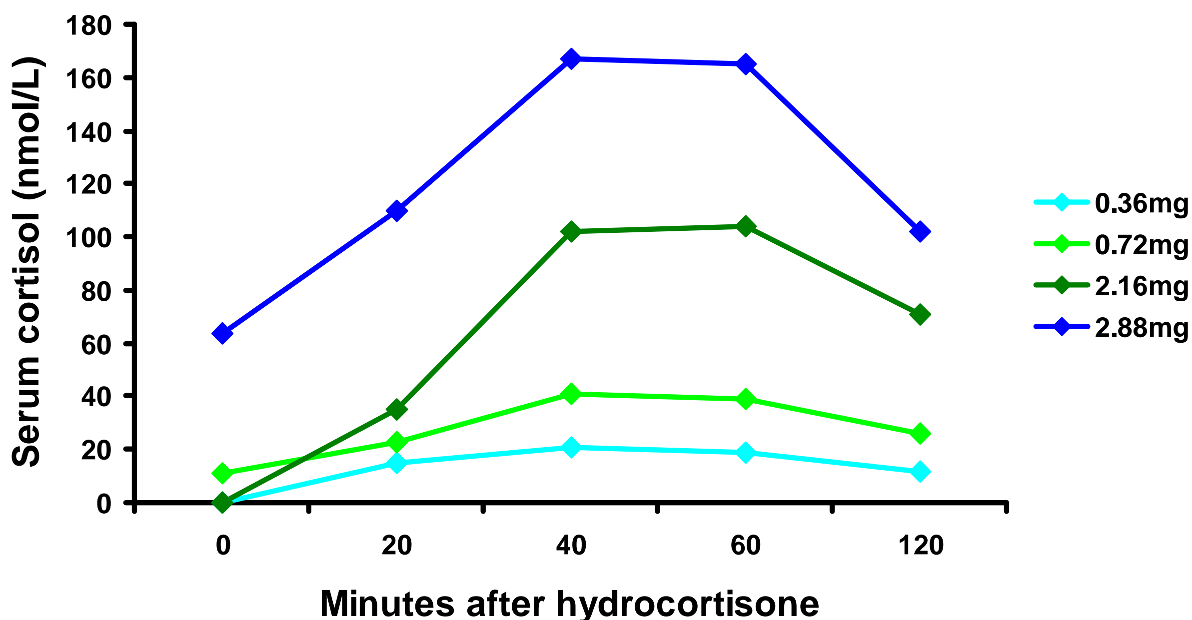


Figure 9.2. The serum cortisol profiles after bolus subcutaneous hydrocortisone in a single patient with Addison's disease. Hydrocortisone was administered subcutaneously using a Medtronic MiniMed 712 pump, as a single bolus. The doses 0.36mg and 0.72mg were evaluated on a single day, and 2.16mg and 2.88mg were evaluated on another day.

The peak measured serum and salivary cortisol occurred forty minutes after the subcutaneous bolus, which mimics the timing of the cortisol peak in response to waking, a mid-day meal or a physical or psychological stress (Pruessner *et al.,* 1997; Quigley and Yen, 1979; Kirschbaum *et al.,* 1996). The baseline serum cortisol for 0.72mg and 2.88mg was not zero, since these boluses were preceded by the 0.36mg and 2.16mg boluses, respectively.

The basal-bolus subcutaneous hydrocortisone dose-response data are shown in Figure 9.3. Continuous subcutaneous hydrocortisone was infused at a constant rate (0.6, 0.8 or 1mg/m^2/h) for 6 hours (between 0900h-1500h), a bolus was administered at 1500h and the infusion rate

was simultaneously reduced to 0.2mg/m^2/h. The serum cortisol level appears to plateau after ≈ 4 hours (1300h) of either 0.6mg/m^2/h or 0.8mg/m^2/h continuous infusion; this is less evident for the 1mg/m^2/h continuous infusion (Figure 9.3). The data also demonstrate that the cortisol increment following a bolus dose is reproducible (3mg bolus).

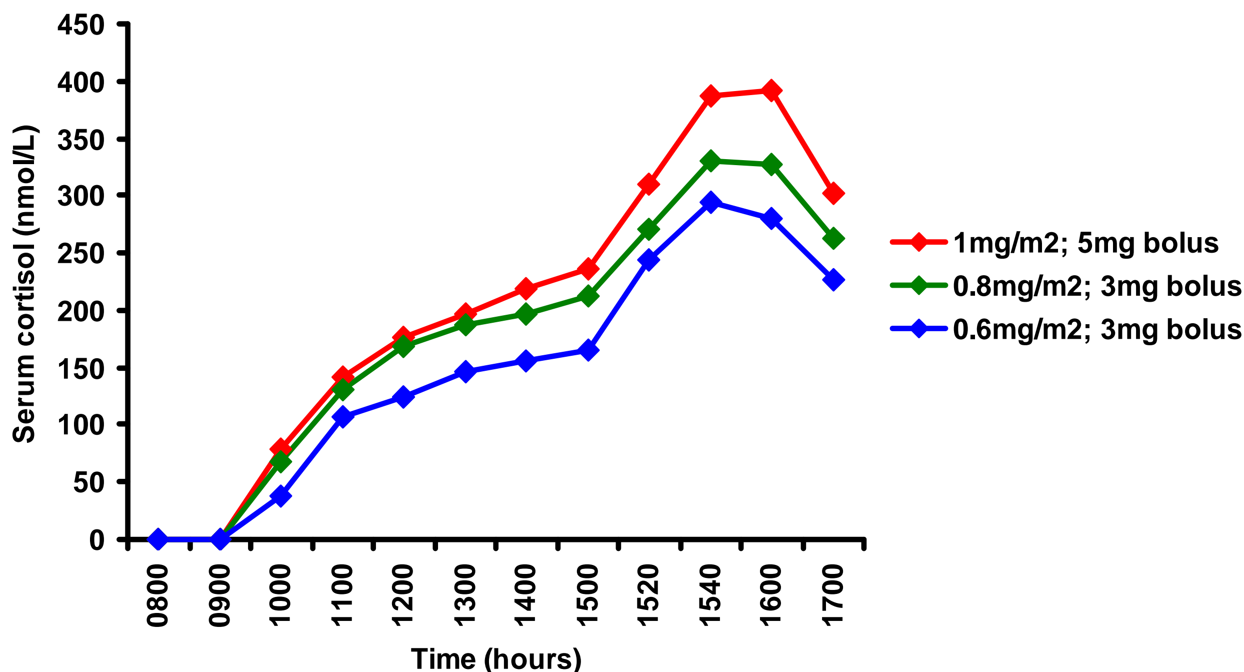


Figure 9.3. The serum cortisol profiles after basal-bolus subcutaneous hydrocortisone infusion in a single patient with Addison's disease. These data were obtained on three separate occasions. Hydrocortisone was infused subcutaneously and continuously from 0900h until 1500h at the specified rate, a bolus dose was administered at 1500h and the infusion rate reduced to 0.2mg/m^2 and continued until 1700h.

Salivary cortisol is a measure of free (unbound) serum cortisol which accounts for about 5% of total circulating cortisol. The parotid gland tissue harbours 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which metabolises cortisol to cortisone (Smith *et al.,* 1996). Thus salivary cortisol concentrations are determined not only by unbound serum cortisol, but also by parotid gland metabolism of cortisol to cortisone. In contrast, salivary cortisone is largely derived from enzymatic conversion of plasma cortisol by the salivary glands, although passive diffusion of circulating cortisone also contributes (Vining and McGinley, 1987). Salivary sampling for cortisol day profiles is a convenient and non-invasive means of

measuring cortisol, compared with serum cortisol profiles. Thus, we are using salivary cortisol as a surrogate for circulating free plasma cortisol.

A recent study has provided preliminary data suggesting that salivary cortisone may be a better biomarker of free cortisol in certain clinical situations (Perogamvros *et al.,* 2010). In this study, salivary cortisone was evaluated during ACTH stimulation testing in healthy controls (*n*=14) and in women receiving oestrogen therapy (either the oral contraceptive pill or hormone replacement therapy) (*n*=12) (Perogamvros *et al.,* 2010). Women receiving oestrogen therapy were evaluated twice – once whilst taking oestrogen and again six weeks after its cessation (Perogamvros *et al.,* 2010). Salivary cortisone was also evaluated during acute hydrocortisone administration (oral and intravenous) in a subgroup of these patients and three individuals with nonfunctional genetic corticosteroid-binding globulin variants (Perogamvros *et al.,* 2010). Overall, salivary cortisone was most highly correlated with, including more so than salivary cortisol, free serum cortisol in the scenarios studied, especially during the oral hydrocortisone study, where measurement of salivary cortisol was frequently contaminated by the medication (Perogamvros *et al.,* 2010). We are not expecting to encounter the problem of salivary cortisol contamination by oral medication in our study because the tablets are encapsulated.

Salivary cortisone has not yet been evaluated in patients with states of cortisol excess or deficiency; this certainly needs to be performed before any claim can be made that evaluation of a cortisol metabolite is superior to measurement of salivary cortisol (Raff and Findling, 2010). Whether measurement of salivary cortisone has utility beyond that provided by measurement of salivary cortisol in our study is not known either. However, clinical samples from our study participants have been stored, and could be retrieved for analysis, if thought necessary, at a later date.

Study progress

At the time of writing this thesis, four participants (all female; age range: 37-56 years, mean age 46.3 years) had completed the study. The mean duration of Addison's disease was 8.75 years (range 5-12 years). Other hormone replacement therapy was: thyroxine (*n*=3), DHEA (mean dose, 25mg daily; *n*=4) and oestrogen for menopausal symptoms (*n*=1). These therapies were continued unchanged throughout the study.

To date, there has been minimal use of the "stress" bolus; most participants did not administer a stress bolus at all during either treatment – citing "there was never a need". This is interesting, since other patients with Addison's disease have reported an altered somatic response to stress; staying calm in stressful situations with a paradoxical decrease in heart rate (Løvås *et al.,* 2010). There have been no infusion-related complications.

Recruitment has been difficult due to the rarity of this disease, high withdrawal rates from the study, study ineligibility and non-consent (Figure 9.4). Five people withdrew from the study: one person did not tolerate the change from dexamethasone to hydrocortisone (he developed minimal peripheral oedema), one person developed an intercurrent problem which we did not believe would be resolved in the short-term, and three participants withdrew after providing informed consent, but prior to study commencement. The participant who did not tolerate the change from dexamethasone to hydrocortisone has resulted in us exercising caution in enrolling participants who have been on long-term dexamethasone. However, since study withdrawal, he has changed his maintenance glucocorticoid replacement to hydrocortisone, using the same dosing as in our clinical trial. At the time of writing this thesis, he has been on thrice daily hydrocortisone for two years, and this has been well-tolerated.

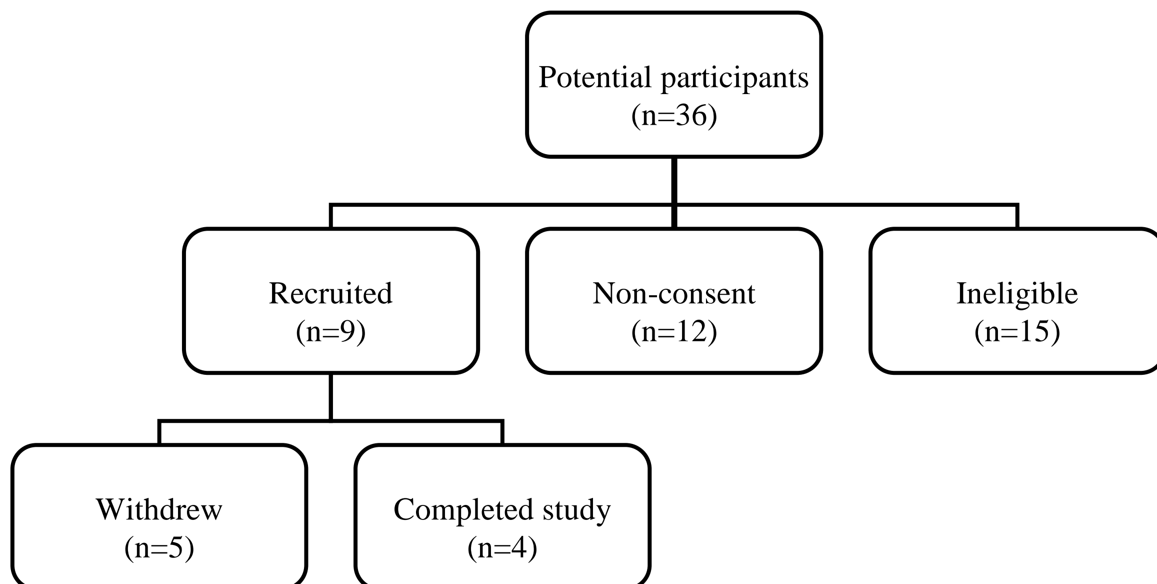


Figure 9.4. Participant recruitment for the Continuous Subcutaneous Hydrocortisone Infusion study. Further details are provided in the text.

Twelve people declined to participate, citing concerns regarding the need for cannula insertion, study duration, impingement of the study on lifestyle, personal high-level stress and the need to maintain a fixed hydrocortisone dose for the study duration, as reasons. One person expressed concern that the infusion pump would draw attention to their disease. Fifteen people were ineligible: coeliac disease, type 1 diabetes mellitus, unstable type 2 diabetes mellitus, shift-work, living outside of metropolitan Adelaide, post-surgical hypocortisolism, diagnosis other than Addison's disease, significant residual adrenal function, long-term dexamethasone treatment, inability to attend review appointments and multiple comorbidities, were amongst the reasons for ineligibility.

We believe that this study will answer an important question – whether circadian (and ultradian) hydrocortisone replacement improves well-being and subjective health status in Addison's disease. This question should be answered before further drug developments and other efforts to provide circadian glucocorticoid replacement are made; since if no objective evidence exists, then such attempts to improve well-being in Addison's disease may be in vain. Until recently, we had believed that recruitment possibilities in South Australia had

been exhausted; however three other patients have expressed an interest in participating. To further facilitate recruitment and study completion, we have an application underway to enable us to recruit from another (interstate) centre.

Attempts to improve quality of life and impaired well-being in patients with Addison's disease often culminate in an increase in the total daily glucocorticoid dose and/or frequency of dosing. Recent data suggest that neither higher total daily doses, nor increased dosing frequency (thrice *vs* twice daily) are associated with better health status; rather, both are associated with worse subjective health status – although cause and effect from this uncontrolled, observational study could not be ascertained (Bleicken *et al.,* 2010). Thus the data suggest that despite optimising oral, intermittent glucocorticoid therapy, significant deficits in well-being persist. Furthermore, in patients with secondary adrenal insufficiency higher total daily glucocorticoid doses (above 20mg hydrocortisone) are shown to be associated with an adverse metabolic profile (higher body mass index, total cholesterol, low density lipoproteins and triglycerides, and glucose intolerance), lower bone mineral density and bone loss (decreased osteocalcin) and elevated intraocular pressure (McConnell *et al.,* 2001; Filipsson *et al.,* 2006; Wichers *et al.,* 1999; Zelissen *et al.,* 1994; Li Voon Chong *et al.,* 2001). Although some patients with secondary adrenal insufficiency may have partially preserved adrenal function, and thus may be more easily overtreated; overtreatment in Addison's disease is possible and similar consequences on metabolic and bone health could be expected.

A recent study reported no benefit of a single, nocturnal, eight hour intravenous hydrocortisone infusion compared with oral glucocorticoid replacement on well-being or cognition in patients with adrenal insufficiency (Harbeck *et al.,* 2009). However due to the patient cohort studied and the study protocol itself these data can not be confidently extrapolated to Addison's disease. The patient cohort comprised an assortment of patients

with secondary adrenal insufficiency due to craniopharyngioma (*n*=2), pituitary adenoma (*n*=6) or Sheehan's syndrome (*n*=1), and primary adrenal insufficiency due to Addison's disease (*n*=3) or bilateral adrenalectomy for Cushing's disease (*n*=2). This could confound the data because: (1) persistent cognitive deficits and impaired health status after Cushing's disease have been described; (2) impaired health status has been reported by patients treated for craniopharyngioma; and (3) other untreated pituitary hormone deficiencies (e.g., growth hormone, sex hormones) could have adversely affected cognition and/or well-being (Tiemensma *et al.,* 2010; Heald *et al.,* 2004; Dekker *et al.,* 2006).

The study protocol was as follows: (1) subjects were admitted to hospital for an overnight eight hour intravenous infusion, followed by their usual oral glucocorticoid, breakfast and evaluation or (2) slept at home, arrived at hospital in the morning, given their usual oral glucocorticoid, breakfast and were evaluated. The concerns we have with the protocol are: (1) it is unlikely that a single, brief overnight infusion could produce detectable differences in health status; (2) the subjects' slept in hospital (unfamiliar environment) for the infusion component but not the oral glucocorticoid only component; these different environments could have adversely affected their performance during cognitive testing; and (3) the evaluation was not performed during circadian infusion; immediately prior to evaluation, the subjects were given their usual oral glucocorticoid (Harbeck *et al.,* 2009). Thus, we believe that the benefit of CSHI therapy on well-being in Addison's disease remains to be determined.

An alternative hypothesis is that impaired subjective health status in patients with Addison's disease is due to hypoglycaemia. The fatigue, faintness and deficits in concentration and in memory also occur in healthy individuals during hypoglycaemia-induced neuroglycopaenia, suggesting that cerebral glucose deficits may contribute to these symptoms in patients with Addison's disease (Mitrakou *et al.,* 1993). Patients with Addison's disease have an increased risk of developing hypoglycaemia, possibly as a result of increased glucose oxidation and

decreased endogenous glucose production due to hypocortisolism, leading to improved insulin sensitivity (Christiansen *et al.,* 2007). Thus, it has been hypothesized that hypoglycaemic episodes accompanied by insufficient cerebral glucose, may induce neuroglycopaenic symptoms (fatigue, faintness and cognitive deficits) in Addison's disease.

The precedent for this hypothesis is based on data from animal studies. In adrenalectomised rats, free-choice ingestion of sucrose diminished the adverse metabolic effects (e.g., poor weight gain, less white adipose tissue) of cortisol deficiency (Bell *et al.,* 2000). When healthy rats had access to "comfort food" (palatable energy-dense food with high fat and glucose content), they displayed dampened ACTH and corticosterone (their principal circulating glucocorticoid) responses to a chronic stressor (restraint) (Pecoraro *et al.,* 2004).

The hypothesis that impaired health status in patients with Addison's disease is due to hypoglycaemia was evaluated recently in a controlled trial (Klement *et al.,* 2010). In this study, age- and BMI-matched controls (*n*=10) and patients with Addison's disease (*n*=10) were evaluated for mood, short-term memory and attention at baseline and following a free-choice high-calorie buffet ("comfort" food) and a low-calorie green salad ("control" meal) on two separate occasions (Klement *et al.,* 2010). The patient group acutely discontinued glucocorticoid replacement for the study. The patient group reported feeling more tired, unwell, restless and less vivid, and content than the control group, regardless of the treatment intervention ("comfort" or "control" food) (Klement *et al.,* 2010). At baseline, the patient group had higher neuroglycopaenic symptom scores (due to difficulty in thinking and faintness) compared with the control group; these symptoms were attenuated by "comfort" food (Klement *et al.,* 2010). There was also a trend to improved attention and short-term memory in the patient group after "comfort" food (Klement *et al.,* 2010).

It was postulated that the improvements in cognition were due to an increase in glucose supply to the brain, after the high-calorie meal, rather than an effect on the hypothalamic-pituitary-adrenal axis, since ACTH and cortisol concentrations were not influenced by the type of meal in either group (Klement *et al.,* 2010). Also, since such improvements did not occur with intravenous glucose infusion, it has been postulated that neuroendocrine signals from the gastrointestinal tract (glucose receptors, vagal afferents, gut peptides) and psychological aspects of ingestion (free choice) may be important (Klement *et al.,* 2009). These studies require replication, to determine whether this is a disease-specific effect.

9.4 Conclusion and Future Studies

At the time of writing this thesis, the effect of CSHI therapy on subjective health status in Addison's disease is not known. Recruitment has been difficult, although we have had some recent interest locally, and we expect that expanding the study nationally, will also assist study completion. Currently, we plan to recruit from one other centre.

In the first instance, the effect of CSHI therapy on subjective health status needs to be determined in a randomised, double-blind, placebo-controlled clinical trial, which is currently underway. If there is no proven benefit, then other explanations for impaired well-being, such as hypoglycaemia, deserve further study. Certainly this question must be answered before this mode of hydrocortisone replacement can be advocated as therapy, since it is not without risk, some inconvenience and potential morbidity (cannula insertion, infection), and there exists only anecdotal evidence of benefit of its use in Addison's disease (Løvås and Husebye, 2007).

Another group is also evaluating CSHI therapy in Addison's disease (ClinicalTrials.gov identifier: NCT01063569; http://clinicaltrials.gov/ct2/show/NCT01063569). This group is attempting to recruit 40 patients over two years. The primary outcome of that study is plasma ACTH; secondary outcomes are health-related quality of life, sleep quality, 24 hour serum and

salivary cortisol profiles, 24 hour glucose profile and insulin sensitivity (euglycaemic clamp). To our knowledge, the protocol does not include bolus doses of hydrocortisone. This study will, however, provide important data that we have not collected – specifically relating to the metabolic (e.g., insulin sensitivity) consequences of circadian cortisol replacement.

An Addison's disease-specific quality of life (AddiQoL) questionnaire has recently been formulated and validated in a small, selected cohort of patients with Addison's disease; although population (unselected patients and controls) and cross-cultural validations have not yet been performed (Løvås *et al.,* 2010). Future studies of well-being might therefore include this as an additional assessment of health status in Addison's disease. Further studies are needed to clarify the clinical utility of salivary cortisone, if any, beyond that provided by salivary cortisol in the diagnosis of states of glucocorticoid excess, deficiency and in monitoring glucocorticoid replacement therapy.

In the event that CSHI therapy does not improve subjective health status, there may still be a role for CSHI therapy in Addison's disease or in other patient groups. For example, an overall dose reduction could be achieved in patients with Addison's disease, thus CSHI therapy may assist those patients with hydrocortisone-associated weight gain (Løvås and Husebye, 2007). There are anecdotal data of improved biochemical (androgen) control in a patient with congenital adrenal hyperplasia; this is another patient group who could benefit from CSHI therapy (Bryan *et al.,* 2009). Further to this, a recent survey of adult patients (*n*=203) with congenital adrenal hyperplasia found significant impairments in subjective health status, inadequate control of androgen levels and metabolic abnormalities consistent with excessive glucocorticoid dosing (obesity, hypercholesterolaemia, insulin resistance and osteopaenia and osteoporosis) (Arlt *et al.,* 2010). The clinical utility of CSHI therapy in patients with secondary adrenal insufficiency has not been evaluated; presumably because concomitant

endocrine deficiencies – growth hormone, gonadotropins – could confound the data. However, this is another cohort in whom CSHI therapy might ultimately be of benefit.

The availability of glucocorticoid replacement therapy in the 1950s revolutionized the management of adrenal insufficiency; data from studies currently in progress should help to clarify whether there is anything more to be gained, in terms of a reduction in some of the morbidity of Addison's disease, from "fine-tuning" glucocorticoid replacement therapy.

Chapter 10: Final Discussion

The studies presented in this thesis have encompassed an area of hypothalamic-pituitary-adrenal (HPA) axis regulation, specifically as it relates to the effects of vasopressin (VP), a less well studied influence at both central and peripheral sites in the axis. The studies have involved a rare disease, ACTH-independent macronodular adrenal hyperplasia (AIMAH), established adrenal incidentalomas and a search for new incidentalomas in cases thought to have an increased incidence of adrenal tumours (metabolic syndrome), and finally, a study of the relevance of HPA axis regulation to overall well-being utilising patients with Addison's disease as a model.

These studies have led to several novel findings and provided rich opportunities for more study using the materials obtained. The new knowledge has generated additional questions requiring exploration in the future. A summary of the intersecting studies performed and their motivating questions, outcomes and suggested further lines of investigation is shown in Table 10.1.

We studied families with vasopressin-sensitive AIMAH (VPs-AIMAH) with the goal of determining the presence of pre-clinical manifestations, determining a chromosomal locus that may harbour a new gene causing VPs-AIMAH, and then sequencing the exomes of affected individuals to find the gene/mutation underlying AIMAH. In addition, we specifically studied VP receptor expression by the AIMAH tumours. Finally, we studied genome-wide gene expression in the tumours to help understand the abnormal steroidogenesis, and to identify other genes/pathways involved, in AIMAH.

Table 10.1. Hypothalamic-pituitary-adrenal axis regulation, relation to vasopressin and relevance to well-being in humans.

Area of study	Questions	Outcomes	Further knowledge required
Familial VP-sensitive AIMAH	Are mild clinical forms present?	Milder forms detected in families, facilitating earlier diagnosis	Application of methods to non-VP-sensitive families
Familial VPs-AIMAH	What is the genetic basis (or its chromosomal locus?	Possible linkage in 14q32.11-32.12	Full sequencing of genes in AIMAH-01 locus
Familial VPs-AIMAH	What is the genetic basis?	Not yet determined by positional candidate or exome sequencing approach	Further sequencing of non-synonymous variants
Familial VPs-AIMAH	What is the mechanism of VP sensitivity?	Eutopic overexpression of AVPR1A and ectopic expression of AVPR1B	Application to other VP-sensitive adrenal tumours
Familial VPs-AIMAH	Which genes are differentially expressed? What pathways are involved in AIMAH? Why is steroidogenesis inefficient?	Novel mechanisms for abnormal adrenal steroidogenesis; NB: incomplete correlation with *in vivo* adrenal stimulation studies	Application to other AIMAH tumours
Adrenal incidentaloma VP-sensitivity study	What is the frequency of VP-sensitivity, and is this correlated with SCS?	VP-sensitivity may predict cortisol secretory autonomy	Further study expansion required
Screening for SCS	What is the rate of VP-sensitive incidentalomas causing SCS in a target population?	Negligible rate of SCS in target population; Incidental finding of low false positive rate of nocturnal salivary cortisol testing	Nil
HPA axis regulation and well-being	What is the role of circadian/ultradian rhythms of cortisol secretion in well-being (Addison's disease as a model)?	Ongoing study	Ongoing study

We found that milder forms of VPs-AIMAH can be detected with the prospect of earlier diagnosis. Linkage studies yielded a single locus of interest in one family. Studies of exomes have led to a large number of potential causative genes. Both study of the relevant locus and genes with non-synonymous variants is needed. Targeted expression studies of VP receptor genes have revealed eutopic overexpression of the type 1a (AVPR1A) and ectopic overexpression of the type 1b (AVPR1B) receptors. Gene expression studies have suggested novel mechanisms for the hallmark inefficient steroidogenesis and tumour formation of AIMAH. We noted some concordance between differentially expressed genes in AIMAH and in adrenocortical carcinoma. Due to the apparently benign nature of AIMAH, the common differentially expressed genes in these two opposing forms of adrenal tumour are unlikely to be key players in the highly malignant and aggressive behaviour of adrenocortical carcinoma.

At the outset, we wished to put ourselves in a position to generalise our findings to more common adrenal tumours, hence we studied the VP-sensitivity of adrenal incidentalomas. Our studies suggest that a response we have defined as aberrant (increase in cortisol, suppression of ACTH) may predict VP-sensitive adrenal tumour cortisol secretory autonomy, although this requires expansion in terms of patient numbers. We sought to ascertain new adrenal incidentalomas, including VP-sensitive forms, by testing a population thought to be at high risk for subclinical Cushing's syndrome (SCS). However, the rates of SCS in the patient population selected were low, a finding reported by us and now also by others. We incidentally found that the nocturnal salivary cortisol test appears to have a low false positive rate in this setting.

Finally, although VP has a physiological role as an ACTH secretagogue and also may be involved in the regulation of cortisol secretion by adrenal tumours, we asked the question of how such precise, multi-regulated HPA axis function related to human well-being. To address this we studied the most important HPA axis product, cortisol, in patients with

endogenous cortisol deficiency, Addison's disease. Our studies have determined that we can produce circadian, and for the first time, ultradian, variations in cortisol levels, by continous subcutaneous hydrocortisone infusion using a proprietary insulin pump. This study is ongoing but will perhaps place HPA axis regulation, whether by VP or other factors that we know are operative, in a new context.

Appendix 1: Laboratory Methods

Table A.1.1 Primers used for RT-qPCR

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size	UPL Probe #
ACTG2	atgggcaggttatcaccatt	gaattccagcggactccat	93	11
AGPAT1	gaaggagcgtcgcttcac	gtgccggactctgtcagc	95	33
AVPR1A	ttgtgatcgtgacggcttac	gatggtagggttttccgattc	110	3
AVPR1B	acaagaatgcccctgatgaa	ggttgcccaaaagcatagag	66	31
AVPR2	ctcatggcgtccaccact	ccacaaagactatggagagcag	145	71
CYP11A1	acatcaaggccaacgtcac	gggtcatggacgtcgtgt	60	51
CYP11B1	gccaggctaagcagcaat	ctggggacaaggtcagca	61	60
CYP11B2	gtgaccgcaggttgcttt	cccttattcctttcccatgc	61	61
CYP17A1	ctatgctcatcccccacaag	ccttgtccacagcaaactca	63	67
CYP21A2	agatgcagcctttccaagtg	ggttcgtacgggagcaataa	132	21
HAS2	accaaatgaaatgccaaagg	tggcatacaaaatttaaaaacacagt	90	76
HIST1H3I	ccaaacgcgtcactattatgc	agacttcttgggctgataggaa	93	42
HPRT1	tgaccttgatttattttgcatacc	caggcaagacgttcagtcct	68	73
HSD3B2	cacctggcttcatacagaagg	tgggcattgtgtgaaagaga	72	18
MMP16	agggcatccagaagatatatgg	ggcactgtcggtagaggtctt	70	39
MYH11	cccaacaggaaacacagaca	aaactcagtgtctgcacaatcc	62	65
NR5A1	ggggacagatttgaggactg	ggagaaaccgtcagctgtactt	67	45
PSMB2	agagggcagtggaactcctt	aggttggcagattcaggatg	71	25
SCN7A	cacatatctgaaaaatgttgaaagc	ccatcggttgtaaagaactgattat	81	72
SKAP1	tgatcatcaaaagttcccactc	gcaaagagaggagtccaagg	66	29
SP1	agggctgcaagtagtgagga	aggcaagatatgggaaagca	67	3
STAR	ggctaaaagtttagtattctgggaga	aagggaatttgctttcaccat	69	11
TDGF1	aaaatgggttacttgattggtga	acttgcccttccatttagcc	72	77
TFPI2	tgctacagtccaaaagatgagg	tcttggattaaaataatagcgagtca	66	53
TGFB2	aggaaggggtgaagtgctagt	caacgatcaaattgtactgctga	76	44
TSPAN8	aaaatgttgctttggctttgtaa	tggtctagctagccgagacatt	93	68

Table A.1.2. Primers used for sequencing of candidate genes in locus of possible linkage

	Forward primer (5'-3')	Reverse primer (5'-3')
Ataxin 3 (ATXN3)		
Exon 1	ggagctggagggggtggttc	gcagacagctccccaccgaa
Exon 2	gaagatactatacaattctg	aaaaagaaatgtgacttagt
Exon 3	ttggaaatgggaatgcacaagata	aacttccgaaggtcgccttgttac
Exon 4	tggtggcgtgtgcttgta	tgagtgaatgaaacagggaaca
Exon 5	ttgtggcacagcatcactaag	agacaggacctccctttgttg
Exon 6	gctttgatgggttgtatctattctta	ccaaatcacagcctatcacca
Exon 7	ttccagtgttctgtgctgccttt	ggtccaaaatagagtcgccaacaa
Exon 8	ttagttggattcaaattgcaacac	cccactatatagctattgcttctgc
Exon 9	atgtgtgagccaccacgc	tcacaggattcaggcagtaacc
Exon 10	atcagactaactgctcttgcattc	gtaactgctccttaatccaggg
Exon 11	ggcaatggaatggttgtgtatc	accaaagtggaccctatgctg
Inositol 1,3,4-triphosphate 5/6 kinase (ITPK1)		
Exon 2	cctgtgggcgatttcctc	cgtttggctccgatctcc
Exon 3	ctgaaaccctctcatggtcct	ctgcacgtgaagaagagagaaa
Exon 4	ttagcaggagagaagagttgtgg	tccagactatacctccagagagc
Exon 5	gctggaggataggacaggaac	ccctctgtctccacacctca
Exon 6	gttattgttcacacacacgagca	gagcttaaatccagagggcacta
Exon 7	ttcctttctagacgtgccaagta	tatctaccaaggagccaggacta
Exon 8	gctgagaaggtggtggattct	ctccatcccaccaagaacac
Exon 9	agtgtggtccctgcattgag	ctaccctgctggagagcttg
Exon 10	agtaggtctgtctggcttcctg	ctggtcacctgaggacagtct
Exon 11A	ctcctcctccctgcatgat	tagtagtagcatcgccgttgg
Exon 11B	ctgtgtctcatgcctgtcaataa	atacagaccccagggacattagt

Table A.1.2. (contd.) Primers used for sequencing of candidate genes in locus of possible linkage

	Forward primer (5'-3')	**Reverse primer (5'-3')**
Tyrosyl-DNA phosphodiesterase 1 (TDP1) Two sets of primers were needed for exon 3.		
	Forward primer (5'-3')	Reverse primer (5'-3')
Exon 3A	ggcagaatctgatagtgga	tgtctttctcctttttgatcacc
Exon 3B	cctcccaaaaggcagaaaag	acacgctctgcctgagttaagtc
Exon 4	tgagtcagatcttgattgtcagtg	cttgagtcaaatgctctcctgat
Exon 5	ctccttatgaactgtttggcaga	gagtggctttcacctgagaaata
Exon 6	tgagcctggccatgaatgat	gactgatgctggagagaggattt
Exon 7	tccagtagatatggatattagtgagttg	ccatgctcagtctctcctaagaat
Exon 8	ccaccaaatactcaacctttctg	aagtgaatgttctggcttttgac
Exon 9	ggagataccgagaattcacctct	aggattcactggctgtcacttta
Exon 10	ggattggctcttagatcatttcttg	tatttgcccagaattgtgcag
Exon 11	gttaattaggctttccaagagcag	ctctccattacaaagaggaccaat
Exon 12	ccttttcttcaacatggtacattc	aatgcagagcttcatattgcctat
Exon 13	cctgttaccttcttgctgtttattg	cagtgacataaataacactctaattcct
Exon 14	gataataggctctttgttttcatgc	ggacacataagagatttgattgctt
Exon 15	aatgcagcaactctgctagaaaat	caagctgttgtgactaaaggaaga
Exon 16	cttcttgaggccttctggtttt	aagggattccaagactaaccagt
Exon 17	cacataagtgtttttatgccatcag	gaaagattttgtaagggtgcaaata
G protein-coupled receptor 68 (GPR68) Three pairs of primers were needed to amplify this single exon.		
Exon 1-1	cttggtagagcttgaaccacctt	agctccttggcccagatgac
Exon 1-2	catcctcctgtacgagaacatct	agcaggagggagaagtggtag
Exon 1-3	ctaccacgtgttgctgctggt	agagcaggcttccggagttac

Table A.1.2. (contd.) Primers used for sequencing of candidate genes in locus of possible linkage

	Forward primer (5'-3')	**Reverse primer (5'-3')**
Calmodulin 1 (phosphorylase kinase, delta) (CALM1)		
Exon 1	cgtgccgttactcgtagtcag	tagaaatctgggaagaaatctcg
Exon 2	cctatgttgaaatcaaccaaaca	acatgtgggcatccttagtttta
Exon 3	attgtgactgggttggataaaga	ggacctgaaagacaatcctgtaa
Exon 4	tctgaaatgattactgcagagca	tgatacggttaccataaggagga
Exon 5	gggcatagctaagtcaaagacct	aaggctgtctagtgaagcagttg
Exon 6	actgcttcactagacagcctttg	tgtagcttgatctttaggggaca
Chromogranin A (CHGA) Two pairs of primers were needed for exons 6 and 7; Nested PCR was performed for exon 1		
Exon 1	tagcgaggggtgaggttaga	tcaggcccaagtgtcttgt
Exon 1 (nested)	ggaggatcgaccgacagac	ccacactgcagggtcagg
Exon 2	aacaaaatcagccctagcctct	cttggctttatgccgaacttatct
Exon 3	tcattgctatcactgtctggcta	ttcaactgtctgtacaccttgga
Exon 4	actgacacataccttggaaagga	ttccaccagattttctgactctc
Exon 5	ggaaatggaggtcttcactctta	agagagggaaataagggattatgg
Exon 6A	tggaagccaagaaacataatgag	cactcaggcccttctctctgt
Exon 6B	agagaaaagtggtgaagccacag	aaggtggaatgaggttatggaga
Exon 7A	agagactgggaaaatggtggtc	cctctttcttctcctcggggta
Exon 7B	cagttccatgaagctctccttc	atctctccatccccagacttc
Exon 8	agctcattatcactgtctccatga	tatgttacagtcaggagttctcagc

Table A.1.3. Primers for exon capture single nucleotide variation validation.

Gene name, Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
Breast cancer 2, early onset, BRCA2	tgttttcacttttagatatgatacgg	ttcagatatatttgcgctcaatg
Catalase, CAT	aaactggtgattcaattctctgc	caccccttcagcttacaaagactc
Endothelial PAS domain protein 1, EPAS1	attagactgccctcccatgc	gttgcgagggttgtagatgac
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian), ERBB2	ctgtctctgccttaggtgtgact	tgtagacatcaatggtgcagatg
Polycystic kidney and hepatic disease 1 (autosomal recessive), PKHD1	ccttccttatctgtctcctagcc	ctgttgagggactggaactgact
Versican, VCAN	cagaaacactaataccagagatgaga	aagacttggtcatttccacagaa
Kinectin 1 (kinesin receptor), KTN1	cattgctttctcaattgcagtc	ttgggaagtatatccctcgtaca
mutS homolog 6 (E. coli), MSH6	aaggtcgttttcctgatttgact	tgttgcgctgtttctctaggtat
RAN binding protein 17, RANBP17	cctacctcttcaagcacatagca	ccagctgctctaagataaattcg
Platelet-activating factor acetylhydrolase 1b, catalytic subunit 2 (30kDa), PAFAH1B2	tgtagaaagtgccaaatggaga	gtatccaggagctgcacgtt
Neuropilin 1, NRP1	ccacagcttacctgttatcttgc	aacctctgctaaagaaaggcact

Table A.1.4. Primers for exome capture variant validation.

Gene name, Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
Myosin, heavy chain 8, skeletal muscle, perinatal, MYH8	caacatgtcctgaactgtgtctc	aacatcttctcgtagacggcttt
Collagen, type VII, alpha 1, COL7A1	ctctgactcctgatccctgaac	gacctctgacctcagggacaac
IQ motif containing GTPase activating protein 1, IQGAP1	atcctctctcctttggtgcag	accttgtatctggtctgttctcg
RAS protein activator like 1 (GAP1 like), RASAL1	acacaatgcccagtacatggtag	cttaagcatacctggcctgaag
A kinase (PRKA) anchor protein 13, AKAP13	aatgctctacaaggtatggctga	tttctcccaatcaagaggtatca
Coiled-coil domain containing 88A, CCDC88A	ttagatgcacttcgagagaaagc	cccaaatgtatttactgtttgttca
Phosphodiesterase 11A (PDE11A)	gttttaatgttccattgcagagc	tcccacaactgctagtttttacc

Microarray Validation

Reverse transcription

Reverse transcription was performed as follows:
1µl 100µM oligoDT (Sigma)
1µl 10mM dNTPi (PCR nucleotide mix, Roche Diagnostics)
500ng total RNA
PCR grade water – for a total reaction mixture of 13µl

This reaction was incubated at 65ºC for 5 minutes and then rested on ice for at least 2 minutes.

To each reaction mixture was added:
4µl 5X First Strand buffer (Invitrogen, Mulgrave, Victoria)
1µl 0.1M DTT (Invitrogen, Ontario, Canada)
1µl SUPERase inhibitor (Ambion, Austin, Texas, United States)
1µl Superscript® III reverse transcriptase (Invitrogen, Victoria)

This was incubated as follows:
50ºC for 30 minutes
55ºC for 30 minutes
70ºC for 15 minutes
The reaction mixture was chilled on ice and stored at -20ºC.

RT-PCR

The reaction mixture comprised:
5µl 2XLC480 mastermix (Roche Diagnostics)
0.5µl forward primer (5µM)
0.5µl reverse primer (5µM)
2.9µl PCR grade water
0.1µl Universal Probe Library probe (Roche Diagnostics)
1µl cDNA template (1/10 dilution)

The primers were designed as for VP receptor RT-qPCR and are listed in Table A.1.1. Standard curves for qPCR were constructed using pooled cDNA from each sample (2µl of each) at 1, 1/5, 1/25, 1/125 dilution. An existing template (Mono Colour Hydrolysis Probe – UPL Probe 384-III) for the LightCycler®480 II was used for the qPCR reaction.

The protocol was as follows:
Preincubation at 95ºC for 10 minutes
Amplification (45 cycles) at 95ºC for 10 seconds
60ºC for 30 seconds
72ºC for 1 second
Cooling at 40ºC for 30 seconds

The data were analysed using LightCycler® 480 software. For each sample the expression of the gene of interest was normalized to the expression of the housekeeping gene, PSMB2.

Candidate Gene/Variant Sequencing

Germline DNA from members (as specified in Chapters 5 and 6) of our three AIMAH families, AIMAH-01, AIMAH-02 and AIMAH-03, was sequenced.

Primer Design

Primers were designed to flank the DNA sequence to be amplified. For exons 4-6 and 8-11 of ATXN3, we used previously published primers (Sjöblom *et al.,* 2006). For the remaining exons of ATXN3 (1-3, 7) and all other candidate genes sequenced, primers were designed using Primer 3 (v.0.4.0) (http://frodo.wi.mit.edu/primer3/) (Tables A.1.2 – A.1.4). The primers were selected for the following criteria: primer length 20-28, optimal 24; primer melting temperature 57ºC-63ºC, optimal 60ºC; primer GC% 30%-75%.

Polymerase chain reaction

Each PCR reaction comprised:

2X LC480 ProbeMaster (Roche Diagnostics, GmbH, Mannheim, Germany)	10µL
Forward primer (10µM)	0.5µL
Reverse primer (10µM)	0.5µL
PCR-grade H_2O	8µL
DNA (10ng/µL)	1µL

The PCR reactions were performed in the MJ Research PTC-200 thermal cycler (GMI, Inc, Minnesota, United States).

Various PCR cycling protocols were attempted to optimise the quantity and quality of the PCR product. The final cycling protocols used are listed below. Unless otherwise specified, the PCR cycling protocol was as follows:

Step 1: 95°C for 10 minutes
Step 2: 95°C for 30 seconds
Step 3: 68°C-60°C for 30 seconds – decrease by 0.8°C per cycle
Step 4: 72°C for 1 minute
Step 5: Return to Step 2, 9 times
Step 6: 95°C for 30 seconds
Step 7: 60°C for 30 seconds
Step 8: 72°C for 1 minute
Step 9: Return to Step 6, 29 times
Step 10: 72°C for 8 minutes
Step 11: 4°C forever
Step 12: End

Ataxin 3

Exon 1
Step 1: 95°C for 10 minutes
Step 2: 95°C for 20 seconds
Step 3: 69.5°C for 20 seconds
Step 4: 72°C for 30 seconds
Step 5: Return to Step 2 for 39 additional cycles
Step 6: 72°C for 3 minutes
Step 7: Hold at 4°C forever
Step 8: End

Exon 2, 3, 7
Step 1: 95°C for 10 minutes
Step 2: 95°C for 20 seconds
Step 3: 64°C for 20 seconds
Step 4: 72°C for 30 seconds
Step 5: Return to Step 2 for 39 additional cycles
Step 6: 72°C for 3 minutes
Step 7: Hold at 4°C forever
Step 8: End

Exon 4, 5, 6, 8, 9, 10, 11
Step 1: 96°C for 2 minutes
Step 2: 96°C for 10 seconds
Step 3: 64°C for 10 seconds
Step 4: 70°C for 30 seconds
Step 5: Return to Step 2 for 2 additional cycles
Step 6: 96°C for 10 seconds
Step 7: 61°C for 10 seconds
Step 8: 70°C for 30 seconds
Step 9: Return to Step 6 for 2 additional cycles
Step 10: 96°C for 10 seconds
Step 11: 58°C for 10 seconds
Step 12: 70°C for 30 seconds
Step 13: Return to Step 10 for 2 additional cycles
Step 14: 96°C for 10 seconds
Step 15: 57°C for 10 seconds
Step 16: 70°C for 30 seconds
Step 17: Return to Step 14 for 40 additional cycles

Step 18: 70 °C for 5 minutes
Step 19: Hold at 4°C forever
Step 20: End

CHGA

Exon 1

Step 1: 95°C for 10 minutes
Step 2: 95°C for 20 seconds
Step 3: 55-70°C for 20 seconds*
Step 4: 72°C for 30 seconds
Step 5: Return to Step 2, 29 times
Step 6: 72°C for 3 minutes
Step 7: 4°C forever
Step 8: End

*Gradient PCR was used to determine an optimal annealing temperature of 57.5°C.

The PCR product (1:100 dilution with PCR-grade water) was then amplified using the nested primers.

PCR Protocol - Exon 1 (nested primers)

Step 1: 95°C for 10 minutes
Step 2: 95°C for 20 seconds
Step 3: 60°C for 20 seconds*
Step 4: 72°C for 30 seconds
Step 5: Return to Step 2, 19 times
Step 6: 72°C for 3 minutes
Step 7: 4°C forever
Step 8: End

PCR clean-up and Sequencing reaction

The PCR products were cleaned using ExoSAP-IT® (USB Corporation, Cleveland, Ohio, United States), according to the manufacturer's protocol and then submitted for sequencing.

Sequencing analysis

The sequence was analysed using Mutation Surveyor® software (Version 2.51, SoftGenetics LLC, Philadelphia, USA). Comparison was made to the reference sequence for each gene in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

High resolution melt analysis

Primer optimization

The primers used were the same as used for sequencing. Primer optimization using 2μM, 3μM and 4μM concentrations of magnesium chloride was performed using gradient and touchdown PCR to determine the optimal annealing temperature.

Each reaction contained:

	2μM	3μM	4μM
PCR grade water	2.04	1.64	1.24
Forward primer (4μM)	0.5	0.5	0.5
Reverse primer (4μM)	0.5	0.5	0.5
DMSO (dimethyl sulfoxide)	0.16	0.16	0.16
$MgCl_2$*	0.8	1.2	1.6
HRM mastermix*	5	5	5
DNA (5ng/μL)	1	1	1
Total reaction volume (μL)	10	10	10

*These were from the LightCycler® 480 High Resolution Melting Master kit (Roche Diagnostics, Germany)
$MgCl_2$ was supplied at a concentration of 25mM
HRM mastermix contained FastStart Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP) and High Resolution Melting Dye

Protocol:

Step 1: 95ºC for 10 minutes
Step 2: 95ºC for 20 seconds
Step 3: 68ºC for 20 seconds
-1ºC per cycle
Step 4: 72ºC for 20 seconds
Step 5: Go to step 2, 3 times
Step 6: 95ºC for 20 seconds
Step 7: 64ºC to 65ºC for 20 seconds
Step 8: 72ºC for 20 seconds
Step 9: Go to step 6, 2 times
Step 10: 95ºC for 20 seconds
Step 11: 63ºC to 65ºC for 20 seconds
Step 12: 72ºC for 20 seconds
Step 13: Go to step 10, 2 times
Step 14: 95ºC for 20 seconds
Step 15: 62ºC to 65ºC for 20 seconds
Step 16: 72ºC for 20 seconds
Step 17: Go to step 14, 2 times
Step 18: 95ºC for 20 seconds
Step 19: 60ºC to 65ºC for 20 seconds
Step 20: 72ºC for 20 seconds
Step 21: Go to step 18, 2 times

Step 22: 95ºC for 20 seconds
Step 23: 58ºC to 65ºC for 20 seconds
Step 24: 72ºC for 20 seconds
Step 25: Go to step 22, 35 times
Step 26: 72ºC for 5 minutes
Step 27: 4ºC forever

The PCR products were run on a 2% agarose gel. 4µM $MgCl_2$ and a touchdown PCR from 68ºC to 61ºC were determined to be the optimal parameters for HRM.

HRM (using 4µM $MgCl_2$)

This was performed using the LightCycler® (LC480) system (Roche Diagnostics).

The protocol was as follows:

1 cycle
    95ºC for 10 minutes
Quantification – 65 cycles
    95ºC for 20 seconds
    68ºC to 61 ºC for 15 seconds
    72ºC for 45 seconds
Melting curve analysis – 1 cycle
    95ºC for 1 minute
    45ºC for 1 minute
    72ºC for 5 seconds
    99ºC continuous acquisition, ramp rate 0.01ºC/second
    50ºC for 10 seconds

Melting curves were analysed using LightCycler® software, according to the manufacturer's instructions.

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

Table A.2.1. Differentially expressed genes: All AIMAH *vs* normal
Table legend is on page 386.

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
7964927	TSPAN8	chr12	3.5589	6.6857	0.0084
8176375	RPS4Y1	chrY	3.4602	4.1537	0.0467
8151686	MMP16	chr8	3.4199	12.9708	0.0008
8176719	EIF1AY	chrY	3.3405	4.5097	0.0364
8141016	TFPI2	chr7	3.2676	9.7286	0.0016
8151684	MMP16	chr8	3.1015	12.4937	0.0010
7948904	SNORD28	chr11	2.9990	6.6006	0.0088
8152617	HAS2	chr8	2.9457	11.3721	0.0011
8124531	HIST1H3I	chr6	2.6941	7.3289	0.0059
8097920	LRAT	chr4	2.6351	9.0090	0.0024
7902398	SNORD45A	chr1	2.5525	5.8581	0.0139
7988767	CYP19A1	chr15	2.4932	4.1780	0.0455
8067969	CHODL	chr21	2.4521	7.9285	0.0043
8126905	CRISP3	chr6	2.4426	9.9396	0.0015
8004545	ATP1B2	chr17	2.4153	11.0612	0.0012
8150877	SNORD54	chr8	2.4068	7.0564	0.0068
7954899	CNTN1	chr12	2.2640	8.4288	0.0035
7935535	CRTAC1	chr10	2.2344	11.5861	0.0011
8127987	SNORD50A	chr6	2.1298	7.0283	0.0069
7948902	SNORD29	chr11	2.0897	9.4840	0.0018
8133876	CD36	chr7	2.0683	5.6627	0.0161
7961524	ERP27	chr12	2.0212	11.3552	0.0011
7960544	VAMP1	chr12	1.9855	9.6773	0.0017
8156290	CKS2	chr9	1.9252	5.5967	0.0171
8080562	IL17RB	chr3	1.9036	13.9175	0.0007
7927803	LRRTM3	chr10	1.9028	7.7325	0.0047
7925457	RGS7	chr1	1.8721	10.2991	0.0014
8010078	SNORD1C	chr17	1.8629	5.2665	0.0219
8044961	RNU4ATAC	chr2	1.8317	4.4902	0.0369
8169598	ZCCHC12	chrX	1.8007	10.8622	0.0012
8068651	PCP4	chr21	1.7972	7.8156	0.0046
8104079	FAT1	chr4	1.7796	10.8211	0.0012
7998722	SNORD60	chr16	1.7717	4.0877	0.0496
8116418	GFPT2	chr5	1.7595	8.3491	0.0035
7961829	BCAT1	chr12	1.7366	10.2265	0.0014
7902400	SNORD45B	chr1	1.7341	11.6556	0.0011
7975076	HSPA2	chr14	1.7103	6.2073	0.0114
7979269	GCH1	chr14	1.7083	12.3146	0.0010
8117330	HIST1H3A	chr6	1.7063	6.2493	0.0111
7929282	HHEX	chr10	1.6995	9.8475	0.0015

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8151871	CCNE2	chr8	1.6972	10.3441	0.0014
8015396	HAP1	chr17	1.6960	11.6538	0.0011
8020653	CABYR	chr18	1.6958	9.9967	0.0015
8138765	HOXA11	chr7	1.6673	10.7732	0.0012
8097928	RBM46	chr4	1.6589	8.3057	0.0036
7972297	ABCC4	chr13	1.6563	5.9827	0.0132
8131944	NFE2L3	chr7	1.6516	8.5625	0.0032
8023259	SNORD58A	chr18	1.6447	5.6718	0.0161
8176384	ZFY	chrY	1.6358	4.4425	0.0379
8134257	GNG11	chr7	1.6111	7.1259	0.0065
7955858	HOXC10	chr12	1.6025	9.9486	0.0015
7926875	BAMBI	chr10	1.5956	4.2951	0.0420
7934997	PPP1R3C	chr10	1.5915	5.3633	0.0204
8122182	TBPL1	chr6	1.5895	7.9706	0.0042
7950391	PGM2L1	chr11	1.5885	6.5215	0.0094
8045637	KIF5C	chr2	1.5738	6.0839	0.0122
7973067	NP	chr14	1.5599	4.2579	0.0430
8045688	TNFAIP6	chr2	1.5399	6.7951	0.0078
8142497	CTTNBP2	chr7	1.5309	7.0958	0.0066
8138749	HOXA9	chr7	1.5013	7.9982	0.0042
8160033	GLDC	chr9	1.4987	5.1120	0.0243
8083246	CPB1	chr3	1.4892	6.6026	0.0088
8122144	SNORA33	chr6	1.4834	5.5643	0.0172
7933442	PTPN20B	chr10	1.4834	4.8252	0.0294
7899502	RNU11	chr1	1.4789	4.5829	0.0347
7922414	SNORD76	chr1	1.4766	4.4648	0.0376
8169115	NRK	chrX	1.4661	7.6886	0.0048
8126891	CRISP2	chr6	1.4643	4.2313	0.0442
8106743	VCAN	chr5	1.4520	5.4344	0.0194
7933437	PTPN20B	chr10	1.4467	5.1132	0.0243
8169949	RP6-213H19.1	chrX	1.4199	9.1530	0.0022
8060805	CHGB	chr20	1.4177	7.8952	0.0044
8078008	LSM3	chr3	1.4135	6.8990	0.0075
7906377	MNDA	chr1	1.3861	9.1921	0.0022
7917304	MCOLN3	chr1	1.3738	4.7552	0.0309
8020702	TAF4B	chr18	1.3661	6.8756	0.0076
8059279	EPHA4	chr2	1.3601	7.7052	0.0048
8120176	C6orf141	chr6	1.3437	6.0779	0.0122
7982889	NUSAP1	chr15	1.3413	7.1989	0.0064
7948908	SNORD26	chr11	1.3280	6.4907	0.0096

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
7955502	SCN8A	chr12	1.3269	5.5937	0.0172
8111234	CDH12	chr5	1.3266	6.3226	0.0108
7978391	NOVA1	chr14	1.2807	7.7594	0.0047
7914212	SNORA61	chr1	1.2773	5.4498	0.0192
7948906	SNORD27	chr11	1.2751	5.3287	0.0210
8096511	BMPR1B	chr4	1.2749	6.6972	0.0083
8081431	ALCAM	chr3	1.2698	7.8503	0.0045
7904843	PDZK1	chr1	1.2595	5.2384	0.0224
7902808	HS2ST1	chr1	1.2463	5.8437	0.0140
7901048	SNORD46	chr1	1.2421	4.3283	0.0408
8107769	SLC12A2	chr5	1.2381	4.1910	0.0452
7957032	YEATS4	chr12	1.2284	5.9262	0.0134
7978542	KIAA1143	chr14	1.2281	6.2409	0.0111
8138466	MACC1	chr7	1.2233	5.1248	0.0242
7963280	LOC57228	chr12	1.2222	6.6142	0.0088
8101762	SNCA	chr4	1.2159	7.5791	0.0051
7922402	SNORD47	chr1	1.2123	4.3146	0.0413
8117535	HIST1H2AG	chr6	1.2082	7.5408	0.0051
8082745	CCRL1	chr3	1.2065	6.6086	0.0088
8050695	SF3B14	chr2	1.2034	5.6935	0.0159
8078187	PLCL2	chr3	1.2016	4.9969	0.0264
8135688	LSM8	chr7	1.1988	5.9142	0.0134
8013272	CCDC144A	chr17	1.1984	6.9093	0.0075
8089329	MYH15	chr3	1.1957	7.7986	0.0046
7933379	PTPN20B	chr10	1.1928	5.4689	0.0189
7909689	SMYD2	chr1	1.1919	7.0998	0.0066
8005204	CCDC144A	chr17	1.1874	7.0363	0.0069
8117377	HIST1H1E	chr6	1.1857	5.5761	0.0172
8163185	TXN	chr9	1.1780	5.8698	0.0138
7933263	PTPN20A	chr10	1.1699	5.1062	0.0244
8091715	LXN	chr3	1.1680	4.6321	0.0338
8014956	NR1D1	chr17	1.1666	4.8444	0.0289
7909789	TGFB2	chr1	1.1627	7.0058	0.0070
8145361	NEFM	chr8	1.1605	4.5470	0.0357
8132399	C7orf36	chr7	1.1585	5.9430	0.0134
8009705	OTOP2	chr17	1.1573	5.1790	0.0234
8023526	LOC100132992	chr18	1.1567	7.6791	0.0048
8122966	CLDN20	chr6	1.1560	5.0725	0.0250
8120239	TMEM14A	chr6	1.1516	4.8306	0.0293
7922410	SNORD44	chr1	1.1428	6.0539	0.0124

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
7956220	OBFC2B	chr12	1.1412	4.6140	0.0342
7986293	MCTP2	chr15	1.1383	7.4147	0.0055
8128284	EPHA7	chr6	1.1267	6.6189	0.0088
7947230	BDNF	chr11	1.1249	6.3631	0.0105
8086498	KIAA1143	chr3	1.1197	6.2760	0.0110
7967034	PLA2G1B	chr12	1.1125	4.7927	0.0302
8131927	MPP6	chr7	1.1094	4.2069	0.0447
8092162	PP13439	chr3	1.1055	7.8044	0.0046
8140500	TMEM60	chr7	1.1034	4.4225	0.0383
8038624	C19orf48	chr19	1.0986	5.4255	0.0196
8060895	RNU105B	chr20	1.0981	4.7875	0.0302
7914202	SNHG12	chr1	1.0968	7.3866	0.0056
8098508	ING2	chr4	1.0888	6.1905	0.0116
8099897	UGDH	chr4	1.0853	8.4250	0.0035
8151587	C8orf59	chr8	1.0801	5.6065	0.0169
7969428	UCHL3	chr13	1.0715	7.3979	0.0056
8106727	FLJ41309	chr5	1.0706	5.3221	0.0211
8066739	ZNF663	chr20	1.0635	8.0093	0.0042
8001178	C16orf87	chr16	1.0623	4.3599	0.0400
7958620	IFT81	chr12	1.0618	4.9243	0.0278
7917019	C1orf173	chr1	1.0616	4.4845	0.0371
7926596	COMMD3	chr10	1.0599	6.8526	0.0077
7939341	CD44	chr11	1.0599	5.0286	0.0257
7903592	KIAA1324	chr1	1.0563	4.4569	0.0376
7957260	GLIPR1	chr12	1.0497	6.9066	0.0075
7938331	ZNF143	chr11	1.0480	7.8562	0.0045
8092134	PLD1	chr3	1.0461	7.2028	0.0064
8095303	LPHN3	chr4	1.0406	5.2130	0.0228
8046201	SSB	chr2	1.0364	5.8970	0.0135
8047174	SLC39A10	chr2	1.0344	5.0774	0.0250
8056890	CHN1	chr2	1.0339	8.1455	0.0039
7990090	hCG_2004593	chr15	1.0299	4.5167	0.0362
7951034	SNORA8	chr11	1.0262	5.9074	0.0134
8139820	ZNF680	chr7	1.0262	4.5542	0.0354
8058147	PPIL3	chr2	1.0126	4.9906	0.0264
7988177	STRC	chr15	1.0092	6.1049	0.0121
8124437	HIST1H3F	chr6	1.0085	5.0215	0.0259
8108251	NPY6R	chr5	1.0065	4.1038	0.0488
7986350	ARRDC4	chr15	1.0058	4.6637	0.0330
8102831	C4orf49	chr4	1.0057	5.0866	0.0248

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8018305	HN1	chr17	1.0051	6.8188	0.0078
8168416	USMG5	chrX	1.0041	4.2993	0.0419
8009685	SLC9A3R1	chr17	-1.0028	-7.1486	0.0065
7994572	RUNDC2C	chr16	-1.0091	-5.0343	0.0256
8008768	PPM1E	chr17	-1.0097	-5.1305	0.0241
7931204	LHPP	chr10	-1.0119	-5.0337	0.0256
8122176	TCF21	chr6	-1.0122	-5.9946	0.0131
8033445	CD209	chr19	-1.0145	-4.5916	0.0344
7904158	OLFML3	chr1	-1.0147	-6.2356	0.0111
8048717	SGPP2	chr2	-1.0148	-4.7652	0.0307
8118890	SCUBE3	chr6	-1.0185	-4.9351	0.0276
8036110	ATP4A	chr19	-1.0200	-4.5344	0.0360
8009875	MYO15B	chr17	-1.0228	-7.1990	0.0064
7908125	RGL1	chr1	-1.0247	-5.4339	0.0194
8145470	DPYSL2	chr8	-1.0265	-6.2281	0.0111
8114938	JAKMIP2	chr5	-1.0280	-5.3575	0.0205
7981142	CLMN	chr14	-1.0295	-6.4500	0.0099
8098060	RXFP1	chr4	-1.0296	-4.3883	0.0391
7991186	NTRK3	chr15	-1.0306	-6.2519	0.0111
8053406	RETSAT	chr2	-1.0331	-6.1762	0.0117
7961275	TAS2R13	chr12	-1.0331	-5.3807	0.0201
8126760	RCAN2	chr6	-1.0346	-4.4891	0.0369
8156706	TMOD1	chr9	-1.0361	-5.8263	0.0141
7961757	ST8SIA1	chr12	-1.0380	-5.5677	0.0172
8112914	DHFR	chr5	-1.0382	-4.6250	0.0340
8076586	SCUBE1	chr22	-1.0383	-5.1324	0.0241
8059580	DNER	chr2	-1.0395	-6.2824	0.0110
7961595	RERGL	chr12	-1.0399	-4.9913	0.0264
7939559	TSPAN18	chr11	-1.0409	-4.6237	0.0340
7931519	KNDC1	chr10	-1.0411	-6.3242	0.0108
7987315	ACTC1	chr15	-1.0412	-4.5208	0.0361
8138888	PDE1C	chr7	-1.0423	-6.7124	0.0082
8086615	LRRC2	chr3	-1.0433	-5.2333	0.0224
8020349	ANKRD20B	chr18	-1.0451	-5.3886	0.0201
8157582	GSN	chr9	-1.0466	-6.6696	0.0085
7922846	FAM129A	chr1	-1.0511	-4.2677	0.0428
8085431	NUP210	chr3	-1.0527	-7.7426	0.0047
8169186	TBC1D8B	chrX	-1.0565	-4.9946	0.0264
7985620	ALPK3	chr15	-1.0567	-5.2362	0.0224
7931226	ZRANB1	chr10	-1.0592	-5.3531	0.0206

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8161964	FRMD3	chr9	-1.0600	-5.7974	0.0144
8066091	C20orf117	chr20	-1.0604	-4.6153	0.0342
8161558	AQP7P1	chr9	-1.0617	-5.2617	0.0219
8090193	HEG1	chr3	-1.0626	-6.1111	0.0121
8111490	PRLR	chr5	-1.0653	-4.5341	0.0360
8095080	PDGFRA	chr4	-1.0692	-6.1598	0.0118
8066985	FAM65C	chr20	-1.0713	-4.9294	0.0277
7923386	LMOD1	chr1	-1.0736	-5.7740	0.0148
8127563	COL12A1	chr6	-1.0786	-4.4614	0.0376
7967386	MPHOSPH9	chr12	-1.0800	-7.6391	0.0049
8110932	SEMA5A	chr5	-1.0819	-6.2383	0.0111
8137264	TMEM176A	chr7	-1.0828	-4.8796	0.0284
7932796	SVIL	chr10	-1.0839	-6.9711	0.0072
7958565	MVK	chr12	-1.0861	-4.7419	0.0313
8111153	MYO10	chr5	-1.0935	-6.8394	0.0077
8165658	ND2	chrM	-1.1028	-4.7886	0.0302
7983393	SORD	chr15	-1.1042	-4.9310	0.0277
8090823	SLCO2A1	chr3	-1.1058	-5.3868	0.0201
8132092	INMT	chr7	-1.1062	-5.9753	0.0132
8133670	POR	chr7	-1.1099	-5.8095	0.0143
8035095	CYP4F11	chr19	-1.1110	-6.4614	0.0098
8032839	SEMA6B	chr19	-1.1141	-7.2383	0.0063
8048749	KCNE4	chr2	-1.1142	-5.5447	0.0176
8079966	SEMA3B	chr3	-1.1152	-9.1573	0.0022
7916986	NEGR1	chr1	-1.1152	-7.4528	0.0054
8132592	OGDH	chr7	-1.1152	-7.1983	0.0064
8050115	C2orf46	chr2	-1.1155	-4.6178	0.0342
8032623	TBXA2R	chr19	-1.1198	-7.6126	0.0050
8044584	PSD4	chr2	-1.1204	-6.0809	0.0122
8124280	FAM65B	chr6	-1.1218	-8.3565	0.0035
8063923	SLCO4A1	chr20	-1.1247	-5.0133	0.0261
8011516	ATP2A3	chr17	-1.1248	-6.3202	0.0108
8037621	EML2	chr19	-1.1261	-7.9466	0.0043
7944082	TAGLN	chr11	-1.1288	-5.9858	0.0132
8030881	PPP2R1A	chr19	-1.1304	-7.7133	0.0048
8013157	TOM1L2	chr17	-1.1331	-6.4221	0.0101
7963880	ITGA7	chr12	-1.1342	-4.6827	0.0324
8110265	FGFR4	chr5	-1.1384	-7.1120	0.0066
7910164	CABC1	chr1	-1.1437	-6.8071	0.0078
8152506	SAMD12	chr8	-1.1464	-5.9428	0.0134

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8170671	ATP2B3	chrX	-1.1464	-4.9103	0.0279
8097080	SYNPO2	chr4	-1.1557	-5.1904	0.0233
8009913	MYO15B	chr17	-1.1570	-6.7843	0.0078
7945712	TH	chr11	-1.1588	-9.5099	0.0018
8102792	PCDH18	chr4	-1.1589	-7.0837	0.0066
8090070	ADCY5	chr3	-1.1590	-8.0314	0.0042
8129924	TXLNB	chr6	-1.1599	-5.5421	0.0176
8098379	WDR17	chr4	-1.1710	-7.5813	0.0051
8106088	CARTPT	chr5	-1.1738	-6.1026	0.0121
8177620	CARTPT	chr5_h2_hap1	-1.1738	-6.1026	0.0121
8129071	FRK	chr6	-1.1739	-8.5426	0.0032
8072413	SMTN	chr22	-1.1790	-8.2484	0.0037
7942007	LRP5	chr11	-1.1798	-8.9263	0.0025
7986385	SYNM	chr15	-1.1804	-7.5565	0.0051
7951662	CRYAB	chr11	-1.1817	-5.3904	0.0201
7917996	LRRC39	chr1	-1.1839	-6.5342	0.0094
8130211	SYNE1	chr6	-1.1866	-6.2483	0.0111
8017378	CYB561	chr17	-1.1895	-8.8701	0.0026
8022045	MYOM1	chr18	-1.1900	-7.9178	0.0043
7940451	DAK	chr11	-1.1983	-5.7567	0.0150
8057418	ZNF385B	chr2	-1.1992	-6.5287	0.0094
8074856	PRAME	chr22	-1.2028	-7.3067	0.0060
8116130	FAM153B	chr5	-1.2116	-4.3654	0.0398
8070681	C21orf84	chr21	-1.2139	-4.2654	0.0428
8115099	PDGFRB	chr5	-1.2145	-9.2028	0.0022
8175319	ZNF75D	chrX	-1.2155	-6.7497	0.0080
8013341	MFAP4	chr17	-1.2179	-4.9819	0.0266
8163202	SVEP1	chr9	-1.2212	-6.1548	0.0118
8139832	ZNF117	chr7	-1.2228	-7.2635	0.0062
8028872	LTBP4	chr19	-1.2321	-5.2740	0.0218
7917503	GBP3	chr1	-1.2369	-6.2700	0.0111
8170602	ZNF185	chrX	-1.2442	-6.3993	0.0103
8080344	STAB1	chr3	-1.2444	-5.0416	0.0255
7926152	ECHDC3	chr10	-1.2474	-8.0847	0.0041
7919645	SV2A	chr1	-1.2525	-8.1672	0.0039
8080714	FLNB	chr3	-1.2549	-5.6777	0.0161
7970329	GAS6	chr13	-1.2554	-5.8806	0.0137
8139840	ERV3	chr7	-1.2581	-5.1348	0.0241
7999909	GPRC5B	chr16	-1.2588	-9.9683	0.0015
7950067	DHCR7	chr11	-1.2626	-5.2246	0.0226

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
7968154	ATP8A2	chr13	-1.2666	-6.7104	0.0082
8170648	BGN	chrX	-1.2683	-6.8792	0.0076
7903162	TMEM56	chr1	-1.2687	-6.1652	0.0118
8105596	RGS7BP	chr5	-1.2695	-6.6678	0.0085
7960529	SCNN1A	chr12	-1.2704	-5.9741	0.0132
8168345	ACRC	chrX	-1.2717	-4.0912	0.0494
7979721	C14orf83	chr14	-1.2817	-5.1221	0.0242
7964872	PTPRB	chr12	-1.2835	-6.7963	0.0078
8066925	PTGIS	chr20	-1.2836	-8.8809	0.0026
8056151	PLA2R1	chr2	-1.2850	-8.5867	0.0031
8099817	FLJ13197	chr4	-1.2896	-4.8618	0.0285
8098344	NBLA00301	chr4	-1.2914	-8.6011	0.0031
8159096	DBH	chr9	-1.2925	-9.1996	0.0022
7922474	KIAA0040	chr1	-1.2944	-6.8006	0.0078
7916493	PPAP2B	chr1	-1.2970	-6.3919	0.0103
8175234	GPC3	chrX	-1.2995	-4.4546	0.0376
7942417	ARHGEF17	chr11	-1.3061	-7.4542	0.0054
7923378	CSRP1	chr1	-1.3076	-7.6635	0.0049
7949719	PC	chr11	-1.3087	-5.5549	0.0174
8163599	DFNB31	chr9	-1.3094	-7.3444	0.0058
8105302	FST	chr5	-1.3179	-8.3836	0.0035
8009443	ARSG	chr17	-1.3227	-6.8043	0.0078
8090098	MYLK	chr3	-1.3257	-7.0058	0.0070
8067055	ATP9A	chr20	-1.3370	-8.1488	0.0039
8039674	ZNF154	chr19	-1.3409	-7.1632	0.0065
7990333	CYP11A1	chr15	-1.3502	-5.7018	0.0158
8132118	AQP1	chr7	-1.3554	-8.3485	0.0035
8139500	TNS3	chr7	-1.3620	-7.1398	0.0065
8126798	GPR116	chr6	-1.3827	-7.7940	0.0046
8155754	MAMDC2	chr9	-1.4078	-9.3193	0.0021
8057677	SLC40A1	chr2	-1.4103	-8.1983	0.0039
7929816	SCD	chr10	-1.4104	-5.8952	0.0135
7969050	CYSLTR2	chr13	-1.4144	-4.4161	0.0383
8029693	FOSB	chr19	-1.4147	-4.8547	0.0287
8096704	NPNT	chr4	-1.4190	-5.2969	0.0214
8154692	TEK	chr9	-1.4211	-6.5177	0.0094
8005048	MYOCD	chr17	-1.4223	-5.3290	0.0210
7928872	SNCG	chr10	-1.4537	-10.6591	0.0012
7937823	KCNQ1	chr11	-1.4687	-11.0897	0.0012
7947274	MPPED2	chr11	-1.4732	-4.8882	0.0283

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8092552	IGF2BP2	chr3	-1.5269	-7.5085	0.0052
7975932	TMEM63C	chr14	-1.5360	-5.3732	0.0203
8098204	CPE	chr4	-1.5476	-7.2350	0.0063
8152522	ENPP2	chr8	-1.5493	-4.7910	0.0302
7926506	CACNB2	chr10	-1.5509	-10.5977	0.0012
7983650	SLC27A2	chr15	-1.5613	-5.2082	0.0228
8162394	ASPN	chr9	-1.5688	-5.8330	0.0141
8159086	ADAMTSL2	chr9	-1.5708	-8.3364	0.0035
8048995	ITM2C	chr2	-1.5839	-10.1873	0.0014
8148029	COLEC10	chr8	-1.5916	-4.5703	0.0351
8044021	IL1RL1	chr2	-1.5924	-4.2799	0.0425
8079433	PTH1R	chr3	-1.6595	-5.9299	0.0134
8160168	FREM1	chr9	-1.6613	-7.5639	0.0051
8140556	HGF	chr7	-1.6791	-5.4871	0.0187
8161418	LOC441416	chr9	-1.6864	-4.2896	0.0420
8156783	COL15A1	chr9	-1.7002	-7.4985	0.0052
8066619	PLTP	chr20	-1.7035	-5.1301	0.0241
7921916	RGS5	chr1	-1.7143	-7.8924	0.0044
8040695	KCNK3	chr2	-1.7205	-10.8978	0.0012
7950162	PDE2A	chr11	-1.7547	-7.1462	0.0065
8121181	FHL5	chr6	-1.7596	-6.0787	0.0122
7925320	NID1	chr1	-1.7605	-7.5843	0.0051
7952205	MCAM	chr11	-1.7882	-6.7278	0.0082
7945680	H19	chr11	-1.7955	-8.0547	0.0041
8140085	MLXIPL	chr7	-1.8281	-9.7257	0.0016
7976322	CHGA	chr14	-1.8383	-10.2768	0.0014
8080511	CACNA1D	chr3	-1.8440	-9.9080	0.0015
8113800	FBN2	chr5	-1.8603	-9.6132	0.0017
8069508	ANKRD20B	chr21	-1.9413	-8.7033	0.0029
7918878	CASQ2	chr1	-1.9651	-7.2916	0.0061
8126153	KCNK5	chr6	-1.9945	-4.1471	0.0469
7983413	DUOX1	chr15	-2.0006	-10.2252	0.0014
8175217	GPC4	chrX	-2.0121	-5.4852	0.0187
8097449	PCDH10	chr4	-2.0457	-9.5559	0.0018
8098441	ODZ3	chr4	-2.0633	-10.6268	0.0012
7912622	LRRC38	chr1	-2.0798	-7.5126	0.0052
8153373	CYP11B2	chr8	-2.1155	-15.8382	0.0005
8080028	DOCK3	chr3	-2.1176	-10.8862	0.0012
7979710	PLEK2	chr14	-2.1637	-8.2643	0.0037
8129677	SGK1	chr6	-2.1798	-4.3094	0.0415

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8169145	MUM1L1	chrX	-2.2097	-6.3951	0.0103
8016414	SKAP1	chr17	-2.3411	-8.7597	0.0028
8056518	SCN7A	chr2	-2.3652	-5.6105	0.0169
8069505	C21orf15	chr21	-2.5738	-14.9176	0.0005
7999674	MYH11	chr16	-2.8125	-6.8476	0.0077
8079422	TDGF1	chr3	-2.9516	-11.7402	0.0011
8042788	ACTG2	chr2	-3.1238	-4.5843	0.0347

FDR = false discovery rate; Log_2FC = log_2(fold-change)

Table A.2.2. Differentially expressed genes: Advanced *vs* early AIMAH.
Table legend is on page 389.

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8033233	TUBB4	chr19	2.3076	9.7346	0.0066
8014956	NR1D1	chr17	2.1813	10.0823	0.0062
8071061	psiTPTE22	chr22	1.8814	5.1091	0.0423
8056222	DPP4	chr2	1.6535	5.3889	0.0382
7937772	IGF2	chr11	1.5840	4.8635	0.0481
7953547	ATN1	chr12	1.5222	8.4394	0.0113
7978272	CIDEB	chr14	1.5046	7.6226	0.0157
7945663	CTSD	chr11	1.4266	7.9068	0.0138
7952116	BCL9L	chr11	1.4116	8.9270	0.0098
8133331	POM121C	chr7	1.4088	8.7494	0.0098
7962689	VDR	chr12	1.3776	10.4559	0.0052
8149825	STC1	chr8	1.3569	8.2324	0.0130
8004545	ATP1B2	chr17	1.3492	6.8776	0.0205
8012349	PER1	chr17	1.3435	6.7898	0.0215
7950391	PGM2L1	chr11	1.3387	6.1176	0.0275
8133326	POM121C	chr7	1.3385	6.7304	0.0219
7955858	HOXC10	chr12	1.2562	8.6810	0.0099
8016718	CHAD	chr17	1.2113	5.1870	0.0410
8158862	SNORD62A	chr9	1.2107	6.9107	0.0205
8158864	SNORD62A	chr9	1.2107	6.9107	0.0205
8026139	NFIX	chr19	1.1984	6.7278	0.0219
8010841	FN3K	chr17	1.1651	5.6728	0.0343
8135915	HIG2	chr7	1.1641	4.8345	0.0496
8025828	LDLR	chr19	1.1599	5.2849	0.0390
7939642	CREB3L1	chr11	1.1585	6.8695	0.0205
8113641	CDO1	chr5	1.1557	5.1582	0.0414
8024623	NFIC	chr19	1.1531	6.8810	0.0205
8131263	RNU13P2	chr7	1.1403	5.1936	0.0409
8052947	CYP26B1	chr2	1.1339	6.3521	0.0253
8074399	CLTCL1	chr22	1.1332	6.7945	0.0215
7920873	SNORA42	chr1	1.1241	6.1874	0.0268
7959957	ULK1	chr12	1.1196	7.8915	0.0138
7967493	NCOR2	chr12	1.1096	6.4230	0.0249
8170716	PLXNB3	chrX	1.1020	7.4902	0.0159
8175947	HCFC1	chrX	1.0917	5.7910	0.0319
8140297	POM121C	chr7	1.0892	7.6296	0.0157
7994889	SRCAP	chr16	1.0880	5.6027	0.0353
7928558	ZMIZ1	chr10	1.0598	6.4989	0.0247
7956470	MBD6	chr12	1.0536	5.0495	0.0438
8064613	SLC4A11	chr20	1.0502	7.3915	0.0159

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
7962951	MLL2	chr12	1.0372	5.5762	0.0363
8143307	HIPK2	chr7	1.0328	7.5603	0.0159
8118498	CYP21A2	chr6	1.0306	6.4814	0.0247
8112176	RNU6ATAC	chr5	1.0275	5.4294	0.0378
8067652	EEF1A2	chr20	1.0272	6.6238	0.0228
7996219	NDRG4	chr16	1.0242	7.1574	0.0189
8043909	NPAS2	chr2	1.0230	5.0382	0.0438
7927091	LOC399744	chr10	1.0228	8.7780	0.0098
8144078	SHH	chr7	1.0220	6.6984	0.0221
8005267	RAI1	chr17	1.0168	6.1317	0.0274
8178177	CYP21A2	chr6_cox_hap1	1.0113	6.4254	0.0249
8179440	CYP21A2	chr6_qbl_hap2	1.0113	6.4254	0.0249
8031992	tcag7.907	chr19	1.0010	6.8520	0.0206
7943867	BCO2	chr11	-1.0064	-7.2954	0.0168
7922343	TNFSF4	chr1	-1.0105	-5.1385	0.0419
7993807	TMEM159	chr16	-1.0135	-5.6824	0.0340
8134263	COL1A2	chr7	-1.0143	-5.5053	0.0373
7971565	P2RY5	chr13	-1.0168	-6.9382	0.0205
8168087	IGBP1	chrX	-1.0175	-6.6202	0.0228
8127544	EEF1A1	chr6	-1.0311	-8.8723	0.0098
7933772	ANK3	chr10	-1.0382	-5.0322	0.0439
8105348	GPX8	chr5	-1.0519	-7.3880	0.0159
8176644	TMSB4Y	chrY	-1.0633	-5.7919	0.0319
8170635	ZNF275	chrX	-1.0806	-6.2120	0.0268
7908459	CFH	chr1	-1.0935	-4.9868	0.0451
8126760	RCAN2	chr6	-1.1009	-5.3170	0.0387
7939137	EIF3M	chr11	-1.1027	-8.1310	0.0130
7926410	MRC1	chr10	-1.1049	-5.5075	0.0373
7926451	MRC1	chr10	-1.1049	-5.5075	0.0373
7933821	RHOBTB1	chr10	-1.1273	-6.6721	0.0225
7925904	AKR1CL2	chr10	-1.1701	-8.2115	0.0130
7988644	ATP8B4	chr15	-1.1824	-5.4269	0.0378
7925918	AKR1C1	chr10	-1.1854	-7.9605	0.0138
7948364	MPEG1	chr11	-1.2037	-5.6332	0.0348
8145293	ADAM28	chr8	-1.2042	-5.0706	0.0438
8109490	SGCD	chr5	-1.2047	-5.4640	0.0377
8057677	SLC40A1	chr2	-1.2266	-7.9364	0.0138
8120176	C6orf141	chr6	-1.2288	-6.1867	0.0268
8088745	FRMD4B	chr3	-1.2802	-8.5506	0.0107
7988414	GATM	chr15	-1.2932	-5.8843	0.0308

Transcript ID	Gene symbol	Chromosome	Log_2FC	t-statistic	FDR
8113790	MARCH3	chr5	-1.2951	-5.0739	0.0438
7948455	MS4A6A	chr11	-1.3649	-5.5592	0.0367
8114612	CD14	chr5	-1.4917	-5.0601	0.0438
8134026	DPY19L2P4	chr7	-1.5171	-7.8296	0.0139
8016438	HOXB2	chr17	-1.5275	-6.1396	0.0274
8101780	PGDS	chr4	-1.5623	-7.0299	0.0205
8016463	HOXB6	chr17	-1.6324	-5.3097	0.0387
8111915	SEPP1	chr5	-1.6399	-7.8684	0.0138
8178811	HLA-DRB3	chr6_cox_hap1	-1.6750	-5.4788	0.0375
8180003	HLA-DRB3	chr6_qbl_hap2	-1.6868	-5.8360	0.0316
7917503	GBP3	chr1	-1.7054	-9.6226	0.0066
8178802	HLA-DRB3	chr6_cox_hap1	-1.7163	-6.0716	0.0287
8096511	BMPR1B	chr4	-1.7913	-10.4744	0.0052
8141016	TFPI2	chr7	-1.8085	-5.9934	0.0300
8163908	GGTA1	chr9	-1.8114	-7.0549	0.0205
7962559	SLC38A4	chr12	-1.8540	-6.5459	0.0241
7915590	RNU5F	chr1	-1.9497	-6.1815	0.0268
8170859	RPL10	chrX	-2.0089	-10.7868	0.0052
8107798	SLC27A6	chr5	-2.0376	-7.3891	0.0159
8156571	C9orf3	chr9	-2.1109	-5.7259	0.0331
8126905	CRISP3	chr6	-3.3013	-14.9527	0.0011

FDR = false discovery rate; $Log_2FC = log_2$(fold-change)

Appendix 3: Ingenuity Pathway Analysis of differentially expressed genes

Table A.3.1. All AIMAH *vs* normal. Differentially expressed gene functions.
Table legend is on page 391.

Category	p-value
Genetic Disorder	1.24E-09-2.09E-02
Drug Metabolism	6.22E-06-2.09E-02
Small Molecule Biochemistry	6.22E-06-2.09E-02
Cardiovascular System Development and Function	7.92E-06-2.09E-02
Tissue Development	1.28E-05-2.09E-02
Cardiovascular Disease	1.59E-05-2.09E-02
Connective Tissue Disorders	1.98E-05-2.09E-02
Organismal Survival	2.45E-05-2.61E-05
Developmental Disorder	2.8E-05-2.09E-02
Carbohydrate Metabolism	2.94E-05-2.09E-02
Endocrine System Disorders	2.99E-05-2.09E-02
Metabolic Disease	2.99E-05-2.09E-02
Skeletal and Muscular Disorders	3.18E-05-2.09E-02
Cell-To-Cell Signaling and Interaction	3.56E-05-2.09E-02
Cellular Assembly and Organization	3.56E-05-2.09E-02
Lipid Metabolism	3.68E-05-2.09E-02
Molecular Transport	3.68E-05-2.09E-02
Neurological Disease	8.76E-05-2.09E-02
Cancer	1.1E-04-2.09E-02
Inflammatory Disease	1.38E-04-2.09E-02
Embryonic Development	1.38E-04-2.09E-02
Behavior	1.66E-04-2.09E-02
Organ Development	2.23E-04-2.09E-02
Organ Morphology	2.55E-04-2.09E-02
Reproductive System Development and Function	2.79E-04-2.09E-02
Nervous System Development and Function	3.03E-04-2.09E-02
Tissue Morphology	3.03E-04-2.09E-02
Immunological Disease	3.85E-04-2.09E-02
Organismal Development	4.35E-04-2.09E-02
Amino Acid Metabolism	4.35E-04-2.01E-02
Cell Morphology	4.35E-04-2.09E-02
Cellular Development	4.35E-04-2.09E-02
Cellular Growth and Proliferation	4.35E-04-2.09E-02
Connective Tissue Development and Function	4.35E-04-2.09E-02
Hematological System Development and Function	4.35E-04-2.09E-02
Hematopoiesis	4.35E-04-2.09E-02
Lymphoid Tissue Structure and Development	4.35E-04-2.09E-02
Organismal Functions	4.68E-04-1.32E-02
Renal and Urological System Development and Function	5.65E-04-2.09E-02
Cellular Movement	8.44E-04-2.09E-02
Tumor Morphology	8.44E-04-2.09E-02
Skeletal and Muscular System Development and Function	8.69E-04-2.09E-02
Psychological Disorders	8.71E-04-1.42E-02
Cellular Function and Maintenance	1.29E-03-2.09E-02
Hepatic System Development and Function	1.32E-03-6.18E-03
Vitamin and Mineral Metabolism	1.71E-03-2.09E-02
Cell Death	2.54E-03-2.09E-02
Infection Mechanism	2.54E-03-2.09E-02
Ophthalmic Disease	2.54E-03-2.09E-02
Gene Expression	4.13E-03-2.09E-02
Cellular Compromise	4.18E-03-2.09E-02
Reproductive System Disease	4.18E-03-2.09E-02

Nutritional Disease	4.5E-03-1.42E-02
Digestive System Development and Function	5.71E-03-2.09E-02
Endocrine System Development and Function	6.18E-03-2.09E-02
Organismal Injury and Abnormalities	6.83E-03-2.09E-02
Inflammatory Response	7.91E-03-2.09E-02
DNA Replication, Recombination, and Repair	8.53E-03-8.53E-03
Nucleic Acid Metabolism	8.53E-03-2.09E-02
Renal and Urological Disease	1.02E-02-1.17E-02
Antigen Presentation	1.42E-02-2.09E-02
Hematological Disease	1.42E-02-2.09E-02
Immune Cell Trafficking	1.42E-02-2.09E-02
Respiratory System Development and Function	1.65E-02-1.65E-02
Infectious Disease	1.75E-02-2.09E-02
Gastrointestinal Disease	1.93E-02-2.09E-02
Auditory Disease	2.09E-02-2.09E-02
Cell Cycle	2.09E-02-2.09E-02
Cell Signaling	2.09E-02-2.09E-02
Cell-mediated Immune Response	2.09E-02-2.09E-02
Dermatological Diseases and Conditions	2.09E-02-2.09E-02
Hepatic System Disease	2.09E-02-2.09E-02
Humoral Immune Response	2.09E-02-2.09E-02
Post-Translational Modification	2.09E-02-2.09E-02
RNA Post-Transcriptional Modification	2.09E-02-2.09E-02
Respiratory Disease	2.09E-02-2.09E-02

p-value – probability pathway over-represented by chance

Table A.3.2. Advanced *vs* early AIMAH. Differentially expressed gene functions.
Table legend is on page 393.

Category	p-value
Skeletal and Muscular System Development and Function	4.21E-06-3.07E-02
Connective Tissue Development and Function	2.65E-05-3.58E-02
Embryonic Development	6.82E-05-3.58E-02
Organismal Development	6.82E-05-3.58E-02
Tissue Development	2.62E-04-3.58E-02
Cell Cycle	3.92E-04-3.58E-02
Digestive System Development and Function	3.92E-04-3.07E-02
Organ Development	3.92E-04-3.07E-02
Protein Synthesis	5.47E-04-2.06E-02
Gene Expression	6.92E-04-3.07E-02
Cancer	1.09E-03-3.58E-02
Genetic Disorder	1.09E-03-3.75E-02
Reproductive System Disease	1.09E-03-3.41E-02
Lipid Metabolism	1.11E-03-3.75E-02
Molecular Transport	1.11E-03-3.75E-02
Small Molecule Biochemistry	1.11E-03-3.75E-02
Drug Metabolism	1.41E-03-3.58E-02
Endocrine System Development and Function	1.41E-03-3.07E-02
Tissue Morphology	2.32E-03-3.19E-02
Cellular Development	2.66E-03-3.58E-02
Nervous System Development and Function	2.66E-03-3.58E-02
Developmental Disorder	2.93E-03-3.58E-02
Cell Death	3.67E-03-3.58E-02
Cardiovascular System Development and Function	3.87E-03-2.57E-02
Organ Morphology	3.87E-03-3.58E-02
Cardiovascular Disease	4.23E-03-3.58E-02
Cell Morphology	4.24E-03-3.58E-02
Antimicrobial Response	5.19E-03-5.19E-03
Auditory and Vestibular System Development and Function	5.19E-03-5.19E-03
Carbohydrate Metabolism	5.19E-03-3.41E-02
Cell Signaling	5.19E-03-3.58E-02
Cell-To-Cell Signaling and Interaction	5.19E-03-3.82E-02
Cellular Assembly and Organization	5.19E-03-3.07E-02
Cellular Compromise	5.19E-03-2.06E-02
Cellular Function and Maintenance	5.19E-03-3.58E-02
Cellular Growth and Proliferation	5.19E-03-3.58E-02
Cellular Movement	5.19E-03-3.07E-02
Dermatological Diseases and Conditions	5.19E-03-1.95E-02
Endocrine System Disorders	5.19E-03-3.58E-02
Energy Production	5.19E-03-5.19E-03
Hair and Skin Development and Function	5.19E-03-2.06E-02
Immunological Disease	5.19E-03-3.58E-02
Infection Mechanism	5.19E-03-2.57E-02
Inflammatory Response	5.19E-03-3.82E-02
Neurological Disease	5.19E-03-3.13E-02
Ophthalmic Disease	5.19E-03-2.06E-02
Organismal Injury and Abnormalities	5.19E-03-3.58E-02
Renal and Urological System Development and Function	5.19E-03-2.57E-02
Reproductive System Development and Function	5.19E-03-2.57E-02
Skeletal and Muscular Disorders	5.19E-03-3.75E-02
Vitamin and Mineral Metabolism	5.19E-03-3.74E-02
Amino Acid Metabolism	5.22E-03-3.07E-02
Behavior	8.17E-03-2.97E-02
Cell-mediated Immune Response	1.03E-02-2.57E-02

Connective Tissue Disorders	1.03E-02-3.75E-02
Gastrointestinal Disease	1.03E-02-2.06E-02
Hematological System Development and Function	1.03E-02-3.82E-02
Hematopoiesis	1.03E-02-2.57E-02
Humoral Immune Response	1.03E-02-3.58E-02
Inflammatory Disease	1.03E-02-3.07E-02
Metabolic Disease	1.03E-02-3.58E-02
Nutritional Disease	1.03E-02-3.07E-02
Organismal Functions	1.03E-02-2.18E-02
Renal and Urological Disease	1.03E-02-1.05E-02
Respiratory System Development and Function	1.03E-02-1.55E-02
Tumor Morphology	1.03E-02-2.57E-02
Antigen Presentation	1.55E-02-2.97E-02
Hypersensitivity Response	1.55E-02-1.55E-02
Immune Cell Trafficking	1.55E-02-3.82E-02
Post-Translational Modification	1.55E-02-3.07E-02
Visual System Development and Function	1.55E-02-1.55E-02
Hepatic System Development and Function	2.06E-02-2.06E-02
Hepatic System Disease	2.06E-02-2.06E-02
Nucleic Acid Metabolism	2.06E-02-3.58E-02
Hematological Disease	2.57E-02-3.58E-02
Infectious Disease	2.57E-02-2.57E-02
Respiratory Disease	2.65E-02-3.52E-02
Lymphoid Tissue Structure and Development	3.58E-02-3.58E-02

p-value – probability pathway over-represented by chance

Table A.3.3. All AIMAH *vs* normal. Pathways overrepresented by differentially expressed genes. Table legend is on page 396.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Cellular Effects of Sildenafil (Viagra)	2.98E00	5.96E-02
Cardiac β-adrenergic Signaling	2.62E00	5.63E-02
Eicosanoid Signaling	2.11E00	6.02E-02
Starch and Sucrose Metabolism	2.03E00	2.56E-02
Pantothenate and CoA Biosynthesis	1.98E00	4.69E-02
Human Embryonic Stem Cell Pluripotency	1.91E00	4.58E-02
Dopamine Receptor Signaling	1.84E00	5.38E-02
Protein Kinase A Signaling	1.61E00	3.45E-02
CDK5 Signaling	1.57E00	5.32E-02
Phospholipid Degradation	1.57E00	4.76E-02
Factors Promoting Cardiogenesis in Vertebrates	1.49E00	5.32E-02
Tight Junction Signaling	1.47E00	4.19E-02
Linoleic Acid Metabolism	1.39E00	3.31E-02
Caveolar-mediated Endocytosis Signaling	1.37E00	4.71E-02
Aminosugars Metabolism	1.37E00	3.31E-02
Folate Biosynthesis	1.37E00	2.5E-02
C21-Steroid Hormone Metabolism	1.23E00	2.78E-02
Arachidonic Acid Metabolism	1.22E00	2.23E-02
VDR/RXR Activation	1.18E00	5E-02
Aldosterone Signaling in Epithelial Cells	1.16E00	4.21E-02
Regulation of Actin-based Motility by Rho	1.08E00	4.35E-02
FXR/RXR Activation	1.06E00	3.88E-02
Relaxin Signaling	1.04E00	3.36E-02
Biosynthesis of Steroids	9.91E-01	1.56E-02
Citrate Cycle	9.64E-01	3.45E-02
Glycerophospholipid Metabolism	9.6E-01	2.6E-02
Calcium Signaling	9.24E-01	2.93E-02
ILK Signaling	9.15E-01	3.23E-02
Pentose and Glucuronate Interconversions	9.12E-01	1.33E-02
Breast Cancer Regulation by Stathmin1	8.61E-01	3.02E-02
RhoA Signaling	8.26E-01	3.64E-02
Cell Cycle Regulation by BTG Family Proteins	8.21E-01	5.56E-02
LXR/RXR Activation	7.97E-01	3.49E-02
Mechanisms of Viral Exit from Host Cells	7.81E-01	4.55E-02
Germ Cell-Sertoli Cell Junction Signaling	7.79E-01	3.16E-02
Crosstalk between Dendritic Cells and Natural Killer Cells	7.57E-01	3.06E-02
Nitric Oxide Signaling in the Cardiovascular System	7.32E-01	3.09E-02
Thyroid Cancer Signaling	7.25E-01	4.26E-02
Tryptophan Metabolism	7.21E-01	1.58E-02
Valine, Leucine and Isoleucine Biosynthesis	6.83E-01	2.27E-02
Virus Entry via Endocytic Pathways	6.62E-01	3.12E-02
VEGF Signaling	6.62E-01	3.09E-02
Wnt/β-catenin Signaling	6.57E-01	2.96E-02
Sphingolipid Metabolism	6.51E-01	2.59E-02
Endothelin-1 Signaling	6.35E-01	2.7E-02
Huntington's Disease Signaling	6.24E-01	2.5E-02
Hepatic Fibrosis / Hepatic Stellate Cell Activation	6.23E-01	2.99E-02
Parkinson's Signaling	6.19E-01	5.88E-02
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	6E-01	2.97E-02
Role of NFAT in Cardiac Hypertrophy	5.93E-01	2.42E-02
Nucleotide Sugars Metabolism	5.91E-01	1.35E-02
Leukocyte Extravasation Signaling	5.67E-01	2.58E-02
Synaptic Long Term Depression	5.54E-01	2.5E-02

Granzyme A Signaling	5.42E-01	5E-02
Cell Cycle: G1/S Checkpoint Regulation	5.39E-01	3.33E-02
Axonal Guidance Signaling	5.37E-01	2.22E-02
Maturity Onset Diabetes of Young (MODY) Signaling	5.2E-01	3.23E-02
Atherosclerosis Signaling	5.2E-01	2.65E-02
Fatty Acid Metabolism	5.2E-01	1.58E-02
Cardiomyocyte Differentiation via BMP Receptors	5E-01	5E-02
Sphingosine-1-phosphate Signaling	4.88E-01	2.59E-02
Nicotinate and Nicotinamide Metabolism	4.88E-01	2.21E-02
PPARα/RXRα Activation	4.67E-01	2.22E-02
CCR3 Signaling in Eosinophils	4.65E-01	2.5E-02
B Cell Development	4.62E-01	2.7E-02
Neurotrophin/TRK Signaling	4.46E-01	2.67E-02
One Carbon Pool by Folate	4.45E-01	2.56E-02
Actin Cytoskeleton Signaling	4.37E-01	2.13E-02
Antiproliferative Role of Somatostatin Receptor 2	4.37E-01	2.56E-02
cAMP-mediated Signaling	4.32E-01	2.48E-02
Agrin Interactions at Neuromuscular Junction	4.28E-01	2.9E-02
Glycine, Serine and Threonine Metabolism	4.19E-01	1.33E-02
Androgen and Estrogen Metabolism	4.19E-01	1.41E-02
Purine Metabolism	4.16E-01	1.59E-02
Glycosphingolipid Biosynthesis - Globoseries	4.14E-01	2.22E-02
IL-15 Production	4E-01	3.33E-02
Riboflavin Metabolism	4E-01	1.82E-02
Phospholipase C Signaling	3.95E-01	1.95E-02
p70S6K Signaling	3.92E-01	2.29E-02
Role of MAPK Signaling in the Pathogenesis of Influenza	3.87E-01	2.67E-02
Tyrosine Metabolism	3.87E-01	9.9E-03
Leptin Signaling in Obesity	3.79E-01	2.44E-02
Glycosphingolipid Biosynthesis - Neolactoseries	3.74E-01	1.45E-02
Cardiac Hypertrophy Signaling	3.73E-01	2.06E-02
BMP signaling pathway	3.72E-01	2.5E-02
Insulin Receptor Signaling	3.69E-01	2.14E-02
G Protein Signaling Mediated by Tubby	3.61E-01	2.44E-02
Inositol Metabolism	3.5E-01	2.06E-02
Glycosphingolipid Biosynthesis - Ganglioseries	3.39E-01	1.56E-02
Circadian Rhythm Signaling	3.39E-01	2.86E-02
Metabolism of Xenobiotics by Cytochrome P450	3.37E-01	9.71E-03
TGF-β Signaling	3.37E-01	2.41E-02
Aryl Hydrocarbon Receptor Signaling	3.32E-01	1.95E-02
Colorectal Cancer Metastasis Signaling	3.22E-01	1.97E-02
Role of CHK Proteins in Cell Cycle Checkpoint Control	3.18E-01	2.86E-02
Serotonin Receptor Signaling	3.18E-01	2.17E-02
Thrombin Signaling	3.13E-01	1.96E-02
Integrin Signaling	3.13E-01	1.98E-02
α-Adrenergic Signaling	3.12E-01	1.89E-02
FAK Signaling	3.12E-01	2E-02
PAK Signaling	3.12E-01	1.92E-02
PTEN Signaling	3.12E-01	1.9E-02
FGF Signaling	3.06E-01	2.27E-02
Notch Signaling	2.99E-01	2.33E-02
IL-6 Signaling	2.94E-01	2.15E-02
MIF Regulation of Innate Immunity	2.81E-01	2.17E-02
Primary Immunodeficiency Signaling	2.81E-01	1.59E-02
IL-1 Signaling	2.78E-01	1.89E-02
Glutamate Metabolism	2.73E-01	1.28E-02
Amyotrophic Lateral Sclerosis Signaling	2.68E-01	1.79E-02
p38 MAPK Signaling	2.58E-01	2.08E-02

Pyrimidine Metabolism	2.49E-01	1.3E-02
Neuropathic Pain Signaling In Dorsal Horn Neurons	2.39E-01	1.94E-02
Alanine and Aspartate Metabolism	2.37E-01	1.14E-02
Rac Signaling	2.3E-01	1.64E-02
Regulation of eIF4 and p70S6K Signaling	2.26E-01	1.52E-02
Fructose and Mannose Metabolism	2.06E-01	6.9E-03

-log(p-value) – probability pathway over-represented by chance
Ratio = number of genes differentially expressed in expression data/number of genes in the pathway

Table A.3.4. Advanced AIMAH *vs* early AIMAH. Pathways overrepresented by differentially expressed genes. Table legend is on page 398.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
TR/RXR Activation	3.03E00	4.12E-02
C21-Steroid Hormone Metabolism	2.37E00	2.78E-02
LXR/RXR Activation	2.32E00	3.49E-02
VDR/RXR Activation	2.19E00	3.75E-02
Arachidonic Acid Metabolism	1.81E00	1.34E-02
Aryl Hydrocarbon Receptor Signaling	1.6E00	1.95E-02
Glutathione Metabolism	1.54E00	2.04E-02
Metabolism of Xenobiotics by Cytochrome P450	1.25E00	9.71E-03
TGF-β Signaling	1.25E00	2.41E-02
Glycosphingolipid Biosynthesis - Lactoseries	1.22E00	3.7E-02
Taurine and Hypotaurine Metabolism	1.22E00	2.13E-02
Cardiomyocyte Differentiation via BMP Receptors	1.05E00	5E-02
Glycosphingolipid Biosynthesis - Neolactoseries	8.98E-01	1.45E-02
Hepatic Fibrosis / Hepatic Stellate Cell Activation	8.97E-01	1.49E-02
Circadian Rhythm Signaling	8.53E-01	2.86E-02
Sonic Hedgehog Signaling	8.53E-01	3.03E-02
Urea Cycle and Metabolism of Amino Groups	8.53E-01	1.25E-02
Complement System	8.4E-01	2.78E-02
Role of CHK Proteins in Cell Cycle Checkpoint Control	8.27E-01	2.86E-02
MIF Regulation of Innate Immunity	7.78E-01	2.17E-02
Clathrin-mediated Endocytosis Signaling	7.75E-01	1.2E-02
Bile Acid Biosynthesis	6.98E-01	1E-02
Toll-like Receptor Signaling	6.8E-01	1.85E-02
Cysteine Metabolism	6.8E-01	1.14E-02
LPS/IL-1 Mediated Inhibition of RXR Function	6.76E-01	9.39E-03
Molecular Mechanisms of Cancer	6.5E-01	8.06E-03
Role of BRCA1 in DNA Damage Response	6.39E-01	1.64E-02
IL-10 Signaling	5.97E-01	1.43E-02
Actin Cytoskeleton Signaling	5.87E-01	8.51E-03
Macropinocytosis Signaling	5.84E-01	1.39E-02
Axonal Guidance Signaling	5.8E-01	7.41E-03
Eicosanoid Signaling	5.77E-01	1.2E-02
Glycine, Serine and Threonine Metabolism	5.53E-01	6.67E-03
Androgen and Estrogen Metabolism	5.53E-01	7.04E-03
LPS-stimulated MAPK Signaling	5.47E-01	1.28E-02
Basal Cell Carcinoma Signaling	5.2E-01	1.37E-02
BMP signaling pathway	5.2E-01	1.25E-02
Arginine and Proline Metabolism	5.09E-01	5.46E-03
Virus Entry via Endocytic Pathways	4.8E-01	1.04E-02
PPAR Signaling	4.8E-01	1.02E-02
PTEN Signaling	4.76E-01	9.52E-03
CTLA4 Signaling in Cytotoxic T Lymphocytes	4.63E-01	1.02E-02
IL-6 Signaling	4.63E-01	1.08E-02
Factors Promoting Cardiogenesis in Vertebrates	4.46E-01	1.06E-02
p53 Signaling	4.38E-01	1.09E-02
Atherosclerosis Signaling	4.19E-01	8.85E-03
Fatty Acid Metabolism	4.19E-01	5.26E-03
14-3-3-mediated Signaling	4.01E-01	8.77E-03
Corticotrophin Releasing Hormone Signaling	3.94E-01	7.35E-03
Estrogen Receptor Signaling	3.87E-01	8.47E-03
Hereditary Breast Cancer Signaling	3.84E-01	7.75E-03
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	3.81E-01	8.77E-03
Type II Diabetes Mellitus Signaling	3.78E-01	6.33E-03
Tryptophan Metabolism	3.65E-01	3.95E-03

Human Embryonic Stem Cell Pluripotency	3.34E-01	6.54E-03
Hepatic Cholestasis	3.32E-01	6.06E-03
Cellular Effects of Sildenafil (Viagra)	3.27E-01	6.62E-03
Dendritic Cell Maturation	3.24E-01	5.38E-03
NF-κB Signaling	3.17E-01	6.54E-03
Germ Cell-Sertoli Cell Junction Signaling	2.87E-01	6.33E-03
PPARα/RXRα Activation	2.83E-01	5.56E-03
Role of NFAT in Regulation of the Immune Response	2.68E-01	5E-03
RAR Activation	2.57E-01	5.52E-03
Calcium Signaling	2.46E-01	4.88E-03
ILK Signaling	2.45E-01	5.38E-03
Role of NFAT in Cardiac Hypertrophy	2.41E-01	4.83E-03
Breast Cancer Regulation by Stathmin1	2.34E-01	5.03E-03

-log(p-value) – probability pathway over-represented by chance
Ratio = number of genes differentially expressed in expression data/number of genes in the pathway

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

Table A.4.1. All AIMAH *vs* normal; positively enriched gene sets

GENE SET NAME	ENRICHMENT SCORE	NORMALISED ENRICHMENT SCORE	FDR q-value
Almeida et al., 2011; Nodule 7 Up	0.5494	2.8101	0.0000
Almeida et al., 2011; Nodule 4 Up	0.5011	2.5772	0.0000
Almeida et al., 2011; Nodule 6 Up	0.4966	2.5411	0.0000
Almeida et al., 2011; Nodule 5 Up	0.4580	2.3549	0.0000
Almeida et al., 2011; Nodule 1 Up	0.4446	2.3271	0.0000
Almeida et al., 2011; Nodule 2 Up	0.4354	2.2466	0.0000
Almeida et al., 2011; Nodule 3 Up	0.4149	2.1291	0.0000
Bourdeau et al., 2004; AIMAH Up	0.5360	2.1146	0.0000
Giordano et al., 2003; ACC Up	0.5600	1.8985	0.0004
Soon et al., 2009; ACT Up	0.4201	1.7249	0.0024
De Reynies et al., 2009; ACC Up	0.3512	1.7049	0.0026
Schimmer et al., 2006; ACTH Down	0.3263	1.7020	0.0025
Scacheri et al., 2006; Menin KO Down	0.3980	1.6919	0.0028
Giordano et al., 2009; ACC vs ACA Up	0.3022	1.6414	0.0050
Giordano et al., 2009; ACC vs Normal Up	0.2870	1.5605	0.0110
Schimmer et al., 2006; PKC Down	0.3497	1.4331	0.0292
De Reynies et al., 2009; Aggressive ACC Up	0.2697	1.4266	0.0287
Horvath et al., 2006; PPNAD Down	0.3580	1.3894	0.0359
Schimmer et al., 2006; PKA Down	0.2525	1.3014	0.0630
West et al., 2009; ACT Up	0.2686	1.2961	0.0623
Bourdeau et al., 2004; AIMAH All	0.2960	1.2311	0.0974

Table A.4.2. All AIMAH *vs* normal; negatively enriched gene sets

GENE SET NAME	ENRICHMENT SCORE	NORMALISED ENRICHMENT SCORE	FDR q-value
Almeida et al., 2011; Nodule 4 Down	-0.6480	-3.2085	0.0000
Almeida et al., 2011; Nodule 7 Down	-0.6426	-3.1537	0.0000
Almeida et al., 2011; Nodule 6 Down	-0.6335	-3.1479	0.0000
Almeida et al., 2011; Nodule 5 Down	-0.6036	-2.9552	0.0000
Almeida et al., 2011; Nodule 1 Down	-0.5943	-2.9226	0.0000
Almeida et al., 2011; Nodule 2 Down	-0.5716	-2.8476	0.0000
Giordano et al., 2009; ACA vs Normal Down	-0.6381	-2.8339	0.0000
Almeida et al., 2011; Nodule 3 Down	-0.5629	-2.7818	0.0000
Giordano et al., 2009; ACC vs ACA Down	-0.5160	-2.7689	0.0000
Giordano et al., 2009; ACC vs Normal Down	-0.5096	-2.7575	0.0000
Lampron et al., 2006; GIPs-AIMAH Down	-0.6282	-2.6193	0.0000
Giordano et al., 2009; ACA vs Normal All	-0.5612	-2.6111	0.0000
Giordano et al., 2003; ACC Down	-0.7388	-2.5979	0.0000
West et al., 2009; ACT Down	-0.5039	-2.5399	0.0000
De Reynies et al., 2009; ACC Down	-0.4549	-2.3278	0.0000
Bourdeau et al., 2004; AIMAH Down	-0.7363	-2.2550	0.0001
West et al., 2009; ACT All	-0.3926	-2.0350	0.0002
Doghman et al., 2007; SF1 Up	-0.5215	-1.9786	0.0003
Lampron et al., 2006; GIPs-AIMAH All	-0.3848	-1.9426	0.0003
Horvath et al., 2006; PPNAD Up	-0.5000	-1.9362	0.0003
Soon et al., 2009; ACT Down	-0.4930	-1.8989	0.0004
Giordano et al., 2003; ACC All	-0.4733	-1.8827	0.0005
Giordano et al., 2009; ACC vs Normal All	-0.3375	-1.8701	0.0005
Giordano et al., 2009; ACC vs ACA All	-0.3347	-1.8427	0.0008
Doghman et al., 2007; SF1 All	-0.4478	-1.8020	0.0013
De Reynies ACC All	-0.3347	-1.7598	0.0018
De Reynies et al., 2009; Aggressive ACC Down	-0.3583	-1.7431	0.0019
Bassett et al., 2005; Adenoma All	-0.5989	-1.7151	0.0025
Laurell et al., 2009; ACC All	-0.4286	-1.6778	0.0036
Almeida et al., 2011; Nodule 6 All	-0.3151	-1.6693	0.0037
Laurell et al., 2009; ACC Down	-0.4686	-1.6266	0.0054

Almeida et al., 2011; Nodule 4 All	-0.3010	-1.5974	0.0068
Almeida et al., 2011; Nodule 6 All	-0.2910	-1.5395	0.0118
Almeida et al., 2011; Nodule 7 All	-0.2897	-1.5230	0.0132
Almeida et al., 2011; Nodule 3 All	-0.2869	-1.5078	0.0149
Lampron et al., 2006; GIPs-AIMAH Up	-0.3010	-1.4864	0.0170
Almeida et al., 2011; Nodule 1 All	-0.2788	-1.4718	0.0187
Almeida et al., 2011; Nodule 5 All	-0.2804	-1.4703	0.0186
De Fraipont et al., 2005; ACC All	-0.4893	-1.4617	0.0196
Schimmer et al., 2006; ACTH Up	-0.2878	-1.4559	0.0196
Horvath et al., 2006; PPNAD All	-0.3239	-1.4170	0.0256
Giordano et al., 2009; ACA vs Normal Up	-0.3311	-1.3292	0.0467
Scacheri et al., 2006; Menin KO Down	-0.3297	-1.2785	0.0643
Aghajanova et al., 2010; PKA-regulated genes Up	-0.3135	-1.2407	0.0840
Soon et al., 2009; ACT All	-0.2811	-1.2342	0.0869
Ye et al., 2009; APA Down	-0.3979	-1.2341	0.0851

Table A.4.3. Advanced AIMAH *vs* early AIMAH; positively enriched gene sets

GENE SET NAME	ENRICHMENT SCORE	NORMALISED ENRICHMENT SCORE	FDR q-value
Almeida et al., 2011; Nodule 2 Up	0.3913	2.0148	0.0007
Horvath et al., 2006; PPNAD Up	0.4583	1.8060	0.0072
Giordano et al., 2009; ACA vs Normal Up	0.4417	1.8013	0.0050
Doghman et al., 2007; SF1 Up	0.4728	1.8013	0.0038
De Fraipont et al., 2005; ACC All	0.5884	1.7746	0.0039
Almeida et al., 2011; Nodule 5 Up	0.3344	1.7080	0.0062
Doghman et al., 2007; SF1 All	0.4080	1.6729	0.0088
Almeida et al., 2011; Nodule 1 Up	0.3094	1.6146	0.0143
Almeida et al., 2011; Nodule 7 Up	0.3048	1.5542	0.0223
Soon et al., 2009; ACT Down	0.3963	1.5428	0.0227
Almeida et al., 2011; Nodule 6 Up	0.2902	1.4823	0.0349
Almeida et al., 2011; Nodule 3 Up	0.2787	1.4373	0.0457
Almeida et al., 2011; Nodule 1 All	0.2675	1.4367	0.0423
Almeida et al., 2011; Nodule 5 All	0.2705	1.4339	0.0401
Almeida et al., 2011; Nodule 7 All	0.2694	1.4317	0.0380
Almeida et al., 2011; Nodule 4 Up	0.2798	1.4269	0.0372
Almeida et al., 2011; Nodule 4 All	0.2675	1.4217	0.0364
Almeida et al., 2011; Nodule 2 All	0.2587	1.3832	0.0482
Giordano et al., 2009; ACC vs ACA Down	0.2468	1.3484	0.0597
Bassett et al., 2005; Adenoma All	0.4629	1.3425	0.0594
Almeida et al., 2011; Nodule 6 All	0.2486	1.3358	0.0591
Horvath et al., 2006; PPNAD All	0.2948	1.3024	0.0730
Almeida et al., 2011; Nodule 4 Down	0.2546	1.2753	0.0857
West et al., 2009; ACT Down	0.2462	1.2564	0.0944
De Reynies et al., 2009; Aggressive malignant Up	0.2372	1.2552	0.0913

Table A.4.4. Advanced AIMAH *vs* early AIMAH; negatively enriched gene sets

GENE SET NAME	ENRICHMENT SCORE	NORMALISED ENRICHMENT SCORE	FDR q-value
Scacheri et al., 2006; Menin KO Up	-0.4917	-2.1794	0.0000
Giordano et al., 2009 ACA vs Normal Down	-0.4284	-2.0120	0.0000
Scacheri et al., 2006; Menin KO All	-0.4099	-1.9538	0.0005
Aghajanova et al., 2010; PKA-regulated genes Down	-0.4608	-1.9145	0.0007
Almeida et al., Nodule 2 Down	-0.3615	-1.9052	0.0006
De Reynies et al., 2009; ACC Down	-0.3186	-1.7352	0.0086
De Reynies et al., 2009; ACC All	-0.3021	-1.6931	0.0104
Bourdeau et al., 2006; AIMAH Up	-0.4064	-1.6884	0.0102
Lampron et al., 2004; AIMAH Up	-0.3136	-1.6164	0.0246
Schimmer et al., 2006; ACTH Down	-0.2932	-1.5941	0.0257
Giordano et al., 2009; ACC vs Normal Down	-0.2726	-1.5568	0.0304
Aghajanova et al., 2010; PKA-regulated genes All	-0.3193	-1.4990	0.0385
Almeida et al., 2011; Nodule 5 Down	-0.2914	-1.4950	0.0371
Almeida et al., 2011; Nodule 3 Down	-0.2699	-1.4204	0.0621
Schimmer et al., 2006; PKA UP	-0.2510	-1.3930	0.0706
Schimmer et al., 2006; PKA All	-0.2430	-1.3912	0.0678
Lampron et al., 2006; AIMAH All	-0.2620	-1.3864	0.0631
Ye et al., 2009; APA Down	-0.4211	-1.3506	0.0761
Laurell et al., 2009; ACC All	-0.3342	-1.3471	0.0747
Giordano et al., 2009; ACC vs Normal All	-0.2276	-1.3372	0.0773
Bourdeau et al., 2006; AIMAH All	-0.3040	-1.3215	0.0823
De Reynies et al., 2009; ACC Up	-0.2622	-1.3208	0.0794
Giordano et al., 2009; ACA vs Normal All	-0.2633	-1.3066	0.0839
Schimmer et al., 2006; ACTH All	-0.2305	-1.2951	0.0869

Appendix 5: Ancillary linkage data

Table A.5.1. Flanking SNPs for chromosomal regions with LOD score < -2 (AIMAH-01).

The flanking SNPs for a locus are defined as the most proximal SNPs for which the LOD score is > -2, or, in cases where the LOD score of the flanking SNP was < -2, this was the most distal SNP studied on that chromosome in the linkage analysis. Flanking SNPs are presented in pairs. The LOD scores of the loci between a pair of SNPs was < -2.

SNP name: Affymetrix 6.0 SNP array ID.
rs name: SNP Reference ID (NCBI)
Pos(bp): Hg 18 reference

Chromosome	SNP name	rs name	Pos(bp)	LOD score
1	SNP_A-8515688	rs11240777	788822	-3.1405
1	SNP_A-2079582	rs6691722	24546453	-1.9862
1	SNP_A-4222352	rs11210617	42536775	0.2053
1	SNP_A-2141731	rs3737741	46773153	-1.774
1	SNP_A-8683911	rs2056899	47380438	-1.893
1	SNP_A-1899674	rs9437408	76527751	-1.7924
1	SNP_A-4223815	rs509258	76969542	-1.6797
1	SNP_A-2151433	rs10922719	90026999	-1.8221
1	SNP_A-4224059	rs161113	96274535	-1.7343
1	SNP_A-8576843	rs1001848	153314003	-1.8763
2	SNP_A-8487936	rs10165221	40814	-2.4281
2	SNP_A-4256444	rs848552	36554084	-1.8052
2	SNP_A-4258529	rs4528740	62552513	-1.8962
2	SNP_A-8292760	rs10206874	120876615	-1.4639
2	SNP_A-8576559	rs895543	149305162	-1.635
2	SNP_A-2314503	rs2432726	208252827	-0.8592
2	SNP_A-4217788	rs7589613	235721211	-1.0467
2	SNP_A-2067809	rs4973680	242625553	-2.497
3	SNP_A-8487244	rs6805267	210605	-1.789
3	SNP_A-8394339	rs11718833	2781287	-1.7905
3	SNP_A-1971433	rs769809	3742212	-1.8182
3	SNP_A-8571369	rs11720292	41283453	-1.7115
3	SNP_A-2087005	rs905940	63510210	-0.96
3	SNP_A-8325017	rs6439400	134482920	-1.9136
3	SNP_A-1949570	rs1159404	136367342	-1.9114
3	SNP_A-2194662	rs7629423	161806709	-1.7077
3	SNP_A-1803001	rs7629050	169274052	-1.7047
3	SNP_A-8349897	rs9877345	199293372	-3.8296
4	SNP_A-8510905	rs7686415	38714496	-0.9724
4	SNP_A-1977138	rs218273	55109929	-0.9825
4	SNP_A-1932984	rs6535382	83857571	-1.0437
4	SNP_A-2156367	rs6851434	150168776	-1.8819
4	SNP_A-2010258	rs7686229	154355038	-1.2858
4	SNP_A-2230569	rs936565	164219205	-0.0697
4	SNP_A-1949647	rs11733039	187951685	-1.9409
4	SNP_A-8434577	rs10015934	191159251	-3.409
5	SNP_A-8323401	rs7727015	238498	-2.3289
5	SNP_A-1791763	rs13178975	71089591	-1.5202
5	SNP_A-1836024	rs2431241	112171922	-1.7739
5	SNP_A-8596800	rs10050777	120626317	-1.7798

Chromosome	SNP name	rs name	Pos(bp)	LOD score
5	SNP_A-8638249	rs10073851	148603410	-1.8837
5	SNP_A-2206417	rs4867646	170512757	-1.7101
5	SNP_A-2076552	rs1563556	174703382	-0.9322
5	SNP_A-8386804	rs13177248	180618506	-3.4393
6	SNP_A-8615528	rs4960096	551568	-3.8704
6	SNP_A-4253224	rs505685	11631872	-1.9248
6	SNP_A-1920817	rs9466976	24013894	-1.9248
6	SNP_A-2114847	rs1771294	40695765	-1.5924
6	SNP_A-1826952	rs9475319	55469837	-1.7714
6	SNP_A-1986427	rs12526735	73705543	-1.686
6	SNP_A-8544898	rs2756324	105817111	-1.8497
6	SNP_A-2129543	rs10872225	122021667	-1.5891
6	SNP_A-4265952	rs9321440	134771095	-0.9367
6	SNP_A-8527001	rs936629	136577063	-1.3387
6	SNP_A-1892805	rs3020306	152107579	-0.718
6	SNP_A-8374664	rs3823298	170719316	-2.1888
7	SNP_A-2069657	rs6972063	284803	-1.6599
7	SNP_A-8460624	rs2074123	92911083	-1.9419
7	SNP_A-8559675	rs4731632	129216900	-1.991
7	SNP_A-2272711	rs243481	148083355	-1.3573
8	SNP_A-8636742	rs2906331	184884	-2.5574
8	SNP_A-8540848	rs12550628	2883931	-1.3646
8	SNP_A-8490866	rs2457432	22807169	-1.9764
8	SNP_A-2038413	rs2139323	78881275	-1.9046
8	SNP_A-8282456	rs13274426	81461333	-1.9822
8	SNP_A-8630996	rs1427090	126009686	-1.7494
9	SNP_A-8366025	rs7035770	658887	-3.6742
9	SNP_A-1996149	rs766576	19650406	-1.4817
9	SNP_A-8527247	rs10811852	23122379	-1.482
9	SNP_A-8680853	rs7849859	134141706	-1.2218
10	SNP_A-8493834	rs7917054	125708	-3.3038
10	SNP_A-8506944	rs2254501	790213	-0.9668
10	SNP_A-1945719	rs7098110	1906190	-1.9766
10	SNP_A-4267996	rs35636518	30235636	-1.7853
10	SNP_A-2131435	rs10763778	30654280	-1.9085
10	SNP_A-2001275	rs2619636	79073940	-1.9543
10	SNP_A-8288575	rs1250961	81276372	-1.9544
10	SNP_A-1922103	rs2862954	101902054	-1.7608
10	SNP_A-8651203	rs2864011	106165579	-1.6371
10	SNP_A-8298396	rs10787464	114542952	-1.9619
10	SNP_A-2056039	rs7915260	133827521	-1.9962
10	SNP_A-2259588	rs3008304	134832113	-1.2684
11	SNP_A-1908529	rs1872276	44931376	-1.8721
11	SNP_A-2230015	rs4945154	70533723	-1.9184
12	SNP_A-4194123	rs10773943	1380766	-1.5753
12	SNP_A-4227590	rs4882631	74231174	-0.3959
12	SNP_A-8320732	rs4882434	81967380	-1.5306
12	SNP_A-8460599	rs7297790	116721273	-1.9065
12	SNP_A-4295944	rs11060771	129237119	-1.954
12	SNP_A-8470265	rs12422899	132202402	-3.1655
13	SNP_A-8527635	rs4608194	18524818	-3.2115
13	SNP_A-4200157	rs9553331	23988168	-1.4614
13	SNP_A-8498195	rs9508347	28786019	-1.3072
13	SNP_A-8519053	rs532167	71796529	-1.668
13	SNP_A-2191731	rs2984850	101278581	-1.2774
13	SNP_A-8315664	rs2701318	114082644	-2.398

Chromosome	SNP name	rs name	Pos(bp)	LOD score
14	SNP_A-1937259	rs7149011	20632400	-1.8168
14	SNP_A-1843696	rs2273171	30451102	-1.5656
14	SNP_A-2202485	rs8017869	31578767	-1.2811
14	SNP_A-2203534	rs1001766	32546272	-0.8183
14	SNP_A-8451361	rs710302	98615972	-1.7931
14	SNP_A-4237231	rs885883	106345097	-3.2992
15	SNP_A-2132755	rs7179358	18451755	-3.0126
15	SNP_A-4298694	rs4028395	22630108	-1.982
15	SNP_A-1850120	rs7173687	24667811	-1.8379
15	SNP_A-1862926	rs2923052	31054268	-1.4373
15	SNP_A-8329085	rs2444969	31222365	-1.4222
15	SNP_A-8318435	rs6495009	31363216	-1.9776
15	SNP_A-4216070	rs11636599	31406521	-1.9617
15	SNP_A-8307967	rs613479	32146482	-1.8192
15	SNP_A-8344743	rs347894	32452256	-1.8942
15	SNP_A-8484313	rs499248	32860759	-1.9387
15	SNP_A-4284682	rs12102243	32929124	-1.926
15	SNP_A-8561181	rs2589536	33341564	-1.2954
15	SNP_A-2269400	rs6495785	33645875	-1.2471
15	SNP_A-8688898	rs1355294	34022328	-1.7601
15	SNP_A-8560533	rs6495834	34503310	-1.91
15	SNP_A-8363216	rs16949673	64282444	-1.9987
15	SNP_A-8401460	rs11855801	66100386	-1.907
15	SNP_A-2305195	rs2283462	90457354	-1.8112
15	SNP_A-8344643	rs1993975	96546307	-1.9771
15	SNP_A-2315039	rs352722	100137265	-2.646
16	SNP_A-8548560	rs216590	41263	-2.562
16	SNP_A-8299520	rs7191175	88622778	-3.1641
17	SNP_A-8349257	rs6502793	1424369	-1.9101
17	SNP_A-8628246	rs10852873	1551330	-1.9637
17	SNP_A-8475283	rs7225156	1842656	-1.9342
17	SNP_A-2253943	rs7221650	14396863	-1.9812
17	SNP_A-8353505	rs8077409	14926212	-1.9948
17	SNP_A-4290956	rs2779212	15817380	-1.9245
17	SNP_A-8296387	rs4646371	17401399	-1.8963
17	SNP_A-8390736	rs2519861	27895953	-1.9707
17	SNP_A-2045583	rs12952139	28614946	-1.998
17	SNP_A-8468300	rs11079107	49444406	-1.9562
17	SNP_A-8396651	rs963955	50741825	-1.9907
17	SNP_A-1864333	rs7212458	50951397	-1.9645
17	SNP_A-8407866	rs11650568	51424042	-1.9768
17	SNP_A-2201441	rs1024637	55393675	-1.9641
17	SNP_A-2103433	rs1197070	55989592	-1.9773
17	SNP_A-8564270	rs11653507	63322526	-1.0019
18	SNP_A-8385140	rs4797876	145566	-4.4966
18	SNP_A-8533435	rs6506577	8354107	-1.61
18	SNP_A-8546304	rs623152	10020105	-1.7189
18	SNP_A-8290233	rs7227395	40909586	-1.6549
18	SNP_A-8588287	rs3816125	42855609	-1.8553
18	SNP_A-2064430	rs9636107	51351115	-1.6336
19	SNP_A-8691926	rs7246500	266788	-3.8373
19	SNP_A-8627982	rs40282	14154835	-1.9987
19	SNP_A-2234716	rs773901	16864789	-1.9943
19	SNP_A-2200469	rs1293703	58834663	-1.9985

Chromosome	SNP name	rs name	Pos (bp)	LOD score
19	SNP_A-8528135	rs306425	61221153	-1.7705
19	SNP_A-2138934	rs9676323	62922432	-1.9554
19	SNP_A-2272319	rs257673	63170308	-1.9508
19	SNP_A-8454839	rs7910	63785276	-2.1227
20	SNP_A-2029409	rs6078732	134405	-4.6971
20	SNP_A-2007377	rs997135	12278296	-1.9665
20	SNP_A-2264667	rs3004111	23430430	-1.4834
20	SNP_A-8616498	rs6057610	30705050	-1.906
20	SNP_A-8588682	rs2425209	34323746	-1.9923
20	SNP_A-8714054	rs6126741	51109167	-1.3676
20	SNP_A-1791994	rs6123669	55180999	-1.9297
20	SNP_A-8420449	rs6062682	62362264	-2.5063
21	SNP_A-2017937	rs2833271	31409620	-1.7698
21	SNP_A-2018498	rs8128036	35022791	-1.9112
21	SNP_A-8339501	rs2835123	36042651	-1.9773
21	SNP_A-1970577	rs2838447	44195316	-1.9962
21	SNP_A-2020433	rs2250238	44556894	-1.993
21	SNP_A-2020781	rs1892692	46762493	-1.8862
22	SNP_A-8605791	rs78914	16377569	-1.0237
22	SNP_A-8504529	rs369081	16666672	-1.9535
22	SNP_A-8652209	rs3180408	17030682	-1.9164
22	SNP_A-2147362	rs6010063	49503799	-4.5024

Table A.5.2. Flanking SNPs for chromosomal regions with LOD score < -2 (AIMAH-04).

The flanking SNPs for a locus are defined as the most proximal SNPs for which the LOD score is > -2, or, in cases where the LOD score of the flanking SNP was < -2, this was the most distal SNP studied on that chromosome in the linkage analysis. Flanking SNPs are presented in pairs. The LOD scores of the loci between a pair of SNPs was < -2.

SNP name: Affymetrix 6.0 SNP array ID.
rs name: SNP Reference ID (NCBI)
Pos(bp): Hg 18 reference

Chromosome	SNP name	rs name	Pos(bp)	LOD score
1	SNP_A-2162268	rs6702898	83238413	-1.5795
1	SNP_A-8389314	rs12039095	113659493	-1.9733
1	SNP_A-8575781	rs4387216	119481819	-1.5973
1	SNP_A-8578881	rs1686191	162927328	-1.8775
1	SNP_A-4248692	rs7413698	204188482	-1.9004
1	SNP_A-2119452	rs2841409	236990388	-1.9962
1	SNP_A-2190178	rs528011	238084930	-1.9972
1	SNP_A-2000845	rs2580238	240628728	-1.8968
2	SNP_A-8500963	rs4637157	19443	-2.03
2	SNP_A-2086633	rs10186254	3901481	-1.7141
2	SNP_A-8573175	rs10929647	5605816	-1.995
2	SNP_A-8549397	rs12052773	5746565	-2.0763
2	SNP_A-4276789	rs2041687	60259124	-1.7909
2	SNP_A-8674066	rs10928446	133666418	-1.974
2	SNP_A-8644269	rs6741098	134333576	-1.9905
2	SNP_A-8653365	rs1017752	143097449	-1.6665
2	SNP_A-4296181	rs12479030	174836918	-1.5808
2	SNP_A-8450099	rs10165928	192740087	-0.7549
3	SNP_A-8586224	rs2600056	2019132	-1.8292
3	SNP_A-1971561	rs6781770	6789988	-1.9847
3	SNP_A-8302994	rs1485144	10844362	-1.9035
3	SNP_A-8358091	rs7652333	72500062	-1.9799
3	SNP_A-4196879	rs11128275	72598650	-1.9905
3	SNP_A-2105281	rs11128317	73334831	-1.9904
3	SNP_A-8299402	rs9813855	149445813	-1.7947
3	SNP_A-8538007	rs1499625	174172170	-1.8686
3	SNP_A-2161510	rs692486	175497804	-1.6711
3	SNP_A-1795654	rs16860081	186700969	-1.8958
3	SNP_A-8442794	rs13084990	194476456	-1.6088
3	SNP_A-8349897	rs9877345	199293372	-2.8599
4	SNP_A-8323074	rs1810606	3579188	-0.7664
4	SNP_A-2272729	rs2870406	10837381	-1.8902
4	SNP_A-8594370	rs2192530	31324189	-1.9863
4	SNP_A-8313942	rs7675065	41377431	-1.9161
4	SNP_A-8517941	rs12508405	65799901	-1.7589
4	SNP_A-2176889	rs7685402	99879787	-1.3648
4	SNP_A-8445461	rs10015186	133190163	-1.698
4	SNP_A-8434577	rs10015934	191159251	-2.7371
5	SNP_A-8323401	rs7727015	238498	-2.4991
5	SNP_A-8457130	rs2905191	9944201	-1.4637

Chromosome	SNP name	rs name	Pos(bp)	LOD score
5	SNP_A-1910603	rs2929724	17253427	-0.3821
5	SNP_A-8694900	rs6881033	67564947	-1.295
5	SNP_A-8638249	rs10073851	148603410	-1.9364
5	SNP_A-4245215	rs11167702	154180456	-1.7579
5	SNP_A-8646874	rs2591576	165340637	-1.8022
5	SNP_A-8562124	rs753860	168428668	-1.9998
6	SNP_A-8614001	rs6933943	129030995	-1.9964
6	SNP_A-8523219	rs2049957	166771310	-1.9709
6	SNP_A-2314170	rs932356	167018425	-1.9587
6	SNP_A-8643632	rs4507568	170738569	-1.8523
7	SNP_A-8463613	rs11763364	174346	-2.7718
7	SNP_A-1911696	rs4283932	13382200	-0.3838
7	SNP_A-1961161	rs11773111	18049332	-0.2369
7	SNP_A-8635903	rs6973443	46620976	-1.2315
7	SNP_A-2150876	rs1079550	69782465	-1.4259
7	SNP_A-2299263	rs6975355	73043391	-1.8959
7	SNP_A-8368268	rs6467158	79893846	-1.7514
7	SNP_A-4266441	rs4464906	81111692	-1.011
7	SNP_A-8431155	rs12704482	89228376	-0.7775
7	SNP_A-8568608	rs264372	103253294	-1.4796
7	SNP_A-8708440	rs2106912	111502892	-0.5163
7	SNP_A-4212597	rs2106900	113909742	-0.3863
7	SNP_A-2208035	rs1038638	130011632	-1.7471
7	SNP_A-8403583	rs2363821	139856282	-1.456
7	SNP_A-2013020	rs6464211	151504786	-1.9649
7	SNP_A-4228805	rs6958122	152553181	-1.9886
8	SNP_A-8697067	rs10108633	1087659	-1.9767
8	SNP_A-2013125	rs7817362	4912668	-0.8108
8	SNP_A-8613278	rs10091985	27796759	-1.9924
8	SNP_A-8616202	rs10958540	39200978	-1.998
8	SNP_A-8524337	rs7832777	41073862	-1.9847
8	SNP_A-2196602	rs1849057	76378720	-1.7545
8	SNP_A-2038413	rs2139323	78881275	-1.8904
8	SNP_A-4241858	rs10956590	91406063	-1.9003
8	SNP_A-8306768	rs13252957	113682979	-1.95
8	SNP_A-8391361	rs3802266	124054889	-1.5763
8	SNP_A-1994974	rs17292662	125185414	-1.7562
8	SNP_A-2013811	rs7834246	127377339	-1.9624
9	SNP_A-8366025	rs7035770	658887	-3.1096
9	SNP_A-4273037	rs6475326	19376565	-1.9222
10	SNP_A-8288324	rs1414767	83962748	-1.9767
10	SNP_A-2050016	rs11016797	131140060	0.3436
11	SNP_A-8291290	rs6484834	36272744	-1.451
11	SNP_A-1857704	rs10897105	60188058	-1.9696
11	SNP_A-4279978	rs7130258	78453840	-1.9985
11	SNP_A-8538895	rs947937	91630656	-1.0761
11	SNP_A-8685885	rs1941863	97424073	-1.2419
11	SNP_A-2092191	rs907645	105823373	0.2083
11	SNP_A-2021432	rs3816621	122323521	-0.6544
11	SNP_A-8486454	rs1290018	123050386	-1.9682
11	SNP_A-8583760	rs2846241	123578972	1.8853
11	SNP_A-4268404	rs7104706	134381320	-2.4541
12	SNP_A-8438901	rs2284328	173404	-2.2222
12	SNP_A-8483297	rs887303	3576792	-1.1457

Chromosome	SNP name	rs name	Pos (bp)	LOD score
12	SNP_A-8320920	rs7485573	7348484	-1.89
12	SNP_A-4234025	rs7957593	50284957	-1.5843
12	SNP_A-8697948	rs2712549	126203877	-1.9629
12	SNP_A-2261470	rs10744327	126902420	-1.925
12	SNP_A-8587128	rs10847481	127003299	-1.9258
12	SNP_A-8470265	rs12422899	132202402	-2.1856
13	SNP_A-8697229	rs9511255	18516304	-2.1955
13	SNP_A-4240546	rs9508716	29844126	-1.6094
13	SNP_A-2173169	rs7322610	91524658	-1.0861
13	SNP_A-8315664	rs2701318	114082644	-2.0138
14	SNP_A-1937259	rs7149011	20632400	-1.8557
14	SNP_A-8300615	rs2146918	24470949	-0.8929
14	SNP_A-8608989	rs178231	25982061	-1.8453
14	SNP_A-2203536	rs10483420	32547001	-1.9491
14	SNP_A-2191831	rs10140669	33517980	-1.958
14	SNP_A-8309466	rs2281653	54324025	-1.6994
15	SNP_A-2132755	rs7179358	18451755	-2.0401
15	SNP_A-2022976	rs2337980	30231488	-1.9865
15	SNP_A-4299546	rs9745297	32564059	-1.8522
15	SNP_A-8628025	rs11634571	87996716	-1.9754
15	SNP_A-1837740	rs6497126	92086173	-1.2269
15	SNP_A-2315039	rs352722	100137265	-2.4534
16	SNP_A-8644386	rs9939418	942527	-1.768
16	SNP_A-1804492	rs2731740	56791457	-1.9752
16	SNP_A-8580756	rs245620	58615396	-1.9111
16	SNP_A-1908117	rs4782872	80986567	-1.9942
17	SNP_A-2107233	rs394747	509245	-1.9402
17	SNP_A-8526217	rs8071759	9185826	-1.6935
17	SNP_A-4229163	rs11078827	9926566	-1.8624
17	SNP_A-2299055	rs1263984	47183329	-1.917
17	SNP_A-8692173	rs888206	50823212	-1.8022
17	SNP_A-8594213	rs8072762	77894537	-1.9029
18	SNP_A-8637940	rs2339102	22051851	-1.8844
18	SNP_A-2067624	rs3865419	54156174	-1.9609
18	SNP_A-1919184	rs9319956	55745048	-1.959
18	SNP_A-1864646	rs8083025	73284898	-1.398
18	SNP_A-8650400	rs517543	73496612	-1.3879
18	SNP_A-2050874	rs1458223	74306903	-1.9955
19	SNP_A-4259673	rs983495	35989883	-1.0224
19	SNP_A-2068989	rs17691999	37146312	-0.7921
19	SNP_A-4288494	rs329929	57693111	-1.8889
19	SNP_A-8454839	rs7910	63785276	-2.1975
20	SNP_A-4303718	rs6075475	19059256	-1.9452
20	SNP_A-8509818	rs456210	44515378	-1.4434
20	SNP_A-2194952	rs99595	55423214	-1.9755
20	SNP_A-8354642	rs944240	59632486	-1.7384
20	SNP_A-8333850	rs1884330	61326335	-1.8541
20	SNP_A-2251704	rs3003137	62301246	-1.9802
21	SNP_A-4215536	rs116136	14786994	-1.9677
21	SNP_A-1970577	rs2838447	44195316	-1.9234

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

Table A.6.1. Regions detected by genomic segmentation

Cytoband	Start	End	Copy number	Mean
1p34.3	39239246	39305795	Deletion	1.12459
2q35	218928254	218946954	Deletion	1.22565
3p14.3	57708441	57739995	Deletion	1.01161
3q13.13	110476697	110519269	Deletion	1.22178
4p14	40356495	40404575	Deletion	1.23141
7q22.2	104780373	105001958	Deletion	1.27223
7q31.1	109228940	109229030	Amplification	4.07057
9p22.1	19092905	19207461	Deletion	1.29106
11q12.3	61920396	61955612	Deletion	1.18079
12q24.31	121524943	121547099	Deletion	0.985099
19q13.2	44155385	44189994	Deletion	1.00493

Table A.6.2. Regions detected by Hidden-Markov Modelling

Cytoband	Start	End	Copy number	Region average
2p15	61702251	61708548	Deletion	0.688773
3p22.3	32823004	32861706	Deletion	1.27964
7q11.23	75804210	75886092	Deletion	1.18384
12q24.31	121398447	121557331	Deletion	1.22382
19p13.2	11123867	11242997	Deletion	1.28173
19p13.3	2919991	2940836	Deletion	0.738378
22q12.1	27558044	27560196	Deletion	0.658943
MT	410	16150	Amplification	2.64065

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

ABCA1	ARHGAP24	BCL6	C15orf55	CDC73	CHEK2	CXCR7	ELF4	ETV6	FGFR2	FSTL1
ABCB1	ARHGAP26	BCL7A	C5	CDH1	CHIC2	CYFIP1	ELL	ETV7	FGFR3	FSTL3
ABCC1	ARHGEF12	BCL9	C5orf44	CDH10	CHN1	CYLD	EML4	EVI1	FGR	FUS
ABCC3	ARHGEF12	BCR	CACNA2D4	CDH11	CHUK	DACH2	ENG	EWSR1	FH	FYN
ABCC4	ARID2	BDP1	CALR	CDH13	CIC	DAD1	EP300	EXT1	FHIT	FZD2
ABCD4	ARNT	BIRC3	CARS	CDH20	CIITA	DAPK1	EPAS1	EXT2	FHOD3	FZD3
ABCG2	ARPP-21	BLK	CARTPT	CDH24	CLK3	DCLK3	EPHA3	EZH2	FIGF	FZD4
ABI1	ASPSCR1	BLM	CASC5	CDH5	CLP1	DDB2	EPHA5	FABP5	FIP1L1	FZD6
ABL1	ATF1	BLNK	CAT	CDH6	CLTC	DDIT3	EPHA6	FAM120AOS	FLCN	GADD45A
ABL2	ATF6B	BMI1	CBFA2T3	CDK4	CLTCL1	DDX10	EPHB6	FAM123B	FLG2	GADD45B
ACSL6	ATIC	BMP2	CBFB	CDK6	CNBP	DDX6	EPS15	FAM160B2	FLI1	GADD45G
ADAMTS6	ATM	BMP2K	CBL	CDK7	CNTNAP5	DEK	ERBB2	FANCA	FLNB	GAS1
ADAMTSL3	ATP8B1	BMP4	CCDC125	CDKN2A	COL1A1	DLGAP5	ERBB2IP	FANCC	FLT1	GAS2
AFF1	AURKA	BMPR1A	CCDC6	CDKN2B	COX6C	DPP6	ERBB4	FANCD2	FLT3	GAS7
AFF3	B9D1	BRAF	CCNB1	CDKN2C	CREB1	DUSP22	ERC1	FANCE	FLT4	GATA1
AFF4	BAD	BRCA1	CCNB1IP1	CDX2	CREBBP	DUSP8	ERCC2	FANCF	FNBP1	GATA2
AKAP9	BAI3	BRCA2	CCND1	CEBPA	CRTC1	DUX4	ERCC3	FANCG	FNDC1	GATA3
AKT2	BARD1	BRD2	CCND2	CEBPB	CSF1R	E2F1	ERCC4	FAS	FOS	GBE1
AKT3	BCAS3	BRD4	CCND3	CEBPD	CSF2RA	EBF1	ERCC5	FBXW2	FOXM1	GLI2
ALDH6A1	BCL10	BRIP1	CCNE1	CEBPE	CSF2RB	EGFL6	ERG	FBXW7	FOXO1	GMPS
ALK	BCL11A	BTG1	CD180	CENPH	CSF3R	EGFR	ERN1	FCGR2B	FOXO3	GNAS
ALKBH3	BCL11B	BTG2	CD44	CENPK	CSNK1E	EIF4A2	ETS1	FCRL4	FOXO4	GOLGA5
APC	BCL2	BTLA	CD47	CEP110	CTNNA2	ELA2	ETS2	FEV	FOXP4	GOPC
ARFRP1	BCL2L11	BUB1B	CDC25C	CHD5	CTNNB1	ELF1	ETV1	FGFR1	FRK	GPC3
ARHGAP18	BCL3	C15orf21	CDC42EP1	CHD7	CTRL	ELF2	ETV4	FGFR1OP	FSCB	GPHN

GPR123	HOXD11	ITPR1	LEF1	MASTL	MNX1	NCKIPSD	NTRK1	PCDH20	PMS2	PTEN
GPR124	HOXD13	JAG2	LHFP	MCCC2	MPL	NCOA2	NTRK3	PCDH24	POLB	PTH2R
GPR183	HRAS	JAK1	LIFR	MCL1	MRE11A	NCOA4	NUMA1	PCDH8	POLR2A	PTPN11
GRIA3	HSP90AA1	JAK2	LIN52	MDM2	MRPS27	NEIL1	NUMB	PCDH9	POU2AF1	PTPN14
GRID1	HSP90AB1	JAK3	LMO1	MDM4	MSH2	NF1	NUP214	PCM1	POU5F1	PTPN6
GRID2	HTR1A	JAZF1	LMO2	MDS1	MSH6	NF2	NUP98	PCSK7	PPARG	PTPRD
GSK3B	ICAM1	KBTBD11	LPP	MEGF9	MSI2	NFAT5	OBSCN	PDE4DIP	PPP2R4	PTPRD
GTF2H2	ID2	KDR	LRP1B	MEIS1	MSN	NFKB2	OCLN	PDGFB	PPWD1	PTPRT
HCK	IDH1	KDSR	LRP6	MEN1	MTCP1	NGRN	OLIG2	PDGFRA	PRAME	PVT1
HIF1A	IGFBP5	KIAA1128	LTBP3	MET	MUC1	NIN	OMA1	PDGFRB	PRCC	RAB3IL1
HIP1	IKZF1	KIAA1549	LTK	MGC42105	MUTYH	NLN	OMD	PER1	PRDM1	RABEP1
HIST1H4I	IKZF2	KIAA1618	LYL1	MGMT	MXD3	NONO	OR2W1	PHF19	PRDM16	RAD17
HLF	IKZF3	KIT	LYN	MKL1	MYB	NOTCH1	PAFAH1B2	PHF2	PRF1	RAD51
HLF	IL2	KLF1	MAD1L1	MKNK2	MYC	NOTCH3	PAK3	PHOX2B	PRG4	RAD51C
HM13	IL21R	KLF11	MAF	MLF1	MYCL1	NOTCH4	PAK7	PICALM	PRKAA2	RAD51L1
HMGA1	IL2RG	KLF3	MAFB	MLH1	MYCN	NPM1	PALB2	PIK3CA	PRKAR1A	RAD51L3
HMGA2	IL3RA	KLF4	MALAT1	MLL	MYCT1	NR3C1	PARK2	PIK3R1	PRKDC	RAD52
HNF1A	IL7R	KLF5	MALT1	MLL3	MYH11	NR4A3	PATZ1	PIM1	PRLR	RAD9A
HNRNPA2B1	ING4	KLF6	MAML1	MLLT1	MYH9	NRAS	PAX3	PIM2	PRMT6	RAG1
HOXA11	INHBA	KNDC1	MAML2	MLLT10	MYST3	NRIP3	PAX5	PKHD1	PRODH	RAG2
HOXA13	IRF4	KRAS	MANEA	MLLT11	MYST4	NRP1	PAX7	PLAG1	PRRX1	RAI14
HOXA3	IRS2	KTN1	MAP1B	MLLT3	MZF1	NRP2	PAX8	PLCB1	PSIP1	RANBP17
HOXA9	ISCA1L	LASP1	MAP2	MLLT4	NACA	NSD1	PBX1	PLK1	PSMD5	RAP1GDS1
HOXC11	ITGA5	LCK	MAP2K4	MLLT6	NAIP	NTN4	PCDH15	PML	PTCD2	RAPGEF1
HOXC13	ITK	LCP1	MARVELD2	MN1	NBN	NTNG1	PCDH17	PMS1	PTCH1	RARA

RB1	SEPT5	SMN1	TBX22	TNFRSF17	VASN	ZEB1
RBM15	SEPT6	SMO	TCEA1	TOM1L1	VCAN	ZEB2
RECQL4	SERF1A	SNAI1	TCF12	TOP1	VEGFA	ZFPM1
REEP6	SET	SNAI2	TCF3	TOX	VEGFB	ZMYM2
REL	SF3B1	SOCS1	TCF7L2	TP53	VEGFC	ZMYND10
REST	SFPQ	SOCS2	TCL1A	TP73	VHL	ZNF155
RET	SFRS12	SOX4	TCL6	TPM3	VNN1	ZNF331
RGS7BP	SFRS12Ip1	SOX6	TEC	TPM4	VSX2	ZNF384
RHOH	SFRS3	SPECC1	TEK	TPR	WAS	ZNF480
RNF180	SGTB	SPI1	TERC	TRAF1	WHSC1	ZNF521
ROBO1	SH3GL1	SRC	TERT	TREM1	WHSC1L1	ZNF668
ROBO4	SH3TC2	SS18	TET1	TRIM23	WNK2	ZNF674
ROS1	SLC15A1	SS18L1	TET2	TRIM24	WNT5B	
RPL22	SLC22A1	SSX1	TFE3	TRIM27	WNT9B	
RPN1	SLC22A2	SSX2	TFEB	TRIM33	WRN	
RPS6KA2	SLC22A3	SSX4	TFG	TRIP11	WT1	
RUNX1	SLC22A5	STIL	TFPT	TSC1	WWOX	
RUNX1T1	SLC30A5	STK11	TFRC	TSC2	XPA	
SALL4	SLC38A3	SUFU	TGFBR2	TSHR	XPC	
SATL1	SLC45A3	SUZ12	THRA	TSPAN33	XRCC2	
SBDS	SLCO1A2	SYK	THRAP3	TSPAN8	XRCC3	
SDCCAG10	SMAD2	TAF15	TLX1	TTL	XRN2	
SDHB	SMAD3	TAF1L	TLX3	TWIST1	YES1	
SDHC	SMAD4	TAL1	TMEM121	UBLCP1	YSK4	
SDHD	SMARCB1	TAL2	TMPRSS2	USP6	ZBTB16	

Appendix 8: Health-related quality of life assessments

Fatigue scale

Please circle the response that most accurately describes your current symptoms.

Do you have problems with tiredness?
Better than usual No more than usual Worse than usual Much worse than usual

Do you need to rest more?
Better than usual No more than usual Worse than usual Much worse than usual

Do you feel sleepy or drowsy?
Better than usual No more than usual Worse than usual Much worse than usual

Do you have problems starting things?
Better than usual No more than usual Worse than usual Much worse than usual

Are you lacking in energy?
Better than usual No more than usual Worse than usual Much worse than usual

Do you have less strength in your muscles?
Better than usual No more than usual Worse than usual Much worse than usual

Do you feel weak?
Better than usual No more than usual Worse than usual Much worse than usual

Do you have difficulty concentrating?
Better than usual No more than usual Worse than usual Much worse than usual

Do you have problems thinking clearly?
Better than usual No more than usual Worse than usual Much worse than usual

Do you make slips of the tongue when speaking?
Better than usual No more than usual Worse than usual Much worse than usual

How is your memory?
Better than usual No more than usual Worse than usual Much worse than usual

Adapted from Chalder, T., 1993. Development of a Fatigue Scale. J Psychosom Res 37, 147-53.

SF-36 health survey

Instructions: This set of questions asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. Answer every question by marking the answer as indicated. If you unsure about how to answer a question please give the best answer you can.

1. In general would you say your health is: **(Please choose one response)**
   Excellent
   Very good
   Good
   Fair
   Poor

2. Compared to one year ago, how would you rate your health in general now? **(Please choose one response)**
   Much better than one year ago
   Somewhat better now than one year ago
   About the same as one year ago
   Somewhat worse now than one year ago
   Much worse now than one year ago

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? **(Please circle one number on each line)**

Activities	Yes, a lot	Yes, a little	Not at all
Vigorous activities (running, lifting heavy objects, strenuous exercise)	1	2	3
Moderate activities (moving a table, pushing a vacuum cleaner, bowling, golf)	1	2	3
Lifting or carrying groceries	1	2	3
Climbing **several** flights of stairs	1	2	3
Climbing **one** flight of stairs	1	2	3
Bending, kneeling or stooping	1	2	3
Walking **more than a mile**	1	2	3
Walking **several** blocks	1	2	3
Walking **one** block	1	2	3
Bathing or dressing yourself	1	2	3

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health? (Please circle one number on each line)

	Yes	No
Cut down on the **amount of time** you spent on work or other activities	1	2
Accomplished less than you would like	1	2
Were **limited** in the **kind** of work or other activities	1	2
Had **difficulty** performing the work or other activities (for example, it took extra effort)	1	2

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (e.g. feeling depressed or anxious)? **(Please circle one number on each line)**

	Yes	No
Cut down on the **amount of time** you spent on work or other activities	1	2
Accomplished less than you would like	1	2
Didn't do work or other activities as **carefully** as usual	1	2

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours or groups? **(Please choose one response)**

Not at all
Slightly
Moderately
Quite a bit
Extremely

7. How much physical pain have you had during the past 4 weeks? **(Please choose one response)**

None
Very mild
Mild
Moderate
Severe
Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)? **(Please choose one response)**

Not at all
A little bit
Moderately
Quite a bit
Extremely

9. These questions are about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that is closest to the way you have been feeling for each item. **(Please choose one response on each line)**

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time
Did you feel full of life?	1	2	3	4	5
Have you been a very nervous person?	1	2	3	4	5
Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5
Have you felt calm and peaceful?	1	2	3	4	5
Did you have a lot of energy?	1	2	3	4	5
Have you felt downhearted and blue?	1	2	3	4	5
Did you feel worn out?	1	2	3	4	5
Have you been a happy person?	1	2	3	4	5
Did you feel tired?	1	2	3	4	5

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives etc)? **(Choose one response)**

All of the time
Most of the time
Some of the time
A little of the time
None of the time

11. How TRUE or FALSE is each of the following statements for you? **(Please choose one response on each line)**

	Definitely true	Mostly true	Don't know	Mostly true	Definitely false
I seem to get sick a little easier than other people I know	1	2	3	4	5
I am as healthy as anybody I know	1	2	3	4	5
I expect my health to get worse	1	2	3	4	5
My health is excellent	1	2	3	4	5

Thank you!

Adapted from Ware, Jr, J.E., Sherbourne, C.D., 1992. The MOS 36-item short form health survey (SF-36). I. Conceptual framework and item selection. Medical Care 30, 478-83.

THE
GENERAL HEALTH
QUESTIONNAIRE
GHQ28

Please read this carefully.

We should like to know if you have had any medical complaints and how your health has been in general, *over the past few weeks.* Please answer ALL the questions on the following pages simply by underlining the answer which you think most nearly applies to you. Remember that we want to know about present and recent complaints, not those that you had in the past.

It is important that you try to answer ALL the questions.

Thank you very much for your co-operation.

Have you recently

been feeling perfectly well and in good health?	Better than usual	Same as usual	Worse than usual	Much worse than usual
been feeling in need of a good tonic?	Not at all	No more than usual	Rather more than usual	Much more than usual
been feeling run down and out of sorts?	Not at all	No more than usual	Rather more than usual	Much more than usual
felt that you are ill?	Not at all	No more than usual	Rather more than usual	Much more than usual
been getting any pains in your head?	Not at all	No more than usual	Rather more than usual	Much more than usual
been getting a feeling of tightness or pressure in your head?	Not at all	No more than usual	Rather more than usual	Much more than usual
been having hot or cold spells?	Not at all	No more than usual	Rather more than usual	Much more than usual

Have you recently

lost much sleep over worry?	Not at all	No more than usual	Rather more than usual	Much more than usual
had difficulty in staying asleep once you are off?	Not at all	No more than usual	Rather more than usual	Much more than usual
felt constantly under strain?	Not at all	No more than usual	Rather more than usual	Much more than usual
been getting edgy and bad-tempered?	Not at all	No more than usual	Rather more than usual	Much more than usual
been getting scared or panicky for no good reason?	Not at all	No more than usual	Rather more than usual	Much more than usual
found everything getting on top of you?	Not at all	No more than usual	Rather more than usual	Much more than usual
been feeling nervous and strung up?	Not at all	No more than usual	Rather more than usual	Much more than usual

Have you recently

been managing to keep yourself busy and occupied?	More so than usual	Same as usual	Rather less than usual	Much less than usual
been taking longer over the things you do?	Quicker than usual	Same as usual	Longer than usual	Much more than usual
felt on the whole you were doing things well?	Better than usual	About the same	Less well than usual	Much less well
been satisfied with the way you've carried out your task?	More satisfied	About same as usual	Less satisfied than usual	Much less satisfied
felt that you are playing a useful part in things?	More so than usual	Same as usual	Less useful than usual	Much less useful
felt capable of making decisions about things?	More so than usual	Same as usual	Less so thanusual	Much less capable
been able to enjoy your normal day-to-day activities?	More so than usual	Same as usual	Less so than usual	Much less than usual

Have you recently

been thinking of yourself as a worthless person?	Not at all	No more than usual	Rather more than usual	Much more than usual
felt that life is entirely hopeless?	Not at all	No more than usual	Rather more than usual	Much more than usual
felt that life isn't worth living?	Not at all	No more than usual	Rather more than usual	Much more than usual
thought of the possibility that you might make away with yourself?	Definitely not	I don't think so	Has crossed my mind	Definitely have
found at times you couldn't do anything because your nerves were too bad?	Not at all	No more than usual	Rather more than usual	Much more than usual
found yourself wishing you were dead and away from it all?	Not at all	No more than usual	Rather more than usual	Much more than usual
found that the idea of taking your own life kept coming into your mind?	Definitely not	I don't think so	Has crossed my mind	Definitely has

Adapted from Goldberg, D.P., 1972. The Detection of Psychiatric Illness by Questionnaire. London: Oxford University Press.

Gastrointestinal Symptom Rating Scale

Please answer the following questions in relation to the past 4 weeks:

1. Abdominal pains (representing subjectively experienced bodily discomfort, aches and pains)
0 No or transient pain
1 Occasional aches and pains interfering with some social activities
2 Prolonged and troublesome aches and pains causing requests for relief and interfering with many social activities
3 Severe or crippling pains with impact on all social activities

If you answered 1-3, please provide a description of the pain:
a. Location____
b. Type – sharp, dull, burning, other________________________________
c. Duration____
d. Pattern when present – constant, colicky, other______________________
e. Relieved by____
f. Made worse by____
g. Factors known to bring the pain on – foods, drinks, lying down, other____

2. Heartburn (representing retrosternal discomfort or burning sensations)
0 No or transient heartburn
1 Occasional discomfort of short duration
2 Frequent episodes of prolonged discomfort; requests for relief
3 Continuous discomfort with only transient relief by antacids

3. Acid regurgitation (representing sudden regurgitation of acid gastric content)
0 No or transient regurgitation
1 Occasional troublesome regurgitation
2 Regurgitation once or twice a day; requests for relief
3 Regurgitation several times a day; only transient and insignificant relief by antacids

4. Sucking sensations in the epigastrium (representing a sucking sensation in the epigastrium with relief by food or antacids; if food or antacids are not available, the sucking sensations progress to aches and pains)
0 No or transient sucking sensation
1 Occasional discomfort of short duration; no requests for food or antacids between meals
2 Frequent episodes of prolonged discomfort; requests for food and antacids between meals
3 Continuous discomfort; frequent requests for food or antacids between meals

5. Nausea and vomiting (representing nausea which may increase to vomiting)
0 No nausea
1 Occasional episodes of short duration
2 Frequent and prolonged nausea; no vomiting
3 Continuous nausea; frequent vomiting

6. Borborygmus (representing reports of abdominal rumbling)

0 No or transient borborygmus (abdominal rumbling)
1 Occasional troublesome borborygmus (abdominal rumbling) of short duration
2 Frequent and prolonged episodes which can be mastered by moving without impairing social performance
3 Continuous borborygmus (abdominal rumbling) severely interfering with social performance

7. Abdominal distension (representing bloating with abdominal gas)

0 No or transient distension
1 Occasional discomfort of short duration
2 Frequent and prolonged episodes which can be mastered by adjusting the clothing
3 Continuous discomfort seriously interfering with social performance

8. Eructation (representing reports of belching)

0 No or transient eructation (belching)
1 Occasional troublesome eructation (belching)
2 Frequent episodes interfering with some social activities
3 Frequent episodes seriously interfering with social performance

9. Increased flatus (representing reports of excessive wind)

0 No increased flatus
1 Occasional discomfort of short duration
2 Frequent and prolonged episodes interfering with some social activities
3 Frequent episodes seriously interfering with social performance

10. Decreased passage of stools (representing reduced bowel motions)

0 Once a day
1 Every third day
2 Every fifth day
3 Every seventh day or less frequently

11. Increased passage of stools (representing increased bowel motions)

0 Once a day
1 Three times a day
2 Five times a day
3 Seven times a day or more frequently

12. Loose stools (representing reported loose bowel motions)

0 Normal consistency
1 Somewhat loose
2 Runny
3 Watery

13. Hard stools (representing reported hard bowel actions)

0 Normal consistency
1 Somewhat hard
2 Hard
3 Hard and fragmented, sometimes in combination with diarrhoea

14. Urgent need for defecation (representing reports of urgent need for defecation, feelings of incomplete control, and inability to control defecation)

0 Normal control
1 Occasional feelings of urgent need for defecation
2 Frequent feelings of urgent need for defecation with sudden need for a toilet interfering with social performance
3 Inability to control defecation

15. Feeling of incomplete evacuation (representing reports of defecation with straining and a feeling of incomplete evacuation of stools)

0 Feeling of complete evacuation without straining
1 Defecation somewhat difficult; occasional feelings of incomplete evacuation
2 Defecation definitely difficult; often feelings of incomplete evacuation
3 Defecation extremely difficult; regular feelings of incomplete evacuation

Adapted from Svedlund, J., Sjödin, I., Dotevall, G., 1988. GSRS – A clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 33, 129-34.

Free-response questionnaire

Name: ____

For completion at the end of the study

Which treatment, if either, did you prefer?

Treatment 1 □
Treatment 2 □
Neither □
Unsure □
Can't remember □

Please provide reasons for your preference: ____

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