Pathophysiology and Novel Therapeutic Approaches in Autoimmune Addison's Disease

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Declaration

This thesis is based on my studies performed in the Institute of Genetic Medicine, Newcastle University from August 2010- August 2013. The contents of this thesis are original. . All the studies were performed solely by myself, apart from some of the immunoblotting and Elisa works, which the research assistant, Katie MacArthur, assisted me with. No part of this thesis has been submitted for the award of any other degree.

Acknowledgements

Abstract

Autoimmune Addison's disease (AAD) is a debilitating condition and affected patients rely on lifelong steroid replacement. Despite treatment, many patients have increased morbidity and mortality. The disease's rarity has precluded large scale genomic or cellular studies in humans, resulting in an incomplete picture of the pathophysiology of AAD. This thesis details my research on the pathophysiology and novel therapeutic approaches in AAD.

I performed two candidate gene studies on susceptibility alleles that have been implicated in other autoimmune diseases to explore potential causal pathways of these genetic determinants in AAD. The common variant 307*Ser allele of *CD226* gene was found to contribute to AAD susceptibility as part of autoimmune polyendocrinopathy type 2. Two genetic variants from a panel of rare and functionally defective variants in the sialic acid acetylesterase (*SIAE*) gene were identified but they were not significantly associated with AAD.

I explored new therapeutic approaches in AAD using a therapeutic regimen of parenteral $ACTH_{1-24}$. This study demonstrated the presence of residual adrenal function in patients with established AAD, which was remediable to $ACTH_{1-24}$ therapy. Clinical remission was induced in 2 of 13 study participants. However, some patients developed cutaneous reaction following $ACTH_{1-24}$ injection, with one failing to sustain clinical remission. I proceeded to investigate the potential immunologic effects induced by ACTH therapy using ELISA and immunoblotting methods. Auto-reactivity against $ACTH_{1-24}$ and $ACTH_{1-39}$ was detected, which could account for the side effects and treatment resistance observed.

Finally, I attempted to characterise adrenocortical progenitor cells through cell cultures, immunocytochemistry and flow cytometry. A mesenchymal stem cell-like (MSC) population was isolated from human adrenals for the first time. My studies have advanced knowledge relevant to regenerative medicine approaches to adrenal failure, and the finding of residual adrenal function in some Addison's disease patient opens an important therapeutic window for this condition.

Abbreviations

α-MEM	α-modified Eagle's medium
21-OH	21-hydroxylase
AA	Adrenal autoantibodies
AAD	Autoimmune Addison's disease
ACSC	Adrenocortical stem cell
ACTH	Adrenocorticotropic hormone
Ad4BP	Ad4 binding protein
AGP	Adrenogonadal primodium
AH	Autoimmune hypothyroidism
APS1	Autoimmune polyendocrinopathy syndrome type 1
APS2	Autoimmune polyendocrinopathy syndrome type 2
ARS	Alizarin Red S
BCR	B cell receptor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CEDAM	Centre for Diabetes and Metabolism
CFU-Fs	Colony forming units-fibroblasts
CM	Complete growth medium
CMV	Cytomegalovirus
CTLA4	Cytotoxic T lymphocyte antigen 4
CXCL10	CXC chemokine ligand 10
CYP	Cytochrome P450
CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1
CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-pheny-lindole
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region of the X chromosome, gene 1
DdH$_2$O	Double distilled water
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	5-α-dihydrotestosterone
DMEM: HamF12	Dulbecco's Modified Eagle Medium/Nutrient mixture F-12
DNAM-1	DNAX accessory molecule-1
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FAdE	Fetal adrenal-specific SF1 enhancer
FBS	Fetal bovine serum
FCS	Forward scatter
FGF2	Fibroblast growth factor 2
GAG	Glycoaminoglycan
GC/MS	Gas chromatography/mass spectrometry
GR	Glucocorticoid receptor
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSD	Hydroxysteroid dehydrogenase

IL-6	Interleukin 6
INF-γ	Interferon γ
MAF	Minor allele frequency
MALDI-TOF	Matric assisted laser desorption/ionisation-time of flight
MC2R	Melanocortin-2- receptor
MCM4	Minichromosome maintenance–deficient 4
MGB	Minor grove binder
MGPM	Mesenchymal stem cell growth promotion medium
MHC	Major histocompatibility complex
MICA	MHC class I chain-related gene A
MSC	Mesenchymal stem cell
MSH	Melanocyte stimulating hormone
MR	Mineralocorticoid receptor
MRAP	Melanocortin 2 receptor accessory protein
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NKG2D	Natural killer group 2, member D
NFκB	Nuclear factor Kb
NLR	NOD-like receptors (NLRs
NNT	Nicotinamide nucleotide transhydrogenase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phophate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDL-1	Programmed cell death ligand 1
POMC	Proopiomelanocortin
PRL	Plasma renin level
Ptch1	Patched 1
PTPN22	Protein tyrosine phosphatase non-receptor 22
RE	Restriction endonuclease
RFLP	Restriction fragment length polymorphism
RoSA	Revival of autochthonous adrenocortical stem cells in autoimmune Addison's disease
SDS	Sodium dodecyl sulphate
SF1	Steroidogenic factor 1
SHH	Sonic hedgehog
SIAE	Sialic acid acetylesterase
SIM	Selected-ion-monitoring
SLE	Systemic Lupus Erythematosus
Smo	Smoothened
SNP	Single nucleotide polymorphism
SSC	Side scatter
STAR	Steroidogenic acute regulatory protein
STAT1	Signal transducer and activator of transcription-1
STAT4	Signal transducer and activator of transcription-4
TMB	3,3',5,5'-Tetramethylbenzidin
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor

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Chapter 1.   Introduction

1.1 Background to Addison's disease

Addison's disease is a chronic condition caused by failure of the adrenal gland to produce the essential adrenocortical hormones, cortisol and aldosterone. These hormones are essential to maintaining homeostasis as well as the physiology and metabolism of multiple organs. It was first described in 1849 by Dr Thomas Addison, who discovered the pathological changes in the 'suprarenal capsule' (adrenal gland) during autopsies performed on patients who presented with a common cluster of symptoms: pigmentation, hypotension and lassitude (Addison 1855). Of the 11 autopsies described by him, at least six of them were due to tuberculous adrenalitis. However, he was intrigued by one of the patients who had adrenal fibrosis of unknown origin, characterised by inflammation of the adrenal gland with tissue contraction and atrophy. This was likely to be the first description of autoimmune adrenalitis. In line with Addison's autopsy results, tuberculosis infection was the most common cause of primary adrenal failure in the Western world, with 70% of adrenal glands examined during autopsy in 1930 found to be infected (Guttman 1930). However, autoimmune adrenalitis has now taken the lead and accounts for more than 80% of the cases in the Caucasian European population following the introduction of effective anti-tuberculosis therapy (Betterle et al. 2002, Arlt & Allolio 2003).

On the other hand, adrenal destruction can be attributed to other causes such as HIV (human immunodeficiency virus) or CMV (cytomegalovirus) infections, infiltrative diseases, adrenal haemorrhage or malignancy. Genetic disorders such as *DAX1* gene (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1) or steroidogenic factor 1(*SF1*) mutation, congenital adrenal hyperplasia, familial adrenocorticotropic hormone (ACTH) resistance syndromes and adrenoleucodystrophy (which is caused by a defect in the very long chain fatty acid transporter gene), are among

the rarer causes of primary adrenal insufficiency. A detailed discussion of the various causes of primary adrenal failure is depicted in table 1.1. As all the works on this thesis are based on autoimmune adrenalitis, the rest of the discussion will be focusing on autoimmune Addison's disease.

1.2   Autoimmune Addison's Disease (AAD)

Autoimmune Addison's disease (AAD) is a relatively rare endocrine condition with a prevalence of 110-140 cases per million people in the western world (Willis & Vince 1997, Laureti et al. 1999, Erichsen et al. 2009) , making it 30 times less frequent than type 1 diabetes and 200 fold rarer than autoimmune thyroid disease. However, in common with several other autoimmune disorders, the incidence of AAD has been rising, with about 8500 people in the UK suffering from AAD and around 200 newly diagnosed people each year (Lovas & Husebye 2002, Arlt & Allolio 2003). The common age of onset is between 30-50 years old and it is more prevalent in females (female to male ratio of 1.5-3.5:1) (Kong & Jeffcoate 1994, Willis & Vince 1997, Laureti et al. 1999, Lovas & Husebye 2002).

AAD is an organ-specific autoimmune condition, caused by selective immune-destruction of the steroid producing cells in the adrenal cortex, leading to a deficiency of the essential steroid hormones cortisol and aldosterone.   It is an inevitably fatal disease in the absence of treatment and affected patients have to be kept on lifelong steroid replacement for survival. This condition displays a wide spectrum of clinical manifestation, ranging from non-specific complaints of lethargy or dizziness to severe symptoms like hypoglycaemia, hypotension or even death in untreated cases.

Table 1.1.Causes of primary adrenal insufficiency

Aetiologies of primary adrenal failure	Pathological conditions
Adrenal destruction	Autoimmune adrenalitis (autoimmune Addison's disease) Infections (tuberculosis, fungal, viral eg CMV, HIV.) Infiltration by sarcoidosis, amyloidosis and haemochromatosis Haemorrhage (e.g Waterhouse-Friderichsen syndrome) Malignancy (metastatic disease) Adrenoleucodystrophy Adrenal thrombosis from systemic lupus erythematosus (SLE), trauma, antiphospolipid syndrome, panarteritis nodosa
Adrenal dysgenesis or hypoplasia	Genetic defects: Congenital adrenal hypoplasia (*DAX1*; *SF1* mutation); Allgrove's syndrome; Familial glucocorticoid deficiency type: Mutations on ACTH receptor (*MC2R*); melanocortin 2 receptor accessory protein (*MRAP*)(Metherell et al. 2005), steroidogenic acute regulatory protein (*STAR*)(Metherell et al. 2009), nicotinamide nucleotide transhydrogenase (*NNT*) (Meimaridou et al.2012) and minichromosome maintenance–deficient 4 (*MCM4*) (Hugher et al.2012) genes.
Impaired steroidogenesis	Congenital adrenal hyperplasia
Drugs	Ketoconazole, rifampicin, etomidate, cyproterone acetate and adrenolytic agents such as mitotane, aminoglutetimide, trilostane

Additionally, patients who suffer from this condition could remain undiagnosed with a prolonged course of subclinical disease, until intervening events spark off an acute adrenal crisis. It is commonly associated with autoimmune thyroid disease (~70%) and/ or type 1 diabetes (~20%), constituting the type 2 polyendocrinopathy syndrome (APS2) (Betterle et al. 2004, Owen & Cheetham 2009). Cross-sectional studies revealed that around 50% of patients with AAD have other autoimmune conditions (Nerup J 1974, Myhre et al. 2002, Falorni et al. 2004).

1.3 Presentation and natural history of AAD

As previously mentioned, the symptoms of primary adrenal insufficiency are mostly non-specific, causing it to be labelled as the 'master of disguise' in medical textbook. Patients with AAD commonly present with weakness, ill-defined fatigue, postural dizziness, weight loss and anorexia. These symptoms are non-specific and occur insidiously, making it challenging for physicians to make the right diagnosis, and many patients have thus been misdiagnosed with depression or anorexia nervosa. Nevertheless, skin or buccal pigmentation secondary to compensatory ACTH excess and salt craving are among the specific features not to be missed in AAD. Their presence in conjunction with other non-specific complaints is highly suggestive of primary adrenal insufficiency.

The clinical diagnosis of AAD is commonly preceded by a prolonged stage of subclinical adrenal hypofunction, although a small number of patients progress more rapidly and present with adrenal crisis at disease onset (Myhre et al. 2002). The finding of serum 21-hydroxylase autoantibody is the hallmark of AAD. It is seen in 20-80% of patients in cross-sectional studies, in comparison with <0.5% in the general population (Chen et al. 1996, Falorni et al.1997, Myhre et al. 2002). For those patients with normal adrenal function, the

presence of 21-hydrolylase antibody indicates a cumulative risk of ~20% of developing overt Addison's disease (Coco et al. 2006).

Based on the observed natural history of patients with positive adrenal autoantibodies, Bettele et al proposed the presence of 4 clinical stages in AAD, delineating the progressive deterioration in adrenal function (table 1.2). The clinical stages are characterised by the initial rise in plasma renin activity (PRA) and ACTH level prior to impaired cortisol response upon ACTH stimulation. This is followed by the manifestation of overt adrenal insufficiency at the final stage, commonly associated with pigmentation and fatigue (Betterle et al. 1988). Baker et al. recently suggested that borderline elevation in ACTH level (11-20 pmol/L) is a good predictor of progression to overt disease in preclinical AAD stage (Baker et al.2012). In addition to the biochemistry abnormalities described, it is not uncommon to find hyponatramia, hyperkalaemia, metabolic acidosis, hypoglycaemia, aneamia, lymphocytosis and mild eosinophilia among patients with untreated adrenal insufficiency.

Similar to many other autoimmune endocrine disorders, the presence of autoantibodies precedes the clinical manifestation of the disease. A few studies have followed up patients with positive adrenal antibodies. However, it is worth noting that the study numbers were relatively small due to the rarity of AAD and most of them did not segregate the type 1 autoimmune polyendocrinopathy syndrome (APS1) cohort from APS2/isolated AAD during analysis. Betterle et al. followed 17 subjects with positive adrenal autoantibodies (AA) and noted the presence of AA at as early as stage 0 (table 1.2), during the preclinical stage of AAD. As high as 41% (7/17) of them progressed to clinically overt disease after a mean follow up period of 3.2 years (range 6 months to 10 years) (Betterle et al. 1988). He also found that AA titre persisted but fluctuated throughout the preclinical stage, with some people demonstrating gradual decline in AA titres after the onset of the disease. In fact, two

patients had negative AA after a mean period of 35 months. De Bellis et al. followed 20

patients with positive AA and 11 of them remained persistently positive for AA with 2/11

(18%) developed clinically overt AD (De Bellis et al. 1993). A case series also reported

spontaneous remission in 6 patients with positive adrenal antibodies, who were at either

clinical stage 0 or 1. The disappearance of adrenal antibodies was accompanied by

spontaneous recovery of plasma renin level, 5 years after follow-up (De Bellis A et al 1991).

A larger study then followed 100 AA-positive and 63 AA-negative patients without AAD

for a maximum of 21 years (mean 6.0 yr, median 4.8). It was shown that 31% of

asymptomatic individuals with positive AA become symptomatic between 3 months and 11

years (Coco et al. 2006). In addition, 1% of the first-degree relatives of patients with AAD

have anti-21hydroxylase antibodies and about 15% of them would have developed overt

AAD over an observational time of 6 years (Coco et al. 2006). Thus, not all patients with

positive AA progressed to clinically overt adrenal failure; indeed some of them will never

develop AAD. Furthermore, a small number of people with positive AA even reverted back

to an autoantibody-negative state.

These highlighted the highly variable individual response towards adrenal autoantibodies. In

keeping with AAD, observational studies showed that some first-degree relatives of patients

with type 1 diabetes who had anti-islet cell antibodies never develop diabetes (Spencer et al.

1984). The disappearance of anti-thyroid antibodies has also been reported in some patients

with autoimmune thyroiditis (Tunbridge et al. 1981). Nevertheless, the presence of 21-

hydroxylase antibody in an individual remains significant as the cumulative risk of

developing overt adrenal insufficiency remains high at 20% (Coco et al. 2006). Hence, a few

risk factors have been identified predicting the development of overt adrenal insufficiency

among asymptomatic patients with positive AA. These include higher 21-hydroxylase

autoantibody index (Laureti et al. 1998), younger age (Betterle et al. 1997), HLA haplotypes,

particularly DRB1*0404-DQ8 and DRB1*0301-DQ2 (Betterle et al. 1988, Yu L et al. 1999), male gender and biochemical evidence of adrenal dysfunction upon discovery of adrenal antibody (Coco et al. 2006).

Although some works have been done to investigate the preclinical/clinical stages of AAD, the natural history of AAD, particularly those relevant to the post-diagnosis stage, has not been studied in detail. AAD has generally been assumed to be a chronic irreversible condition in which patients will rely on exogenous steroid replacement for life after diagnosis. This disorder has also been assumed to be a monophasic condition, whereby immune-mediated destruction of adrenal gland will inevitably lead to permanent adrenal failure. However, the presence of 'remitting-relapsing' phases or complete remission following a single episode of clinical manifestation in other organ-specific immune disorder i.e multiple sclerosis, autoimmune thyroid diseases highlights the fact that current assumptions on the natural history of AAD could be faulty. Indeed, there are three individuals reported in the literature with spontaneous recovery from established autoimmune Addison's disease (Smans & Zelissen 2008 , Chakera & Vaidya 2012, Baxter et al.2013). There is certainly a knowledge gap in the natural history of established AAD, which is worth exploring as this could potentially revolutionise patient management in AAD.

1.4   Health burden of AAD

AAD is a debilitating condition and carries a significant health burden to the developed world. Although steroid replacement therapy saves the lives of those affected by AAD and makes them feel significantly better than prior to diagnosis, but for most of them, their quality of life is not fully restored. The long term outcome is also compromised by many potential complications associated with chronic steroid treatment, particularly osteoporosis

and type 2 diabetes. It is not uncommon to hear AAD patients complaining of weariness, fatigue, reduced stamina and poor ability to maintain work performance despite optimal steroid therapy. This has resulted in some patients having to utilise more sick leave, unable to carry out full-time jobs or even end up with redundancy.

According to a Norwegian study using the SF36 health assessment (n=79), general health and vitality perception were significantly impaired among all AAD patients irrespective of gender and age of diagnosis (Lovas & Husebye 2002). Role-physical and physical functioning was reduced among affected females, which are strongly associated with the likelihood of receiving working disability benefits and higher fatigue scores. Furthermore, they discovered that Norwegian men treated for AAD (mean age 39 years) had general health equal to men older than 70 years in the general population, whereas affected women have a general health perception on par with Norwegian patients suffering from mild rheumatoid arthritis and American patients with congestive heart failure. A high proportion of patients (41%) aged between 40-67 years were also found to receive working disability benefits despite steroid replacement.

On the other hand, chronic exogenous steroid replacement has been associated with risk of osteoporosis and type 2 diabetes mellitus. Pharmacological doses of glucocorticoids are known to increase osteoporosis and fracture risk, especially in trabecular bone (Reid 1997). This is due to the inhibition of calcium absorption, reduction in bone formation and increased bone resorption. Furthermore, it has been shown that the conventional glucocorticoid therapy doses used in AAD are higher than the endogenous cortisol concentration produced by the adrenal gland (Esteban et al. 1991). This could potentially exaggerate the adverse bone effect among AAD patients. In a large population-based cohort study from Sweden comprising 4007 patients with AAD, a 45% excess risk of hip fracture independent of age at AD diagnosis, sex or duration of diagnosis was found. The relative

Table 1.2. Clinical stages of autoimmune Addison's disease

(Adapted from Betterle et al.1988 & 2002, Baker et al.2012)

Clinical stages	Biochemical and clinical presentation					
	PRA	*Aldosterone*	*ACTH-stimulated cortisol*	*ACTH*	*Basal cortisol*	*Clinically overt Addison's disease*
0	Normal	Normal	Normal	Normal	Normal	None
1	High	Normal/low	Normal	Normal	Normal	None
2	High	Normal/low	Low	Normal	Normal	None
3	High	Normal/low	Low	High	Normal	None
4	High	Low	Low	High	Low	Yes

risk of hip fracture was highest in the year before and the first year after disease diagnosis (HR 4.1; 95% CI: 2.9- 5.8). The risk of hip fracture declined thereafter but remained significant for more than 5 years after diagnosis (HR=1.3, 95% CI, 1.1-1.6) (Bjornsdottir et al. 2011).

Although the life expectancy of AAD patients has been considered normal once the disease is diagnosed and steroid replacement therapy is started. The analyses from large population-based studies showed increased mortality among them. Data from the Swedish National Hospital Registry, comprising 1675 patients with a 14-year study period, demonstrated increased all-cause mortality with a risk ratio of 2.19 for men and 2.86 for women (Bergthorsdottir et al. 2006). The rise in mortality is predominantly ascribed to infectious disease, malignancy and cardiovascular disease (Bergthorsdottir et al. 2006). Similar findings were uncovered by another Swedish study, showing more than a 2-fold increased mortality risk among patients with autoimmune primary adrenal failure (SMR 2.5; 95% CI: 2.3-2.7). Nevertheless, the increased malignancy risk was only observed among APS1 patients when tumours observed during the first year of follow-up were excluded (Bensing et al. 2008). In line with these studies, analyses from the Norwegian group also highlighted the risk of premature death among young AAD patients before the age of 40, commonly due to acute adrenal crisis, infection or sudden death, despite conventional glucocorticoid replacement (Erichsen et al. 2009a).

In addition to the complications discussed above, patients have to prepare for the ever-present risk of an unexpected adrenal crisis, which is inevitably fatal without prompt medical intervention. A survey from Germany involving 254 patients with primary adrenal insufficiency, reported an incidence rate of 6.6 crises per 100 patient years. Around 46.9% (108/254) of patients reported at least one adrenal crisis since their diagnosis (Harner et al. 2010). In another survey carried out by the UK Addison's Disease Self-Help Group, 47% of

the participants reported adrenal crises requiring hospital admission since their diagnosis. Of them, 10% had had four or more occasions of hospital admission with adrenal crisis (White & Arlt 2009). The main precipitating factors for adrenal crisis include gastrointestinal infection and fever, stressful events such as surgery, pregnancy and change of temperature or heat during travel. Despite the extensive observational studies on adrenal crisis, no modifiable risk factors have yet been identified that could effectively prevent this event. Indeed, Hahner et al showed that adrenal crisis was not affected by glucocorticoid dosage, age at diagnosis, body mass index, educational status or DHEA treatment (Hahner et al.2010). Adrenal crisis has certainly increased health costs from hospital stays or even intensive care admission. Although the lack of epidemiological data from UK and European countries makes it hard to estimate the health cost of AAD, data from north America, the National Adrenal Disease Foundation in 2011 reported the annual cost to a patient suffering from AAD amounts to USD23,230 per patient per year, summed up to $11.6 billion to treat the five to six million patients living with AAD. (http://www.aarda.org/pdf/cbad.pdf) The data published included the costs for daily steroid replacement and cost of physician and specialist consultation prior to the diagnosis of AAD being made. In general, AAD results in many years of intensive healthcare consumption and reduced workforce capacity as the average age of diagnosis is 39 years, which could significantly impact the country's economy and health service.

1.5   Normal development of adrenal gland

The adrenal gland is composed of two functionally discrete tissues derived from two embryologically distinct origins. The adrenal cortex is formed from cells of intermediate mesoderm and the inner medulla is derived from neural crest cells of the neuroectoderm origin (Evelyn 1927). Following organogenesis, the adrenal cortex differentiates into 3

distinct zones. Zona glomerulosa, the outermost layer cells arranged in rounded clusters surrounding glomeruli, produce mineralocorticoids that regulate serum sodium concentration and blood volume. The middle zone comprises cells that form the zona fasciculata that synthesises and secretes glucocorticoids, the stress-response hormones. Zona reticularis, residing in the innermost region of the adrenal cortex, is responsible for the production of sex steroid precursors (Keegan & Hammer 2002).

During the 4th week of human gestation, adrenogonadal primodium (AGP) is formed from the proliferation of mesoderm-derived cells of the coelomic epithelia and underlying mesonephros (mesonephric mesenchymal cells) and resides in between the urogenital ridge and the dorsal mesentery (Luo et al. 1994, Hatano et al. 1996), defined by expression of nuclear receptor NR5A1 (Steroidogenic factor 1, SF1). The bipotential AGP separates into the gonadal primordia and adrenal primordial (fetal adrenal cortex) at the 8th week of the embryonic days, under the influence of Wilm's tumour 1and Cited2-mediated upregulation of SF1 expression (Keegan & Hammer 2002). Once separated from the AGP, SF1 expression is maintained via the recruitment of the homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox) and pre B-cell leukemia transcription factor (Pbx1) to a fetal adrenal-specific SF1 enhanceer (FAdE) (Zubair et al. 2006). This FAdE-dependant expression of SF1 is then maintained in the fetal adrenal cortex through autoregulation of SF1 expression. Proliferation of fetal adrenal cells was believed to be under the influence of ACTH derived from fetal pituitary (Mesiano & Jaffe 1997).

Following the formation of the fetal adrenal cortex, the neural crest derived chromaffin progenitor cells will migrate through the cortex to form the medulla, and the mesenchymal encapsulation of the fetal adrenal cortex ensues (figure 1.1). The initiation of the development of the adult definitive cortex will take place in week 8 or 9 of gestation, which becomes evident between the capsular cells and fetal adrenal cells (Uotila1940, Mesiano &

Jaffe 1997). The fetal adrenal cortex regresses rapidly after birth through apoptosis and expansion of the adult cortex. Two layers of differentiated zones in the adult adrenal cortex –zona glomerulosa and zona fasciculata can be distinguished at birth. The zona reticularis becomes evident 3 years after birth and is completed at puberty (Hui et al. 2009).

1.6 Adrenal steroidogenesis

Adrenal steroidogenesis is controlled by a complex orchestration of the hypothalamic-pituitary-adrenal axis and the paracrine signalling of the adrenocortical stem cells and adrenocytes. The pulsatile secretion of ACTH from the pituitary gland is the key regulator of adrenal steroidogenesis. Upon ACTH stimulation, the steroidogenic acute regulatory proteins (StAR) shift cholesterol from the outer mitochondria membrane (OMM) to the inner mitochondria membrane (IMM), making them available for rapid steroidogenesis according to the zonal-specific expression of steroidogenic enzymes (Monna et al.2003). The cholesterol side change cleavage reaction is the initial rate limiting step in steroids biosynthesis, involving the cytochrome P450 side chain cleavage enzyme (P-450$_{scc}$) which resides at the inner mitochondrial membrane (Churchill et al. 1978), that converts the cholesterol side chain to pregnenolone. The pregnenolone produced then travels to the endoplasmic reticulum, where it undergoes further transformation according to the distinct steroidogenic enzymes in each adrenal zonation (figure 1.2).

Figure 1.1. Adrenal gland organogenesis

(Reproduced from Wood and Hammer 2011)
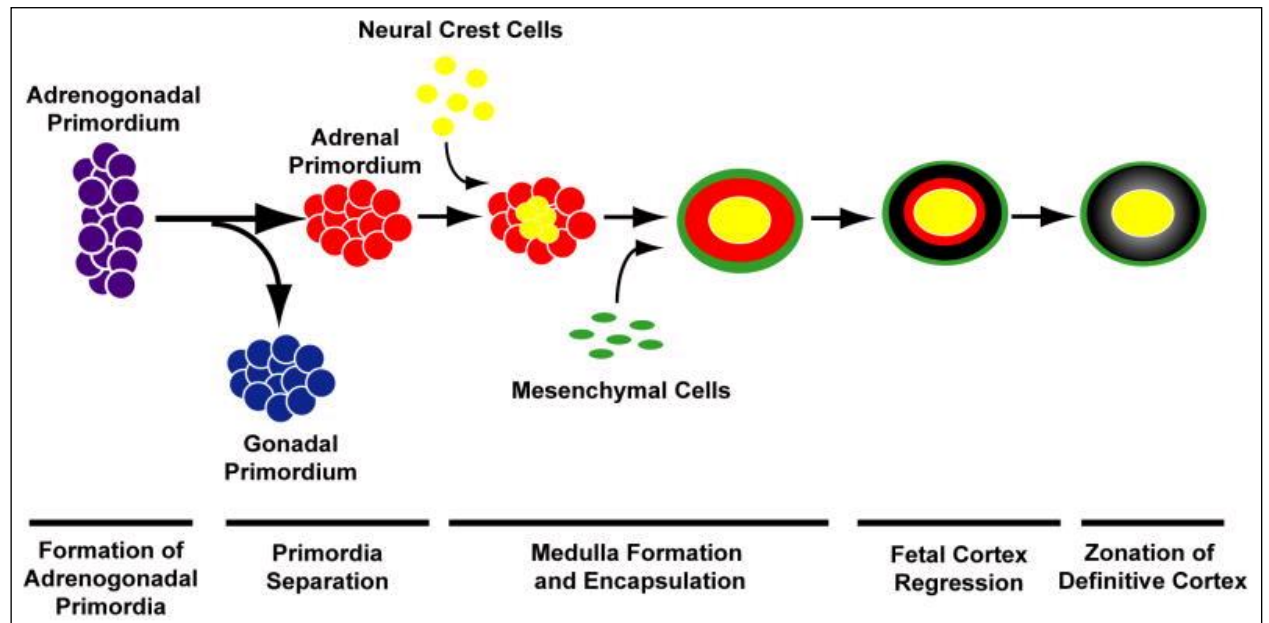
Model of adrenal gland formation from the adrenogonadal primordium to the zonation of the definitive cortex.

(Note colours of the respective cell types: adrenogonadal primordium, purple; fetal adrenal cells, red; gonadal cells, blue; neural crest/medullary cells, yellow; mesenchymal/capsular cells, green; definitive cortex, black; fully differentiated and zonated definitive cells, gray.)

Figure 1.2. Biosynthesis of steroids in adrenal and gonads

(Reproduced from Payne AH & Hales DB 2004)
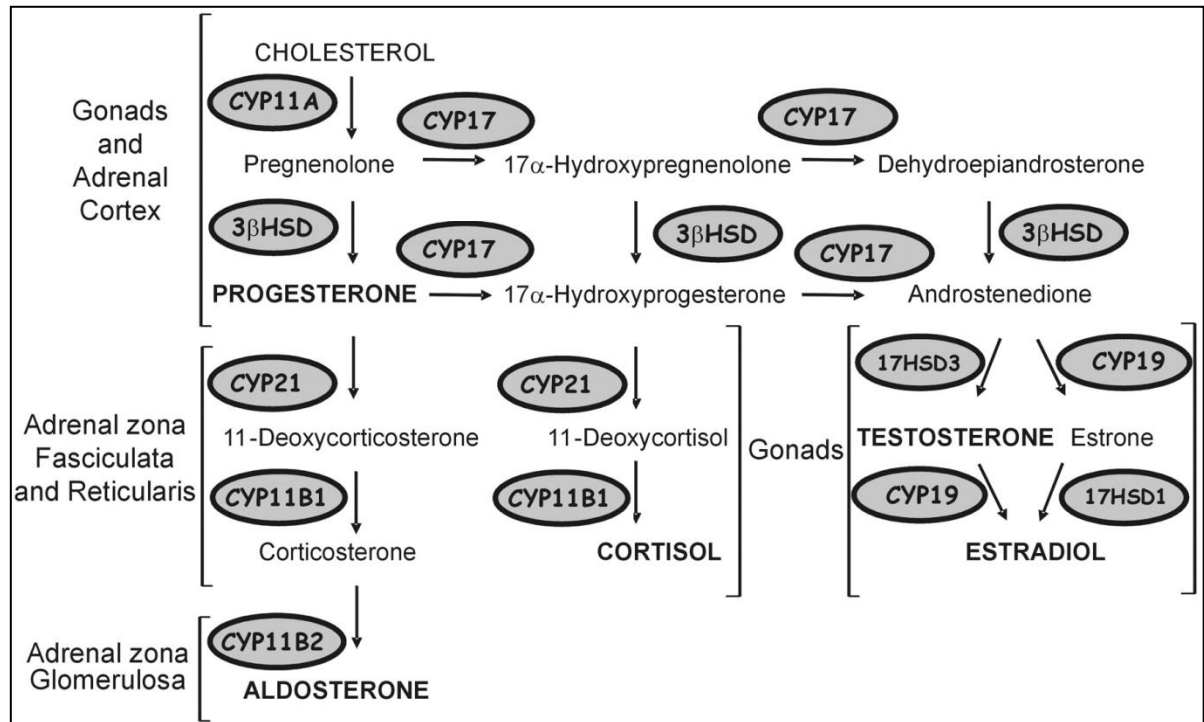
Figure 1.2 depicts the major biosynthesis pathway of steroid hormones in adrenal cortex and gonads. Individual enzymes are highlighted in circle whereas the final steroid hormone products are in capital letters. The rate limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A), taking place in both adrenal cortex and gonads. Each step of the steroidogenesis pathway is regulated by a specific enzyme. Each zone of the adrenal cortex has distinct enzyme activity leading to the final production of aldosterone, cortisol and adrenal androgens. Pregnenolone is first converted to either progesterone or 17α-hydroxypregnenolone , by 3-β-hydroxysteroid dehydrogenase (3β HSD) and steroid 17α-hydroxylase (CYP17) respectively. These intermediate precursors are converted to 11-deoxycorticosterone and 11-deoxycortisol by steroid 21α-hydroxylase (CYP21), an enzyme unique to the adrenal cortex. This is followed by the conversion to aldosterone and cortisol by

aldosterone synthase (CYP11B2) and steroid 11β-hydroxylase (CYP11B1), respectively. Adrenal androgens (dehydroepiandrosterone and androstenedione) are formed from the hydroxylation of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. The presence of 17 β-hydroxysteroid dehydroxysteroid (17HSD) and the aromatase complex (CYP19) in the gonads allow formation of testosterone and estradiol.

1.7 Current concept on the pathogenesis of AAD

Since the discovery of Addison's disease in 1849, various studies comprised of animal models and post-mortem examinations have been undertaken to unravel its underlying mechanism. Although we learned that aberrant immune response toward steroid producing cells in the adrenal cortex results in AAD, the exact mechanism that triggers adrenal autoimmunity remains enigmatic. I will discuss the current understanding of the pathogenesis of AAD from histopathology, immunopathology and genetics perspective.

1.7.1 Histopathology

A lot of valuable histology information was obtained from post-mortem examinations performed when AAD was still a fatal condition. AAD had been labelled as a disease of 'inflammatory contracted suprarenals' back in 1930. In a post-mortem examination of a patient who died of adrenal insufficiency, Saphir & Binswanger noticed that the adrenal glands were hypoplastic, with only remnants of cortical tissue remaining. The remnants were represented by yellow streaks or dots, with a thin capsule enclosing only the reddish grey medulla (Saphir & Binswanger 1930). Histology studies performed in later years concluded that autoimmune adrenalitis is characterised by widespread diffuse infiltration of monoculear cells comprising lymphocytes, plasma cells and macrophages (Carperter et al. 1964, Irvine et al. 1967 & 1968). In addition, the affected adrenal cortex lost the normal 3-zonal architecture, with adrenocortical cells demonstrating necrosis and pleomorphism. Interestingly, islands of hyperplastic adrenocortical cells or regenerating nodules were observed in the midst of mononuclear cell infiltration (Guttman 1930). These residual adrenal nodules eventually became overwhelmed by the inflammatory process; the cortex would then be replaced entirely by fibrous tissue (Jackson et al. 1988). The slow inflammatory process within the adrenal cortex characterised by continuous destruction and

self-regeneration is in keeping with the insidious onset of AAD. It has also been suggested that this disease will only become clinically apparent when 90% of the functioning adrenocortical cells have been destroyed (Rosenthal et al. 1978).

Despite these early histology data, the exact sequence of the pathogenesis of autoimmune adrenalitis in human remains elusive. A similar immune form of Addison's disease occurs in several breeds of domestic dog (Klein & Peterson 2010). The adrenal inflammation in canine was found to be associated with cortical atrophy and lymphoplasmacytic adrenalitis. However, zona glomerulosa was partially spared (Frank et al. 2013). Various studies have tried to induce autoimmune adrenalitis in several animal species, including guinea pigs, rabbits, mice and rats, by injection of autologous or heterologous adrenal homogenates mixed with various adjuvants, as well as transferring spleen or lymph node cells from actively immunised rats or mice into their naive companion (Barnett et al. 1963, Colover & Glynn 1958, Steiner et al. 1960, Fujii et al. 1992). Although these models managed to produce lymphocytic infiltration in the adrenal gland, none of them demonstrated diffuse atrophy of the adrenal cortex, or regeneration nodules that were seen in humans. Hence, animal models are of limited relevance to human disease.

1.7.2 *Immunology*

The role of adaptive immunity in AAD has been highlighted since 1960s, when Nerup and colleagues demonstrated that circulating T lymphocytes from patients with autoimmune adrenalitis were reactive towards fetal adrenal extracts or to a mitochondrial fraction from adrenals (Nerup J et al. 1969 & 1970). However, the mechanistic profile of the immunopathogenesis of AAD has yet been fully unravelled, mainly due to the limitations of obtaining fresh adrenal samples from patients with active autoimmune adrenalitis and poor animal models.

Both humoral and cell-mediated immune responses are believed to contribute to disease pathogenesis. The presence of adrenal autoantibodies is among the potential factors that trigger an immune response towards adrenocortical cells. These steroid-producing cell antibodies have been found to target 21-hydroxylase, 17 hydroxylase and side-chain cleavage enzymes (Bednarek et al. 1992, Krohn et al. 1992, Winqvist et al. 1993). These autoantibodies were found to bind to subcellular fractions of adrenocytes enriched for microsomal enzymes and mitochondria (Ludwig et al. 1976, Nerup et al. 1970, Bright et al. 1990). In contrast to 21-hydroxylase autoantibodies, which are only found in the adrenal cortex, autoantibodies against 17α-hydroxylase and cholesterol side-cleavage enzymes were also found to bind to the ovary, placenta and testis, contributing to autoimmune premature ovary failure (Sotsiou et al. 1980, Anderson et al. 1968). Interestingly, although these 3 steroid-producing enzymes are closely related and their amino acid sequence is partially homogenous, they are independent molecular targets of autoantibodies in AAD (Peterson et al. 1997, Uibo et al. 1994).

Among the identified adrenal autoantibodies, 21-hydroxylase (21-OH) enzymes are the dominant antigens in either isolated Addison's disease or AAD in the context of APS2 (Erichsen et al. 2009, Soderbergh et al. 2004). This autoantibody may persist in patients years after the diagnosis (Dawoodji et al. 2014). The 21-hydroxylase enzyme is an intracellular enzyme localised in the endoplasmic reticulum and comes from the family of p450 cytochrome. It consists of 496 amino acids with a calculated molecular weight of 56kDa (Furmaniak et al. 1988). The enzyme activities are dependent on NADPH, a flavoprotein, P450 reductase. It converts 17α-hydroxyprogesterone into 11-deoxycortisol, and also converts progesterone into 11-deoxycorticosterone. The central and the C-terminal region of 21-hydroxylase enzymes (amino acid 241-494) were thought to be important for autoimmunity (Song et al. 1994; Wedlock et al. 1993). A mutation within this region

(Pro453-->Ser) has resulted in partial loss of autoantibody binding, suggesting that Pro453 has a key role in forming an autoantigenic epitope on 21-hydroxylase (Asawa et al.1994).

On the other hand, AAD is likely due to a T-cell mediated immune response. AAD and APS 2 have been associated with DR3-DQ2 and DR4-DQ8 HLA haplotypes, which are known to predispose to other T cell-mediated autoimmune diseases such as type 1 diabetes (Myhre et al. 2002; Gombos et al.2007). The class II major histocompatibility complex (MHC class II) molecules are also found to be highly expressed in the adrenocortical cells of AAD patients as compared to the normal healthy population. This suggests extensive exposure of the adrenal cortex to interferon-γ (IFN-γ), secreted by activated CD4+ and CD8+ T lymphocytes, which then perpetuate further lymphocytic proliferation and immune response towards the adrenal cortex (Skoskiewicz et al. 1985, Todd et al.1985). In addition, defects in T cell functions had been described in APS2. Impairment of activation-induced cell death with persistence of autoreactive T cells were reported among AAD patients who had compromised CD4+CD25+ regulatory T cell and caspase 3 functions (Kriegel et al. 2004, Vendrame et al. 2006). Recent studies had demonstrated that T cell response are clustered to a few immunodominant 21-OH epitopes, such as HLA-B8–restricted epitope 21-OH$_{431-438}$ (Rottembourg et al. 2010) and HLA-A2–restricted dominant epitope at position 21-OH$_{342-350}$. (Dawoodji et al. 2014). However, there is still limited information with respect to 21-OH specific T cell response in AAD. A more comprehensive epitopte mapping on 21-OH are required to inform the role of T cell mediated immune response in AAD.

Despite the clear association between adrenal autoantibodies and AAD, the exact triggers and reasons for adrenalitis being perpetuated remain enigmatic. Although *in-vitro* studies showed a marked dose-dependent inhibition of 21-hydroxylase enzyme activity with sera from patients with positive 21-hydroxylase autoantibodies (Furmaniak et al.1994), there is

no evidence that the same effects take place *in vivo*. In fact, a study has shown that the serum steroid profile of pre-clinical AAD patients with positive 21-hydroxylase antibodies remained unchanged, in which an increase in 17α-hydroxyprogesterone levels would be expected from reduced 21-hydroxylase enzyme activity (Boscaro et al.1996). Besides, these autoantibodies which have been shown to cross placenta and are present in neonatal blood, did not result in transient or permanent neonatal hypoadrenalism (Winqvist et al.1996). This autoantibody is clearly different from those seen in autoimmune thyroid disease, whereby the presence of thyroid stimulating hormone receptor antibodies that cross the placenta has been shown to lead to neonatal hypothyroidism or hyperthyroidism (Weber et al.1998). Nevertheless, this clinical discrepancy is thought to be reasonable as 21-hydroxylase enzyme is located in the smooth endoplasmic reticulum which could preclude its direct interaction with circulating autoantibodies (Mitchell & Pearce 2012). Nevertheless, some studies have suggested their more plausible role in mediating direct adrenocortical cell destruction (Khoury et al.1981; Bright & Singh 1990). The 21- hydroxylase autoantibodies are IgG1 isotype and target carboxyl terminal of this enzyme. This means that they have the potential to destroy adrenal cells through cellular cytotoxicity by perpetuating complement or antibody-dependant processes directed at membrane or intracellular antigen (Gattoni et al. 2006). Furthermore, the auto-reactive B cells might also act as antigen-presenting cells, which in turn enhances the generation of T cell mediated autoimmune destruction process. Indeed, it has been shown that the level of 21-hydroxyase autoantibodies correlates with the degree of adrenal destruction/ failure at diagnosis (Laureti et al. 1998). Their presence in asymptomatic healthy controls can also predict the risk of progression to clinical disease (Yu L et al. 1999; Falorni et al. 1997).

Apart from the well described adaptive immunity, studies in the last few decades have started to shed light on the potential role of innate immunity in AAD. The adrenal gland is a

highly vascular and dynamic organ. It constantly interacts with immune systems through various mediators such as resident macrophages, lymphocytes and dendritic cells which are present in the adrenal glands (Bornstein & Chrousos 1999; Marx et al. 2000). Although the exact roles of these immune cells have yet to be confirmed, it has been suggested that they are involved in the regulation of steroid hormone production by means of cytokines and chemokines secretion (Marx et al. 1998). For instance, Toll-like receptors (TLR), a group of receptors which are essential for the innate immune system, are expressed in adrenocortical cells under normal conditions. The stimulation of TLR ligand induces strong inflammatory response, leading to the secretion of various chemokines, including TNF-α and interleukin-6 (IL-6) (Kanczkowski et al. 2009). This response is found to be important in regulating stress responses upon infection or bacteraemia but its role in adrenal autoimmunity remains unclear (Bornstein et al. 2006). Additionally, steroid-producing cells could produce cytokines and chemokine, such as IL-1, IL-6, IL-8, IL-18 and TNF-α, which have a key role in the immune-endocrine cross talk to regulate steroidogenesis (Bornstein et al. 2004, Rotondi et al. 2007a). A specific chemokine implicated in AAD and several other autoimmune endocrine disorders is the CXC Chemokine Ligand 10 (CXCL10, also known as interferon-inducible protein 10, IP10) (Rotondi et al. 2007b). They were found to be elevated in the serum of AAD patients and are produced by adrenal cells following cytokines TNFα and interferon (IFN)-γ exposure during viral infection (Rotandi et al. 2005). *In-vitro* studies suggest that toll-like receptor 3, nuclear factor κB (NFκB) and signal transducer and activator of transcription-1 (STAT1) could induce CXCL10 expression in the adrenocortical cell line (Bratland E et al 2013), suggesting the combination of environment (viral), endogenous and innate immunity in the pathogenesis of AAD.

It is clear from the discussion above that the full mechanistic picture of immunopathogenesis remains obscured although parts of them have slowly been revealed. Nevertheless, two

potential models for the immunopathogenesis in AAD have been developed which will be discussed below.

Hypotheses of the immunopathogenesis of AAD

Hypothetical model 1

Bratland & Husebye has delineated a detailed model on the potential pathogenesis of AAD (Bratland & Husebye 2011). They suggested that viral infection or intense stress that induced apoptosis of adrenocotical cells could possibly lead to excessive recruitment of dendritic cells that take up elements released by the apoptotic cells, including genomic DNA and steroid-producing enzymes such as 21 hydroxylase enzymes. If there is a breakdown in central tolerance or a lower set point of clonal selection, Th1 cells might be activated to perpetuate a series of antigen-presenting T cell process. This will include interferon –γ induced B cell expansion, memory B cell and autoantibody production. Additionally, interferon-γ secreted by Th1-CD4+ cells will also lead to activation and expansion of auto-reactive cytotoxic lymphocyte and macrophages, leading to cellular destruction. The autoantibodies generated by B cells that bind to the target antigen in adrenal cells will also result in complementary or antibody-dependant cytotoxic process. In addition, the generation of various chemokines will also induce cell death through radical molecule production (figure 1.3).

Hypothetical model 2.

Mitchell & Pearce suggested another model based on the breakdown of glucocorticoid-induced immune privilege. It was suggested that the local steroidogenesis suppresses adrenal

antigen presentation within the adrenal cortex in normal situation (Mitchell & Pearce 2012).

However, if immune tolerance to adrenal antigen is lost, antigen presentation will be

enhanced followed by inflammatory infiltration in the adrenal cortex (stage 1 disease).

Adrenal cells will then be damaged by the inflammatory process results in reduced local

glucocorticoid production (stage 2 disease). This will then promote further adrenal antigen

presentation, inflammatory cell recruitment and adrenal cell destruction. Adrenal cortex will

eventually destroy and steroidogenesis ceases, correspond to the overt clinical phase of

AAD (figure 1.4).

Figure 1.3. A highly simplified schematic overview of possible events leading to autoimmune adrenalitis and adrenocortical insufficiency

(Reproduced from Braland & Husebye 2011)
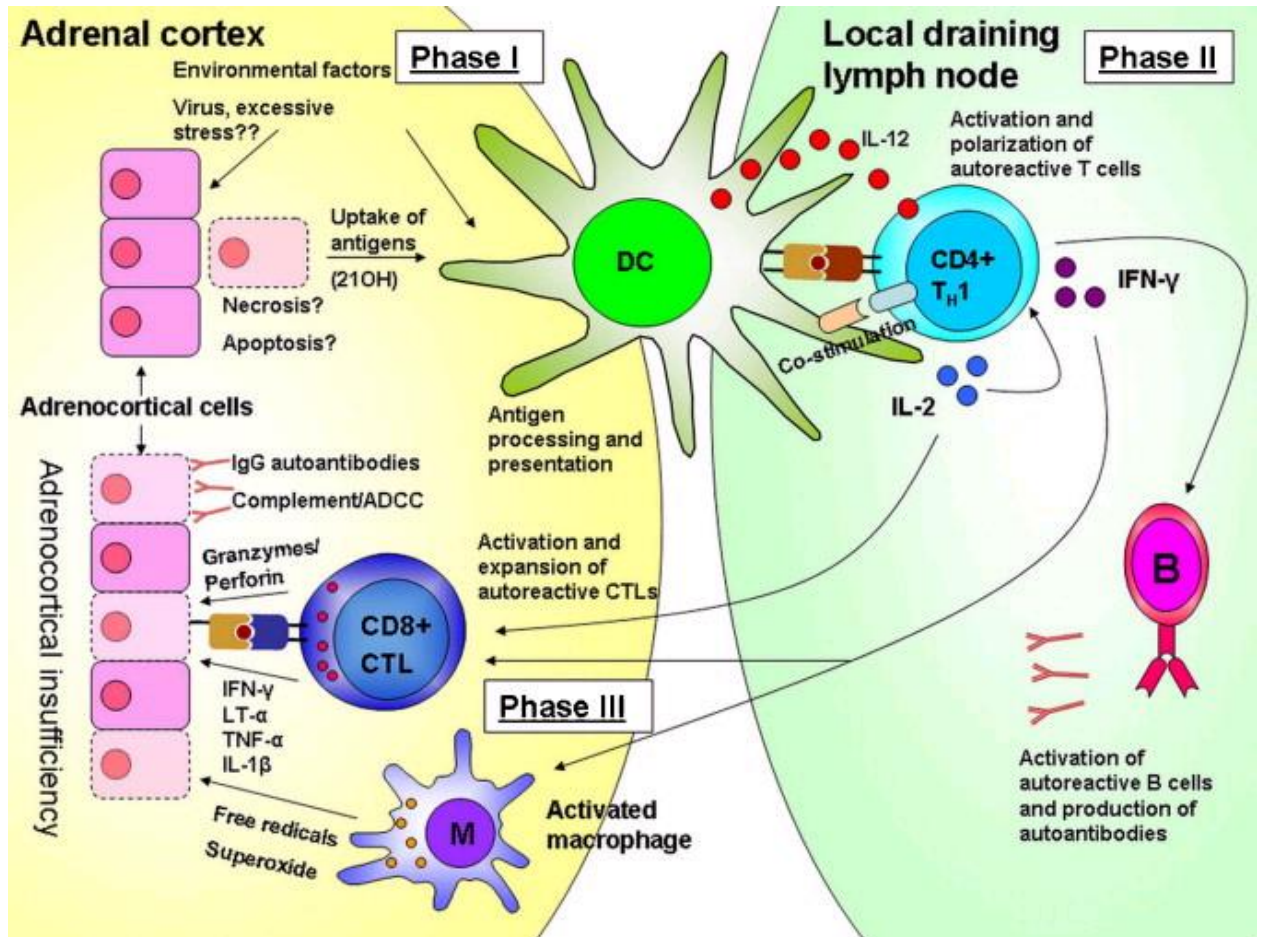
Figure 1.4. A hypothetical model of the events leading the overt autoimmune Addison's disease

(reproduced from Mitchell & Pearce 2012)
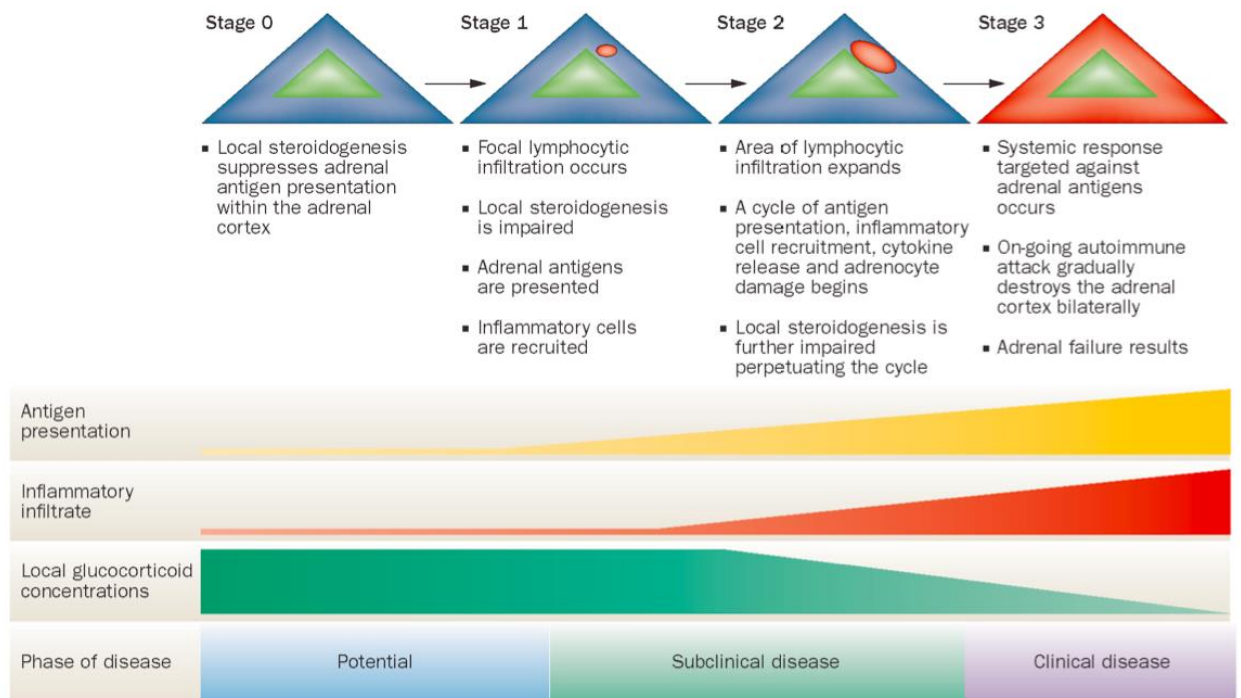
1.7.3 Genetics of AAD

In common with other autoimmune disorders, AAD is believed to have a complex genetic basis, with a risk to first-degree relatives of about 2% (Mitchell & Pearce 2012). AAD with APS2 or isolated AAD is to be distinguished from those in conjunction with APS1, which is a monogenic condition, inherited in a Mendelian fashion owing to *AIRE* gene mutation. Data incorporating AAD probands from the UK and Norway has revealed a sibling recurrence risk ratio (the risk of disease in a sibling of an individual with the disease compared to the population prevalence of the disease) of between 160 and 210 (Mitchell & Pearce, 2012). This is significantly higher than the sibling recurrence risk ratio in autoimmune thyroid disease and type 1 diabetes, which are approximately 10 and 15 respectively (Risch 1987, Vaidya et al. 2002).

The rarity of AAD has precluded large-scale genetic analysis and the genomic studies carried out thus far are based on candidate gene association and case-control design conducted in small cohorts of patients. The number of known risk genes for AAD has increased steadily in the past years and have a dominant influence on various immunological pathways, both the adaptive and innate immune systems. In common with other autoimmune endocrinopathy, the MHC locus on chromosome 6p21 harbouring the class II HLA alleles is strongly associated with AAD. Several studies involving both eastern and western populations have confirmed the risk alleles as HLA-DRB1*03-DQA1*0501-DQB1*0201 (DR3/DQ2) and DRB1*0404-DQA1*0301-DQB1*0302 (DR4/DQ8) haplotypes, with a risk ratio of 36.7 (Erichsen et al. 2009,Gombos et al. 2007, Maclaren and Riley 1986, Myhre et al. 2002, Yu et al. 1999). In contrast, HLA-DRB1*01-DQA1*01-DQB1*0501 (DQ5) is associated with protection against AAD (Myhre et al. 2002). On the other hand, the presence of a highly conserved extended haplotype DR3 and HLA-B8 has been shown to confer the highest risk of progression to AAD in patients with adrenal autoantibodies (Baker et al.

2010). This study showed that 97% of the multiplex individuals (86 AAD patients from 68 families;12 multiplex and 56 simplex) had both HLA-DR3 and HLA-B8 vs. 60% of simplex AD patients (P = 9.72×10^{-4}) and 13% of general population controls (P = 3.00×10^{-19}). The DR3-B8 haplotype of AD patients were less often associated with HLA-A1 compared with controls (47% vs.81%; P = 7.00×10^{-5}).

HLA proteins are expressed on the surface of antigen-presenting cells and display both self and non-self-peptides to T lymphocytes during immune surveillance. The strong association between various class II HLA alleles and AAD suggested that alteration in the MHC class II protein renders the peptide epitope on the steroid 21 hydroxylase to be presented more effectively to the autoreactive T cells, in the presence of certain at risk groups. For instance, DRB1*0404 was more commonly found in AAD patients, compared to DRB1*0401. The protein encoded by HLA-DRB1*0404 differs from the one encoded by HLA-DRB1*0401 in only two amino acid residues (Gregersen et al 1987; Hammer et al. 1995). The lysine residue, which was found at position 71 of the DR β-chain of DR0401, was replaced by arginine in DR 0404 alleles. This significantly affected the peptide binding capabilities of the HLA molecules. Indeed, a significant association was found between carriers of the DRB1*0404- DQ8 haplotype and cellular immune response against a subgroup of peptide found in 21 hydroxylase enzymes among AAD patients (Bratland & Husebye 2011). Nevertheless, only 15-20% of patients are carriers of DRB1*0404, hence other at risk alleles should have significant roles in AAD as well (Erichsen et al. 2009).

Although many other risk alleles identified within the MHC region reflect only the effect of neighbouring genes in linkage disequilibrium, the A5.1/A5.1 genotype of the MHC class I chain-related gene A (*MICA*) seems to be independently associated with progression to clinical adrenal insufficiency. It gives a ratio of 18.0 and absolute risk of 1 per 1131 for AAD (Gambelunghe et al. 1999). *MICA* is considered to confer independent risk to AAD as

the strength of the association was greater than might be expected from linkage disequilibrium with the HLA-DR3/DR4 haplotype alone (Park et al. 2002, Triolo et al. 2009). Polymorphisms of the *MICA* gene are based on the number of triplicate GCT repeats in exon 5. The translated protein interacts with the NKG2D receptor, which is important for thymic maturation of T cells (Hue et al. 2003). However, the debate of whether *MICA* as an independent risk gene remains unsettled, as its role in autoimmunity was challenged in a large-scale genotype analysis in type 1 diabetes (Field et al. 2008).

In addition to the MHC class II alleles, *CTLA4, PTPN2, PDL1, FCRL3, NLRP1, CLEC16A, CIITA, CYP27B1, STAT4* and *GATA 3* polymorphisms have been identified as contributing to AAD susceptibility (Pani et al. 2002, Skinningsrud, et al. 2008, Mitchell et al.2009, Roycroft et al. 2009, Jennings et al. 2009, Magitta et al. 2009, Brozzetti et al. 2010, Zurawek et al. 2010, Mitchell et al.2014) (Figure 1-5). These genes are involved in either adaptive or innate immunity and their alteration could lead to the breakdown of immune-tolerance. Cytotoxic T lymphocyte antigen 4 (*CTLA4*) gene on chromosome 2q33 encodes a costimulatory molecule expressed on the surface of activated T lymphocyte (Brunet et al. 1987). It competes with the co-stimulatory molecule CD28 for interaction with B7/B7.1 and thus down-regulate T cell activation (Husebye & Lovas 2009). Polymorphism of *CTLA4* has been implicated in numerous autoimmune diseases, including Graves's disease, coeliac disease, rheumatoid arthritis and type 1 diabetes (Yanagawa et al. 1995, Nistico et al. 1996, Djilali-Saiah et al.1998, Vaidya et al. 2004). In AAD, a positive association has been shown in polymorphism of an A or G single nucleotide polymorphism in exon 1, AR repeat in the 3'untranslated region of exon 3 and A or G alleles of the JO30 SNP downstream of this gene. These polymorphisms are thought to have reduced the amount of soluble CTLA4 and hence increased T cell activation by enabling CD28 to access more of its ligand (Blomhoff et al. 2004).

The other immune genes that are involved in the modulation of T cell receptor signalling are protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) and *CD274* genes. PTPN22 is located on chromosome 1p13 and encodes a negative regulator of T cell signalling, the lymphoid tyrosine phosphatase (LYP). The *PTPN22* polymorphism 1858 C>T results in Arg620Trp substitution and has been implicated in AAD, as well as type 1 diabetes, rheumatoid arthritis and Graves' disease (Begovich et al. 2004, Velaga et al. 2004, Kahles et al. 2005, Skinningsrud et al. 2008, Roycroft et al. 2009). The increased risk can be explained by the loss-of-function effect of 1858T-allele, leading to unstable LYP that is more susceptible to degradation and results in the survival of autoreactive T cells during thymic selection (Zhang et al. 2011). On the other hand, the variants of *CD274* gene on chromosome 9p24 which encodes programmed cell death ligand 1(PDL1) has also been associated with AAD and other autoimmune conditions (Brown et al. 2003, Hayashi et al. 2008, Mitchell et al. 2009) This gene is responsible for regulating T-cell associated ligands with co-stimulatory roles.

Recently, the signal transducer and activator of transcription 4 (STAT4) and GATA3 polymorphisms have been implicated in AAD (Mitchell et al.2014). These susceptibility genes were previously found to be associated with rheumatoid arthritis (Remmers et al. 2007, Eyre et al. 2012). STAT 4 is a transcription factor known to have a role in CD4$^+$ cell fate, with respect to T_H1 responses generation and T_H17 cell differentiation. GATA3 on the other hand has been shown to have a role in the homeostasis and regulation of CD8+ T-lymphocytes (Tai et al. 2013). Therefore, their polymorphism could contribute to primary T cell dysregulation in AAD (Mitchell et al. 2014).

Apart from T lymphocytes, B lymphocytes can also act as antigen-presenting cells and express cell-surface antigens that regulate T cell response. For instance, FCRL3 is one of the Fc-receptor-like family members, serving as B cell surface receptors of the immunoglobulin

receptor superfamily. They are encoded by the *FCRL* gene located in chromosome 1q21 and were expressed predominantly on B lymphocytes, as well as on natural killer cells and regulatory T cells. A polymorphism of *FCRL3* with the T allele in the *FCRL3* promoter (FCLR3_3*T) has been associated with AAD in the UK cohort as well as several other autoimmune conditions (Capon et al. 2001, Kyogoku et al. 2002, Kochi et al. 2005, Owen et al. 2007). Intriguingly, the same variant was found to be protective for multiple sclerosis in the Spanish cohort, reflecting the complexity of genetics in autoimmunity across different populations (Matesanz et al. 2008).

Although polymorphisms in genes involved in adaptive immune system have been the focus of genomic study in AAD, an increasing number of loci related to innate immunity have been identified as conferring risk to AAD. The NOD-like receptors (NLRs), one of the cytoplasmic pattern-recognition receptors, is thought to trigger an adaptive immune response and activate pro-inflammatory cytokines upon sensing microbial products. NLR polymorphism might result in the failure of differentiating self from non-self antigens and result in autoimmunity. Indeed, polymorphisms in various NLR genes have been implicated in type1 diabetes and vitiligo (Jin et al. 2007, Magitta et al. 2009). The Leu155His polymorphysim in *NLRP1*was also found to be associated with AAD (Magitta et al. 2009, Zurawek et al. 2010). However, the exact functional consequence of this genetic variant has yet to be demonstrated.

On the other hand, a Norwegian study found that polymorphysims in *CLEC16A* and *CIITA* are significantly associated with AAD. These genes can be found in chromosome 16p13 but they are not in linkage disequilibrium, suggesting their independent risks in AAD (Skinningsrud et al. 2008). *CIITA* is also known as MHC class II transactivator (MHC2TA) and encodes the master control of MHC II expression. The variation in this gene might lead to alteration in tissue selectivity of MHC II expression and increase AAD susceptibility

(Ghaderi et al. 2006). *CLEC16A* is a C-type like lectin gene and the intronic polymorphism has been implicated in AAD. Although the function of the protein encoded remains unknown, it is expressed in antigen-presenting cells and has a C-type leptin-binding domain, suggesting its potential role as a cell-surface receptor (Hakomarson et al. 2007, Mitchell &Pearce 2012).

The initiation of adrenal autoimmunity in AAD ascribes to a complex interplay between genetics, environment and endogenous factors. There are limited studies on the environmental influence, although the immunomodulatory role of vitamin D or sun exposure in autoimmune conditions has raised considerable interest. A variation in the *CYP27B1* gene in chromosome 12q13 (1260 C>A) which encodes 1-α hydroxylase, an enzyme that hydroxylase 25-hydroxyvitamin D3, has been shown to increase the risk of AAD in both German and UK cohorts, as well as Graves' disease, Hashimoto thyroiditis and type 1 diabetes (Lopez et al. 2004, Jennings et al. 2005, Bailey et al. 2007).

Although AAD has a strong genetic contribution and an increasing number of risk alleles have being identified in the last few decades, most of them have only a modest effect on disease susceptibility. In fact, MHC is the only single known locus confirmed to make a major contribution to an individual's genetic susceptibility to AAD. The majority of the genetic components of AAD remain undefined. Hence further genomic studies are required to unravel the exact aetiologies and pathogenesis of AAD.

Figure 1.5. Schematic representation of candidate-gene loci that confer susceptibility to AAD

(Reproduced from Mitchell & Pearce 2012)
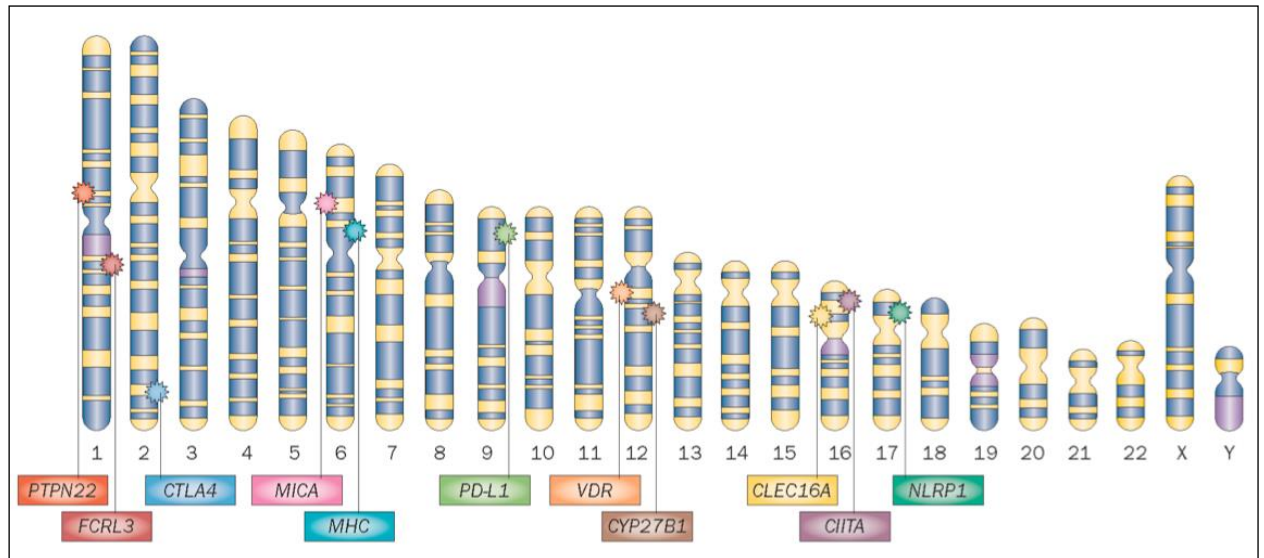
1.8 Current therapeutic approaches in AAD

The synthesis of 11-deoxycortisone in 1937 and eventually cortisone and fludrocortisone in 1946 and 1950 has transformed AAD from a lethal condition to a chronic but manageable disease. The treatment for primary adrenal failure has been virtually unchanged since then, and patients with AAD have a lifelong dependency on daily steroid replacement with the ever-present risk of adrenal crisis. The common glucocorticoid replacement regimen is hydrocortisone (15-20mg) or cortisone acetate (20-30mg) taken twice or three times daily. The use of synthetic glucocorticoids such as prednisolone which is deemed to give a more stable glucocorticoid effect throughout the day has also been advocated. However, their variable pharmacodynamics effects (Diederich et al. 2002) and potential long term undesirable metabolic effects have rendered them less favourable as the first line of treatment. The synthetic mineralocorticoid, fludrocortisone, is usually taken once daily at the dose of 50-200mcg, guided by blood pressure, electrolytes and plasma renin level. Therefore, for most AAD patients, daily steroid replacement is adequate to control symptoms but is not a perfect solution to restore them to full health and normal life-expectancy. Cessation of this medication or inappropriate dose adjustment during intercurrent illness could result in hospital admission or death. Hence, efforts to explore different therapeutic approaches to improve the outlook and health in AAD patients have emerged in the recent decades.

Traditionally, only glucocorticoid and mineralocorticoid are replenished during adrenal failure. The clinical relevance of adrenal androgens deficiency in AAD patients has nonetheless become a hot topic of debate in recent years. The adrenal cortex produces high amounts of dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEAS), estimated to be 4-14mg and 20-25mg respectively on a daily basis (Longcope 1986, Baulieu & Robel 1996). These adrenal androgens are regarded as the inactive precursor steroids, which are

then converted to testosterone and estrogen in peripheral tissues. It has been estimated that approximately 30-50% of androgens in men and a high proportion of androgens in post-menopausal women are contributed by adrenal androgens (Labrie et al. 2003). Nevertheless, the clinical significance of DHEA(S) deficiency in patients with adrenal insufficiency remains unclear. Several studies using oral DHEA replacement therapy in Addison's disease or secondary adrenal insufficiency have been conducted, but the benefits in sexuality or subjective health status were not consistent (Arlt W et al.1999, Hunt et al. 2000, Gebre-Medhin et al. 2000, Johannsson et al. 2002, Lovas et al. 2003, Dhatariya et al. 2005, Bilger et al. 2005, Nair et al. 2006, Christiansen et al. 2007). The latest and largest DHEA trial (n=106) showed beneficial effects on bone by reversing ongoing loss of bone mineral density at the femoral neck, without significant improvement in quality of life (Gurnell et al. 2008). Hence, although adrenal androgen replacement with DHEA 20-50mg has been advocated in AAD, the evidence remains weak and inconsistent.

On the other hand, it is well known that the secretion and metabolism of endogenous glucocorticoid is highly complex. The normal cortisol secretion is characterized by pulsatile ultradian and diurnal rhythm. The cortisol level peaks in the early morning, within 1 hour prior to awakening, and falls progressively during the day to reach a nadir in the evening (Kerrigan et al. 1993). Automated frequent blood sampling of serum cortisol had also revealed a pulsatile ultradian rhythm throughout a 24-hour cycle, in which the amplitude generally decreases during the diurnal trough (Veldhuis et al. 1990, Chrousos 1998). This ultradian rhythm may have the theoretical implication on the generation of tissue-specific signals. The 2 glucocorticoid receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptors have different ligand affinities, and the pulsatility may have a particular effect on the occupancy of the lowered-affinity GR receptor (Young et al. 2004). Furthermore, metabolic abnormalities and increased cardiovascular risk have been implicated in

conditions that disrupt the normal circadian rhythm, such as depression and insomnia (Plat et al. 1999, Spiegel et al. 1999). Hence, the lack of the physiological rhythm in the conventional replacement regimes could explain partly the increased mortality and cardiovascular risk among AAD patients. Cortisol day curves have indeed shown that the conventional twice daily hydrocortisone replacement therapy resulted in a supra-physiological rise of cortisol concentration upon steroid ingestion followed by a rapid fall in serum levels in 4-6 hours, resulting in a sub-physiological level at pre-dose (Trainer et al. 1993).

Hence, continuous hydrocortisone infusion regimen that can mimic and reestablish the normal circadian rhythm of cortisol was developed (Lovas & Husebye 2007). Oksnes et al. showed that continuous subcutaneous hydrocortisone infusion using proprietary insulin pump was well-tolerated by patients and the circadian pattern of ACTH and cortisol level was re-established (Oksnes et al. 2014). However, contrasting results with respect to subjective health status were demonstrated by two randomized trials involving small patient cohorts (n= 33 and 10, respectively) (Oksnes et al. 2014, Gagliardi et al. 2014).

On the other hand, a novel modified-release hydrocortisone formulation and a combined immediate/delayed release hydrocortisone have also been introduced (Debono et al. 2009, Johannsson et al. 2009 & 2012, Verma et al. 2010). The modified-release hydrocortisone formulation (Chronocort) allows delayed and sustained release of hydrocortisone to replicate normal physiological cortisol levels under unstressed conditions (Verma et al. 2010). The once-daily dual-release hydrocortisone tablet (Duocort), on the other hand, is made up of an immediate-release coating together with an extended-release core. This formulation has been shown to mimic the physiological diurnal rhythm of cortisol better, by providing a sustained serum cortisol profile 0-4 hours after the morning dose (Johannsson et al. 2009). A 20% reduction in the late afternoon and 24 hour cortisol exposure was also demonstrated

(Johannsson et al. 2009). Although these novel hydrocortisone formulations have been shown to improve quality of life and biometabolic parameters, the trials involved have either been carried out on highly selected patient groups or were lacking randomized trials with prednisolone (Verma et al. 2010, Johannsson et al. 2012).

The novel therapeutic approaches discussed thus far have been based on steroid replacement therapy which, realistically, will not be able to mimic the physiological circadian rhythm of serum cortisol profile completely. Hence, re-establishment of endogenous adrenal steroidogenesis will be the ultimate curative approach in AAD. This treatment approach has recently been attempted by Pearce and colleagues using B lymphocyte-depleting anti CD20 antibodies (Pearce et al. 2011). One out of six patients demonstrated steady improvements in serum cortisol and aldosterone secretion, and eventually discontinued steroid replacement therapy for 12 months. This study suggested that the natural history of AAD is modifiable and disease-modifying drugs targeted at the immune response could lead to a cure. This is in line with type 1 diabetes mellitus and Graves'opthalmopathy, in which immunomodulatory drugs administered early on in natural history have shown some success (Pescovitz et al.2014, Salvi 2014). An interesting observation from this study was the rapid decline in the serum cortisol level following commencement of exogenous steroid treatment within one month of diagnosis. On average, there was a reduction in serum cortisol level of more than 50% in 3 weeks (Pearce et al. 2012). It was suggested that the removal of trophic influence of ACTH following exogenous steroid could partly lead to the rapid decrease in adrenal steroid biosynthesis. This phenomenon demonstrates the plasticity of the adrenal cortex. I believe that a good understanding of this concept is the key to unlocking the next step towards regenerative medicine in Addison's disease.

1.9 Adrenal plasticity

The adrenal cortex is one of the most plastic tissues in the human body. For more than 60 years, it has been known that bilateral subcapsular enucleation in a rodent resulted in the regeneration of adrenal mass within 4-6 weeks (Ingle & Higgins 1938, Greep & Deane 1949). Steroidogenic function was detected as early as day-8 following enucleation, while full steroidogenic capacity could continue developing for up to 6 weeks (Ingle & Higgins 1938, Ennen et al. 2005). A similar picture was also depicted when the adrenal cortical tissue with capsule attached were auto-transplanted to a flank muscle pocket in a rodent model undergoing bilateral adrenalectomy (Schaberg 1955, Saxe & Connors 1985, Beuschlein et al 2002). Interestingly, administration of exogenous glucocorticoids to the rodent model following adrenal enucleation inhibited the regrowth of adrenal glands and suppressed steroidogenesis (Ingle & Higgins 1938). This suggests that both adrenal size and steroidogenic frunction are primarily regulated by ACTH secreted from the pituitary gland.

In humans, ACTH exhibits diurnal pulsatile secretary pattern. The ACTH secretion starts during sleep and is characterised by a series of plasma peaks for 8-10 hours, followed by a trough level in the afternoon and evening (Veldhuis et al. 1990, Horrocks et al. 1990). Both the adrenal size and steroidogenic function are strongly regulated by this daily pulsatile ACTH secretion. During chronic exogenous glucocorticoid therapy, ACTH secretion is suppressed leading to adrenal atrophy, and functional adrenal failure ensues if exogenous steroids are rapidly withdrawn. Conversely, adrenal gland hyperplasia is seen during Cushing's disease following excessive pituitary ACTH secretion. These lines of evidence suggest that adrenocortical tissue is highly plastic and ACTH is the main regulator of adrenal cell mass and steroidogenesis. The adrenal plasticity is likely owing to the presence of adrenocortical progenitor or stem cells, which continuously repopulate the adrenal cortex in an ACTH-responsive fashion. With this in mind, understanding the characteristics and

behaviour of adrenocortical stem cells is crucial to enabling us to extrapolate the regenerative potential of cortical adrenocytes as a potential therapeutic approach in adrenal failure.

1.10 Adrenocortical stem/progenitor cells

The widely accepted model generated from rodent studies suggests that adrenocortical stem cells (ACSCs) are located in or adjacent to the adrenal capsule, which is also known as the blastema layer. They have the ability to proliferate and differentiate into various steroidogenic lineages upon stimulation of their trophic peptide hormone ($ACTH_{1-39}$) (Pignatelli et al.2002, Kim & Hammer 2007,). However, the identity or molecular phenotype of the adult ACSCs remains uncharacterised in both rodents and man, largely due to the lack of specific markers that define this unique group of cells.

In fact, the 3 separate zonal organisations of adrenal cortex were initially thought to have separated histogenesis but pulse chase studies using bromodeoxyuridine (BrdU) and thymidine-H^3 suggest otherwise. These experiments exhibited gradual dilution of signals, with the strongest signals detected at the peripheral followed by an increasingly centripetal localisation over time (Ford and Young 1963, Zajicek et al. 1986). A transgenic mice model, utilising beta-galactosidase reporters under the control of a steroidogenic promoter, also revealed variegated expression of reporter genes in cord-like radial stripe pattern (Iannaccone et al. 2003). This radial clonality of adrenocytes, which originates from the capsular progenitors and extends radially from the periphery to the corticomedullary junction, had a transit-time of about 104 days in the rat (Zajicek et al. 1986). Furthermore, a histology study using proliferating cell nuclear antigen (PCNA) markers showed that most adrenocortical cells with mitotic DNA were populated at the peripherals of the adrenal

cortex and not the capsule (Mitani et al. 1999, Schulte et al. 2007). The evidence suggests that the adrenocortical cells originating from the ACSCs proliferate and undergo centripetal displacement with a continuous, zone-specific differentiation programme and eventually undergo apoptosis at the corticomedullary junction (Mitani et al. 1999, Pignatelli et al. 2002, Kim & Hammer 2007). The daughter cells will first take on the aldosterone-secreting phenotype while in transit through the outermost layer, the zona glomerulosa, followed by cortisol and adrenal androgen secretion when migrating through zone fasciculata and reticularis respectively.

In recent years, genetic lineage tracing studies of the various morphogenic signalling pathways and transcription factors in mice have consolidated the evidence supporting the presence of stem/progenitor cells in the capsule or subcapsular region. The details of this phenomenon will be discussed below.

1.11 The origin of adrenocortical stem/progenitor cells

Although the adrenal capsule has been ascertained to be the stem cell niche for adult progenitor/stem cells, the source and phenotypic characteristics of ACSCs have yet to be fully defined. As previously discussed, the fetal adrenal enhancer (FAdE) is responsible for SF1 expression in the fetal adrenal cortex during embryogenesis, which is then maintained by SF1 itself through feed-forward autoregulation (Zubair et al. 2006). Further studies showed that FAdE was abrogated at E14.5 in mice, followed by the regression of the fetal cortex (Zubair et al. 2006). Lineage tracing studies in mice using an inducible Cre transgene under the control of FAdE showed that all SF1 positive adult adrenocortical cells were derived from the fetal adrenal zone. (Zubair et al. 2008). Nevertheless, the nature of the definitive SF1 enhancer that activates SF1 transcription in adult adrenal remains elusive, and

there is still a knowledge gap in terms of the molecular and cellular mechanism that coordinates this transition from fetal to the definitive adult adrenal cortex.

The second potential source of ACSCs was unravelled following the discovery of sonic hedgehog (SHH) signalling pathway. This morphogenic signalling pathway has been found to be essential for adrenal gland development and maintenance (King et al. 2009, Ching & Vilain 2009, Huang et al 2010,). SHH co-localises with SF1 at the subcapsular region of the adrenal cortex. They are not found in the rest of the adrenal cortex that expresses both SF1 and differentiated steroidogenic markers such as CYP11B1 and CYP11B2 (King et al. 2009). Interestingly, further lineage tracing studies revealed that SHH expressing cells at the subcapsular region give rise to the differentiated adrenocortical cells (King et al. 2009). In addition, a lineage tracing study carried out by Kim et al. showed that the descendants of the downstream activator of the SHH pathway, GLI1, which is only found in the adrenal capsule and does not express SF1, gives rise to GLI1 negative, SF1 or CYP11B1/ CYP11A1 positive adrenal cells. Consistent with the finding of Kim et al., Huang et al. demonstrated centripetal detection of lacZ activity in the adrenal cortex of mice, after a long period of Gli1-Cre activation, in an inducible Gli1-Cre transgene crossed with a lacZ reporter (Huang et al. 2010).

Hence, it appears that there are 2 potential adrenocortical stem/progenitor cell groups: 1. ACSCs originating from the fetal adrenal cortex; 2. GLI1-positive SF1-negative adrenal cells and SHH-positive, SF1-positive cells from the capsule/subcapsular region, all of which give rise to SF1-positive, differentiated adrenocortical cells. One could also hypothesize that GLI1-postive SF1-negative cells are adrenocortical stem cells which give rise to the intermediate, SHH-positive, SF1-positive progenitor cell group, and these cells act as transient amplifying progenitor cells that differentiate into SF1 positive adrenocortical cells, but further studies are required to prove this hypothesis.

Wood and Hammer then proposed a two-lineage unifying model combining these potential sources of adrenocortical stem/progenitor cells (figure 1.6). The model involves a primary lineage established from SF1 positive, coelomic epithelium which is delaminated into the mesenchyme which forms the fetal adrenal cortex. The secondary lineage derived from SF1 negative mesenchymal cells resides at the capsule giving rise to SF1 positive adult adrenocortical cells (Wood & Hammer 2011). The SF1 positive fetal adrenal-derived primary cell lineage, which is independant of SHH signalling, may initiate SHH expression (via an unknown mechanism) and induce GLI1 expression among nearby SF1 negative mesenchymal cells, converting them into SF1 negative, GLI1 positive cells that serve as an intermediate between SF1 expressing cells originating from the fetal adrenal cortex and SF1-positive adult adrenal cells directed by definitive SF1 enhancer. Although genetic studies have shown that capsular cells are capable of giving rise to differentiated adrenal cortical cells (King et al. 2009, Ching and Vilain 2009, Huang et al. 2010), no studies have shown that these cells are derived from fetal adrenal cell thus far. Hence, the mechanistic and molecular basis for the definitive transition between all these cells remains elusive. Nevertheless, it has recently been revealed that the steroidogenic Leydig cells of the testes are derived from both coelomic epithelium and mesonephric mesehcyme (DeFalco et al 2011), suggesting the same might hold true for adrenal steroidogenic cells.

Figure 1.6. Unifying Model of Adrenocortical Stem/Progenitor Cells

(Reproduced from Wood & Hammer 2011)
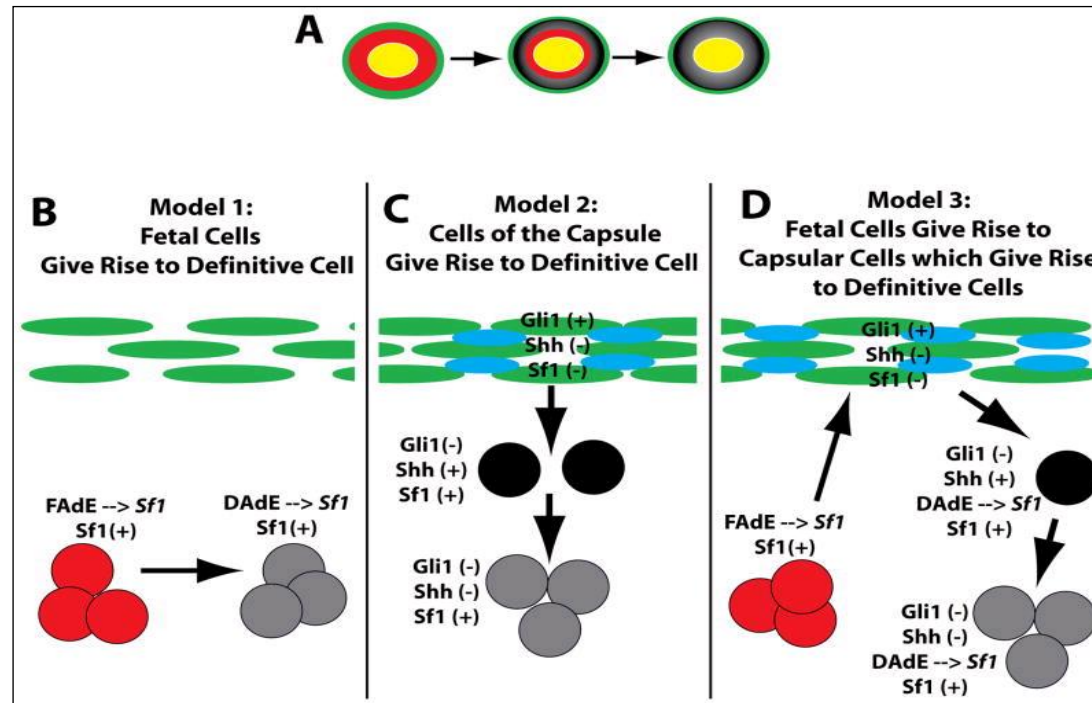
Model 1. Definitive adrenal cells arise from fetal adrenal cells. Model 2. Definitive adrenal cells arise from capsular stem/progenitor cells or subcapsular stem/progenitor cells. Model 3.Proposed unifying model of adrenal stem cells in which fetal adrenal cells give rise to a transition cell in the capsule which in turn gives rise to the definitive adrenal cells.(Colours of respective zones: capsular cell, green; fetal zone, red; capsular stem/progenitor cell, blue; subcapsular/progenitor cell, black; definitive zone, gray)

1.12 Adrenocortical stem cell niche and mediators for stem cell maintenance, proliferation and differentiation

I will next discuss the details of the sonic hedgehog morphogenic signalling pathways (SHH) and endocrine transcription factors (eg. *SF1*, *DAX1*) involved in the development and maintenance of adrenocortical stem cells. Major interest has been drawn to the molecular biology of adrenocortical stem cells in the recent years, largely due to their implication in the development of adrenocortical tumours. Most of these studies were carried out in murine models and have given sound evidence suggesting their essential roles in adrenocortical growth and differentiation, as well as the regulation of adrenocortical stem cells.

1.12.1 Steroidogenic factor-1(SF1)

Steroidogenic factor-1(SF1) or Ad4BP (Ad4 binding protein) is an orphan nuclear receptor paramount in transcriptional regulation of various genes encoding the ACTH-activated steroidogenic enzymes (i.e cytochrome P450 side-chain cleavage enzyme, 17α-hydroxylase , 3β-hydroxysteroid dehydrogenase ,21-hydroxylase , 11β-hydroxylase), as well as several additional genes involved in the steroid biosynthetic pathway, such as the melanocortin 2 receptor (MC2R) and the steroidogenic acute regulatory (STAR) protein (Luo et al. 1994, Parker & Schimmer 1994, Zhang et al. 1995) . It plays a pivotal role in adrenocortical proliferation and differentiation. Adrenal agenesis was demonstrated in homozygous *Sf1* knocked-out mice which died shortly after death from adrenal insufficiency; whereas mice heterozygous for *Sf1* were viable with small adrenal glands (Bland et al. 2000& 2004). Similarly, no homozygous loss of

function mutation in *SF1* have been reported in human to date, likely due to the fact that complete loss of *SF1* is not compatible with life (Ferraz-de-Souza et al. 2011).

SF1 is expressed in various steroidogenic tissues including the adrenal cortex, ovarian theca and granulosa cells, as well as testicular Leydig cells (Hammer & Ingraham 1999). All cortical adrenocytes express SF1 but the adrenal capsule is devoid of its expression (Morohashi & Omura 1996). Upon ACTH stimulation, SF1 is actively recruited to the promoters of a variety of steroidogenic enzyme genes (Winnay et al. 2006). A cyclical occupancy of SF1 on MC2R gene promoter was also exhibited, which coincides with the phosphorylation of SF1 and leads to the recruitment of co-activator proteins resulting in transcriptional activation (Winnay & Hammer 2006).

Interestingly, SF1 has been shown to serve as both a transcriptional activator and repressor of ACTH-dependant target genes, which is well depicted in its action on pre-B-cell transcription factor 1 (*PBX1*) and *DAX1* transcription factor. PBX1 is a direct target of SF1 and acts as the downstream activator of SF1-dependant steroidogenesis (Lichtenauer et al. 2007). DAX1 is another common target gene for SF1 and the gene product was found to serve as a repressor of SF1-dependant transactivation, leading to the inhibition of steroidogenesis in the subcapsular region of adrenal cortex (Lalli et al. 2003).

1.12.2 DAX1(dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region, on the chromosome X, gene 1) transcription factor

DAX1 is an orphan transcriptional factor specifically enriched in the subcapsular region of the adrenal cortex (Swain et al. 1998, Babu et al. 2002). It appears to be necessary for the maintenance of ACSCs in the undifferentiated state with proliferative capacity, via functional repression of SF1 (Ito et al. 1997, Niakan & McCabe 2005, Niakan et al. 2006). DAX1-deficient patients usually exhibit adrenal hypoplasia resulting in adrenal insufficiency (Zanaria et al. 1994, Phelan & McCabe 2001). The widely accepted model suggests a synergistic glucocorticoid receptor (GR)/SF1-dependant induction of DAX1 expression, which in turn leads to the repression of SF1-mediated steroidogenesis (Lalli & Sassone-Corsi 2003). However, the binding of ACTH to MC2R stimulates adenylyl cyclase and induces cAMP production which then unblocks both GR and SF1 from DAX1 promoters. This will then shut off DAX1 transcription and initiate adrenal steroidogenesis (Kim A et al. 2009). Hence, as DAX1 is only present in the subcapsular region (potential stem cell niche), steroidogenesis is inhibited among the undifferentiated progenitor cells and the 'stem-ness' of ACSCs is maintained. Additionally, DAX1 is vital in the fate decision (adrenal vs gonad) and differentiation process of fetal adrenal cells through regulation of GATA 6 transcription (Tremblay & Viger 2001, Jimenez et al. 2003). This is clinically evident from the autopsy of patients with adrenal hypoplasia congenital (AHC- caused by hypofunctional mutations of DAX1), in which the adrenal glands fail to establish an adult ACSC population and broadly show the histological appearance of the fetal adrenal gland (MacMohon et al. 1957). Interestingly, young Dax1 knockout mice initially exhibited a hyperfunction adrenal state consistent with the loss of Dax1-dependant inhibition of steroidogenesis

(Schey et al. 2011) but was followed by attenuation of steroidogenic capacity when they aged, with profound loss of the subcapsular region and concomittent adrenal dysplasia were observed (Yu et al. 1998, Scheys et al. 2011). This model is in keeping with a number of patients with *DAX1* mutations demonstrating enhancement of steroidogenic function state prior to the development of adrenal failure (Lin et al. 2006, El-Khairi et al. 2011).

1.12.3 The sonic hedgehog signalling pathway

The sonic hedgehog (SHH) signalling pathway, which was first discovered following a mutational screen in Drosophilia, was found to be vital in the regulation of embryonic development and adult stem cell maintenance (Ingham & McMahon 2001, McMahon et al. 2003, Xie & Abbruzzese 2003, Gupta et al. 2010, Ingham et al. 2011). SHH is a secreted signalling protein that acts upon target cells by binding to a twelve pass transmembrane binding protein, the Patched1 (Ptch1) membrane receptor (Allen et al. 2011, Izzi et al. 2011). In the absence of the SHH protein, Ptch1 inhibits the accumulation of the seven pass transmembrane protein, the Smoothened (Smo). When the SHH ligand binds to the cell surface receptor Ptch1, it relieves the Ptch1-mediated repression of the Smo protein, leading to downstream activation and expression of the GLI family transcription factors (Ingham and McMahon 2001, Bai et al. 2004).

SHH colocalises with SF1 within the cortical cells in the subcapsular region, but fully differentiated adrenocytes in the rest of the adrenal cortex are devoid of SHH expression (Ching and Vilain 2009, King et al. 2009). When genetic lineage analysis was performed in mice using constitutive Cre expressed from the Shh locus in combination

with conditional floxed reporter, it was shown that the Shh expressing cells give rise to cells in the differentiated steroidogenic adrenal cortex, but not capsule or medulla (King et al 2009). This signifies its role in the proliferation and maintenance of the adrenal cortex but not adrenal differentiation (Ching and Vilain 2009, Huang et al. 2010, King et al. 2009). On the other hand, conditional deletion of *Shh* in steroidogenic adrenocortical cells (under the *Sf1* promoter) demonstrated marked adrenal hypoplasia, with the adrenal gland being 5-10 times smaller than the wild type with normal-size medulla (Ching & Vilain 2009, king et al 2009, Huang et al. 2010). Despite the clear size reduction with a thin capsule, the adrenal gland managed to maintain proper zonal organisation and steroidogenic cell differentiation (Ching & Vilain 2009, King et al. 2009, Huang et al. 2010). These observations suggest that the SHH pathway does not influence the initiation of adrenal differentiation but is essential for the optimal growth of the adrenal capsule, to maintain the pluripotency of adrenocortical stem cells. Nevertheless, it remains undetermined if this proliferative ability of capsular cells is due to the direct effect of SHH as a capsular cell mitogen or a secondary signal that acts on the capsule mesenchyme. Interestingly, the *Shh* mutant mice suffered from adrenal insufficiency when they aged despite having normal glucocorticoid levels when they were young (Huang et al. 2010). Hence, it remains a possibility that humans with impaired SHH signalling might suffer from adrenal insufficiency, particularly when they grow older or when elevated steroid production is required during stress (Laufer et al. 2012).

Summary

The regulation of adrenocortical stem/progenitor population is predicted to be a dynamic process involving both activation and inhibition pathways, combining the exquisite orchestration of paracrine and endocrine signals to achieve an integrated homeostatic mechanism for organ maintenance. Although genetic lineage tracing studies performed on rodent models have started to shed light on this field, there is still a significant knowledge gap on the regulation of ACSC proliferation and differentiation in humans. More work is required to define this unique group of cells.

Figure 1.7 A schematic overview of the signalling pathways involved in the regulation of adrenocortical stem/progenitor cells
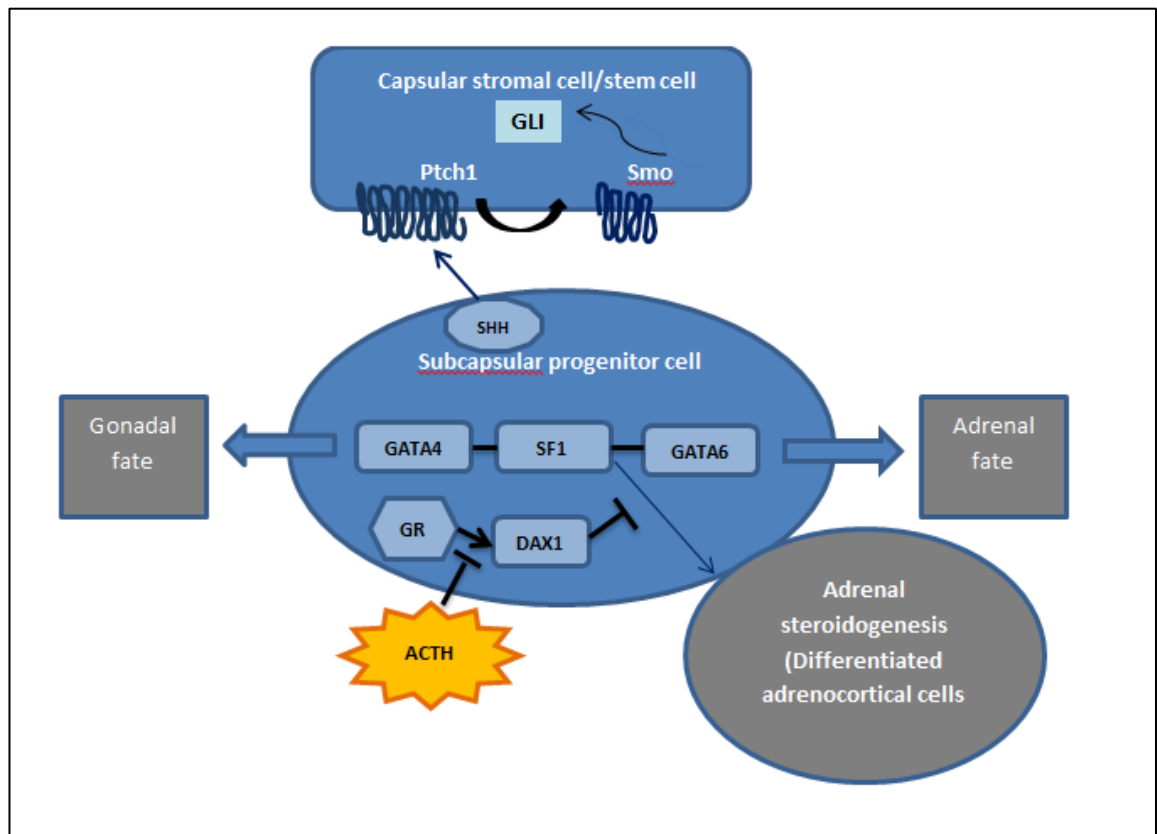
Figure 1.7 depicts the signaling pathways involved in the maintenance and differentiation of adrenocortical stem/progenitor cells. DAX1 inhibits the activation of SF1-mediated steroidogenesis and maintain the cells in an undifferentiated state. ACTH induces the clearance of GR from the DAX1 promoter, resulting in transcriptional silencing of the DAX1 gene and initiation of SF1-mediated steroidogenesis. DAX1 also participates in tissue fate decisions through dynamic regulation of GATA4/GATA6 transcription factors in adrenals and gonads. SHH is secreted from the subcapsular cell to activate SHH signaling in the capsule by binding to the Ptch1 transmembrane receptor, leading to downstream activation and expression of the GLI family transcription factors.

Table 1.3. Potential molecules involves in ACSC maintenance and development

Molecule	Expression	Proposed function	Clinical disease
DAX1	Subcapsular	-Activated by Wnt signalling and glucocorticoids -Potential mediator of undifferentiated state in ACSC -Inhibits SF1-mediated steroidogenesis	X-linked cytomegalic adrenal hypoplasia congenita
SHH	Subcapsular/ peripheral cortex	-Required for adrenal development and differentiation of SHH(+)/ SF1(+) cells into differentiated SF1(+) adrenocortical cell types	None/unknown
GLI1	Capsule	-Potential ACSC [SF1 (-)/ GLI(+) capsular cell] gives rise to underlying cortex -GLI(+)/SF1(−) cells differentiate into all other SF1(+) adrenocortical lineages during fetal development	None/unknown
Wnt ligands & β-catenin (Effector of Wnt signalling)	Subcapsular region and fetal cortex	-Contributes to multipotency of undifferentiated cells (ACSC) -Initiation of adrenal tumorigenesis	Nuclear accumulation and mutation in adrenocortical adenoma/carcinoma

Molecule	Expression	Proposed function	Clinical disease
SF1	Cortex	-Transcription of steroidogenic enzymes Induction of adrenal cell fate (in progenitor cells?)	Adrenal hypoplasia, upregulated in childhood and adult adrenocortical carcinoma
Inhibin-α	Subcapsular	-Prevents TGF II-dependent initiation of transcription -Gatekeeper of adrenal cell fate	Adrenocortical tumour in children, ovarian/testicular germline tumour
Pod1	Capsule	-Inhibition of SF1 transcription in the adrenal capsule (potential ACSCs)	None/unknown
ACTH	Endocrine signals	-Steroidogenesis differentiation -SF1 transactivation -SF1 phosphorylation	Familial glucocorticoid deficiency (ACTH receptor mutation)

Aims of the thesis

It is clear from my overview that there is still a substantial gap in our understanding of the pathophysiology of AAD and human ACSC biology, which needs to be addressed in order to advance this field further. This could be achieved by direct examination of patients with active diseases through either clinical or genetics studies, as well as cellular studies using human adrenal tissues. This approach can overcome the limitations encountered in rodent models due to the substantial differences between rodent and human adrenal. The suboptimal health status among AAD patients also calls for a better therapeutic approach. The general aim of this studentship is to explore the pathophysiology of AAD and to pursue alternative treatment options using a regenerative medicine approach to augment adrenal steroidogenesis, with the ultimate aim of improving the health and long-term outlook of AAD patients.

Study aims:

- To explore the potential causal pathway of common and rare genetic determinants in AAD, that has been implicated by susceptibility in other autoimmune conditions.

- To pursue new treatment options in AAD via re-establishment of adrenocortical steroidogenesis using a therapeutic regimen of parenteral $ACTH_{1-24}$.

- To study the natural history and latency of endogenous ACSC revival, tolerability and potential side effects of the new treatment.

- To elucidate the cellular phenotypic markers of human adrenocortical stem cells (ACSC).

Chapter 2.   Genetics of Autoimmune Addison's disease

2.1 Background

In keeping with many autoimmune conditions, AAD has a strong genetic predisposition.
Detailed understanding of the various genetic determinants in AAD could potentially
uncover causal pathways that have been implicated in this rare but debilitating disease.
Recent genome-wide studies in the commoner autoimmune conditions have identified
novel disease-susceptibility alleles at an unprecedented rate. However, the contribution
of these alleles to AAD has yet to be fully elucidated. Furthermore, most of the
susceptibility alleles identified thus far was found to have a modest disease effect.
Therefore, there are potentially many more unexplored genetic variants implicated in
AAD.

The non-synonymous polymorphism in exon 7 of the gene encoding the lymphocyte
cell-surface CD226 (DNAM1) receptor, Gly307Ser (rs763361), has recently been
identified as conferring risk to type 1 diabetes, Graves' disease, multiple sclerosis,
systemic lupus erythematosus (SLE), systemic sclerosis and rheumatoid arthritis (Hafler
et al. 2009, Maiti et al. 2010, Han et al. 2010 Dieudé et al. 2010, Alcina et al.2010), and
has yet to be investigated in AAD. The variant involves the serine-encoding allele of a
polymorphism in the intracellular tail of the cell-surface CD226 molecule (*CD226
307*Ser*) (Todd et al. 2007). CD226 is also known as the DNAX-accessory molecule-1
(DNAM-1), and is widely expressed in cells of haematopoetic origin including CD4$^+$
and CD8$^+$ T lymphocytes, natural killer (NK) cells, platelets, subset of B cells and
natural killer T lymphocytes, where it plays the role of a costimulatory molecule
(Shibuya et al. 1996). It is possible that the 307*Ser variant alters splicing of the CD226
transcript (Todd et al. 2007, Hafler et al. 2009), or that the change in residue itself
affects downstream signalling. The addition of Ser307 to the adjacent serine-threonine
phosphorylation sites Ser302, Ser305 and Thr306, which are present in the cytoplasmic

tail of the CD226 molecule, might have an effect on the protein level and lead to increased signalling for the activation of T and NK cells (Lofgren et al. 2010). *CD226 307*Ser* is a common genetic variant and the minor allele frequency (MAF) established in the UK 1958 birth cohort controls was 0.47 (Hafler et al. 2009).

On the other hand, a panel of rare and functionally defective genetic variants in the sialic acid acetylesterase (*SIAE*) gene were identified as being strongly associated with many autoimmune conditions, including Crohn's disease, type1 diabetes, SLE, Sjogren's syndrome, juvenile idiopathic arthritis, multiple sclerosis, mixed connective tissue disease, rheumatoid arthritis and ulcerative colitis (Odds ratio>8) (Surolia et al. 2010). This study originally described 19 rare variants of *SIAE* gene of which 11 were catalytically defective. A subsequent larger study (n=66,924) of more prevalent autoimmune and inflammatory disorders, including type 1 diabetes, coeliac disease, Crohn's disease and autoimmune thyroid disease failed to replicate this finding (Hunt et at. 2011). Nevertheless, *SIAE* represents one of the very first associations of rare genomic variants with common autoimmune disorders. Interestingly, loss-of-function rare variants in the *TREX1* (the major mammalian 3'-5' exonuclease) and *CYP27B1* (vitamin D 1α-hydroxylase) genes have also been described in SLE and MS patients respectively, suggesting that in contrast to the common disease-common variant hypothesis, there may be a greater role for rare genetic variants in the susceptibility to less prevalent autoimmune diseases (Namjou et al. 2011, Ramagopala et al. 2011).

Sialic acid acetylesterase (SIAE) is a negative regulator of B lymphocyte signalling. It removes acetyl moieties from the 9-OH position of sialic acid, allows sialic acid on B cell glycoproteins to interact with CD22 (B cell inhibitory receptor) and results in attenuation of B-cell receptor (BCR) signalling (Cariappa et al. 2009). Enhanced BCR signalling with spontaneous anti-chromatin antibodies production had been

demonstrated in *SIAE* mutant mice on a C57Bl/6 background, suggesting that defects in SIAE function conferred by *SIAE* variants might contribute to human autoimmunity (Pillai et al. 2009). Although genome-wide studies have not demonstrated any association or linkage with SNPs at the SIAE locus in patients with autoimmunity, this might not be expected under a multiple rare variant model (common disease/ rare variant). Nevertheless, autoimmune Addison's disease is rarer than all of the previously studied disorders and hence rare variants might have a significant contribution to its pathogenesis.

With this in mind, I have examined the role of the *CD226 307*Ser* variant (*rs763361*) and rare *SIAE* variants on the susceptibility to AAD.

2.2 Subjects and Methods

2.2.1 Subjects

Autoimmune Addison's disease subjects

The diagnosis of AAD was confirmed by either a low basal cortisol with a high adrenocorticotropic hormone (ACTH) level, or a subnormal response to the $ACTH_{1-24}$ stimulation test (short synacthen test, using 250 mcg of parenteral synthetic $ACTH_{1-24}$). Patients with autoimmune polyendocrinopathy syndrome type 1 (APS1), primary adrenal failure owing to infiltrative or infective causes or secondary adrenal failure were excluded. Caucasian subjects with AAD were recruited between 1996 and 2012 through outpatient endocrinology services in the North East of England, and through the UK Addison's disease self-help group. In the CD226 association study, I investigated 326 Caucasian subjects with AAD and the details of the subgroup of AAD are depicted in

table 2.1. On the other hand, 378 Caucasian subjects with AAD were studied in the SIAE association study.

Healthy control subjects

Healthy controls were used to determine the background population of allele frequencies. They were healthy local Caucasian controls with no clinical features or family history of autoimmune diseases. However the autoantibody status was not known and was not tested. Hence, the presence of subclinical autoimmune conditions could not be completely excluded. 311 and 387 healthy local Caucasian controls were used for comparison in the association study for CD226 and SIAE candidate genes respectively. These studies were carried out with the approval of the Leeds (East) Research Ethics Committee (Ref 05/Q1206/144).

2.2.2 Methods

2.2.3 Polymerase chain reaction (PCR) and gel electrophoresis

Polymerase chain reaction (PCR) was used to amplify a region of deoxyribonucleic acid (DNA) around a single nucleotide polymorphism (SNP) or a specific nucleotide region of interest for sequencing. This was achieved through a measurable Taq DNA polymerase which could add nucleotides to pre-existing 3'-OH groups of a primer pair designed for specific DNA template strands.

I followed the generic rules when designing primers for PCR reactions. In general, the primers were 20-25 bp long with > 50% GC content. Repetitive sequences or runs of bases were avoided and the primer pairs were balanced for annealing temperature. Primer pairs were also examined to avoid the presence of hairpin loops or risk of annealing to each other. Finally each primer was assessed to ensure they were suitable

for a unique part of the genome using the BLAST (Basic Local Alignment Search Tool) programme. The final products were between 200-500bp.

The PCR reaction mixture and conditions were according to the recommended manufacturer's protocol, as detailed in table 2.2. Three water blanks were included in every PCR set-up to ensure that there would be no contamination. The PCR would be discarded if the water blank was positive. The PCR reaction conditions were optimized by adjusting the annealing temperature, magnesium concentration or the duration for annealing and extension steps. PCR was performed on the Tetrad 2 thermal cycler and the cycling parameters for each experiment are detailed in the following section.

The PCR products were then checked by electrophoresis (120v) on a 2% agarose gel. 5μl of sample was loaded with 1μl of DNA loading dye (Thermoscientific) in each well and 5μl DNA marker (100bp size ladder) was run in the first lane. Gels were stained with SafeView^{TM} (Newlife BioChemEx, Bethesda) and visualized under UV light.

2.2.4 CD226 genotyping:

I used a TaqMan allelic discrimination assay to genotype the *CD226* variants, Gly307Ser (*rs763361*) for disease susceptibility in AAD. TaqMan genotyping technique involves 2 allele-specific minor grove binder (MGB) probes containing distinct fluorescent dyes and a PCR primer pair to enable detection of a specific SNP target as it accumulates during PCR cycles. The probes also contained a non-fluorescent quencher and a MGB which allows for shorter probe sequences to be designed. The possible genotypes for rs763361 were CC,CT and TT. Two primer (forward and reverse) and probe pairs were used in each reaction to genotype the two possible variants at the SNP site. The probes and primers were provided by Taqman SNP genotyping assay (Applied

Biosystems; assay ID:C_1464836_20). The principle of Taqman allele discrimination is depicted in figure 2.1. I used the genomic DNA previously extracted from the venous blood of each subjects in my supervisor's laboratory. PCR was performed to multiply the DNA sequence of interest with CD226 variants and the parameters for the PCR mixture and reactions are outlined in table 2.2 & 2.3. Allelic discrimination plate read and analysis was carried out using the 7900 HT Fast Real-Time PCR system and SDS 2.3 software. Ten percent of all samples were re-genotyped blind to ensure fidelity of genotyping (>99%).

Table 2.1 The number of isolated AAD vs AAD patients with associated autoimmune conditions in CD226 candidate gene studyThe number of isolated AAD vs AAD patients with associated autoimmune conditions in CD226 candidate gene study

CD226 association study (n=326)	
Isolated AAD	44% (143)
APS2	56% (183)
• Autoimmune hypothyroidism (106) • Graves'disease (30) • Premature ovarian failure (30) • Type 1 diabetes (17) • Pernicious anaemia (14) • Rheumatoid arthritis (8) • Vitiligo (8) • Coeliac disease (7) • Alopecia (2)	

2.2.5 SIAE genotyping

I carried out the genotyping of *SIAE* rare variants using Sequenom iPLEX for single nucleotide polymorphisms (SNPs). The results were verified by either restriction enzyme digestion (RFLP) or direct DNA sequencing where appropriate.

2.2.6 Primer extension-MALDI-TOF genotyping (SEQUENOM-iPLEX)

In contrast to the Taqman genotyping method, Sequenom employs a label-free primer extension reaction to produce allele specific oligonucleotides with distinct masses. The mass differences allow allele discrimination for each of the SNPs to be multiplexed in one single assay, leading to high-throughput genotyping. The high-throughput detection capacity is due to the speed and accuracy of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry. It is based on the principles that the time of flight of each molecule travelling through the vacuum is proportional to the molecule size and the analysis is done in real-time when the molecules hit the detector. The allele discrimination in the iPLEX assay is conferred by primer extension with a high-fidelity polymerase enzyme across the SNP site, with universal reaction conditions for all SNPs. This results in allele-specific differences in mass among the extended products.

Assay design: I studied 9 germline variants within the *SIAE* gene (chromosome 11), which are among the 11 rare and 1 common non-synonymous SIAE variants demonstrated by Surolia et al. to be functionally defective in esterase activity or enzyme secretion (table 2.4). These rare SNPs comprise 21 of the 24 cases (88%) of functionally defective rare SIAE variants reported by Surolia et al. The SNPs selected include C196F, T312M, C226F, F404S, R230W, R479C, W48X, Y349C and M89V. These SNPs were genotyped in Caucasian individuals with AAD and healthy controls in the

UK. The location of these 9 SNPs was identified from ENSEMBL (chromosome 11: 124.503009-124.565.603). The DNA sequence (300-400bp) incorporating the SNP of interest were imported into the MassARRAY assay design software (www.MySequenom.com). The programme multiplexed suitable SNPs together and determined a single base extension primer for each SNP. PCR primers are designed in a region of approximately 100 base pairs around the SNP of interest and an extension primer is designed immediately adjacent to the SNP. There were 9 out of 14 SNPs in SIAE rare variants found to be suitable for a single multiplex sequenom assay design. The primer sets were purchased from Metabion international AG. The details of the primers of the 9 SNPs assayed are outlined in table 2.5.

Table 2.2 PCR reaction mixture for CD226 genotyping (rs763361)

Components (final volume 5 µl/well)	Volume/well (µl)
Genomic DNA (20ng)	1.5
Taqman Universal Mastermix (No AmpErase UNG)	2.5
Taqman SNP Genotyping Assay 20x	0.25
Sterile water	0.75

Table 2.3 PCR thermal cycling condition for CD226 genotyping (rs763361)

Thermal cycling step	Conditions
1. AmpliTaq gold enzyme activation	Hold at 95 ^{o}C (10 minutes)
2. DNA denaturation	92°C (15s)
3. Primer annealling/polymerase extension	60 ^{o}C (1 minutes)
Cycles (2-3)	40 times

.
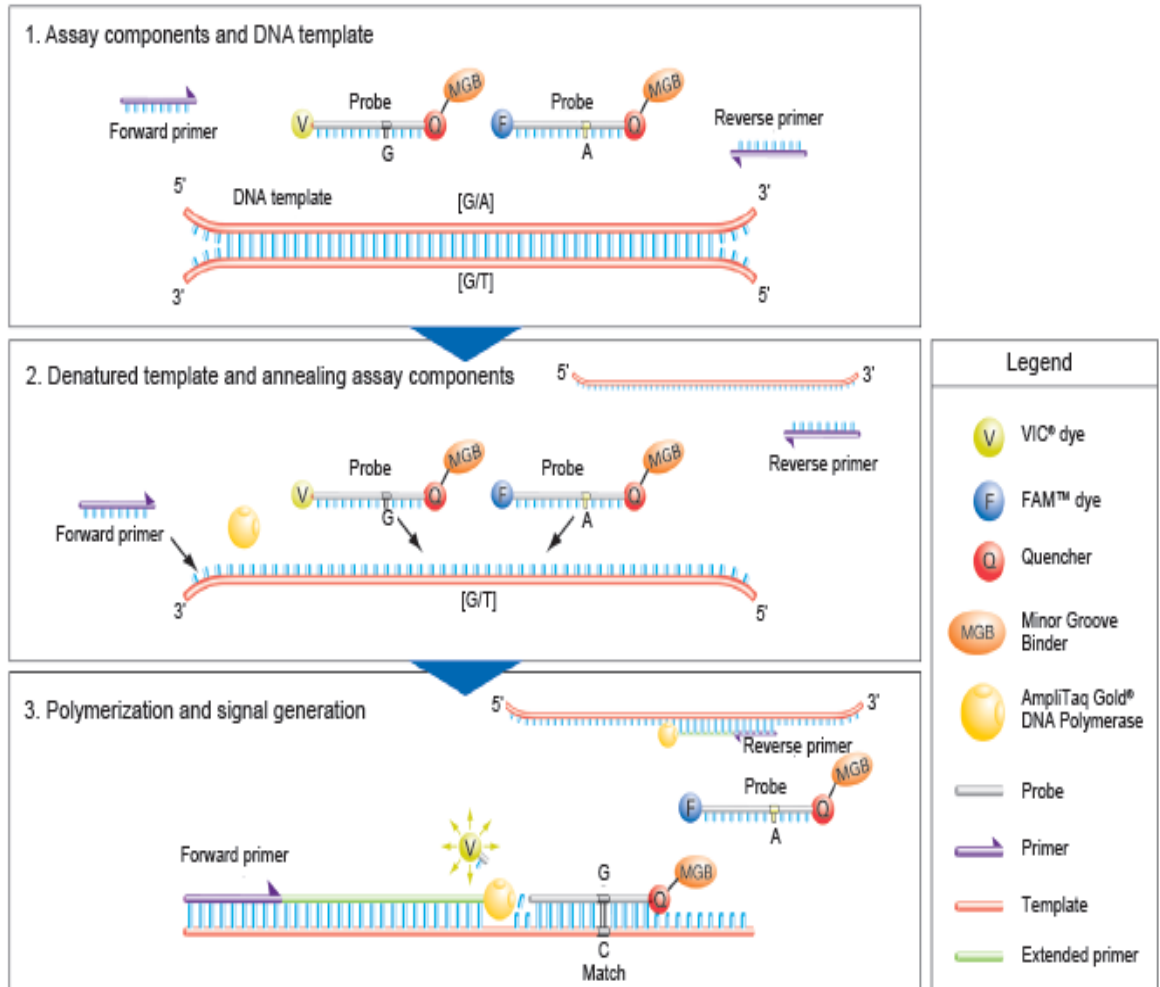
Figure 2.1 Taqman allelic discrimination assay

Taqman allelic discrimination assay was used in genotyping CD226 polymorphism.
This is achieved by the selective annealing of allele specific oligonucleotide (MGB-
labelled) probes upon specific targeted SNP. One probe that perfectly matched to the
wild-type sequence variant labelled with VIC and the second probe matched to the
mutant (SNP) sequence variant labelled with 6-carboxyfluorescein (FAM). The probe
for allele A was labeled with FAM™ dye; the probe for allele G was labeled with VIC®
dye. These TaqMan® probe and primer sets uniquely align with the genome to provide
unmatched specificity for the allele of interest.

Table 2.4 The 12 functionally defective SIAE variants reported and the number of cases found (%) by Surolia et al. (Surolia et al.2010)

Amino acid change	Sequence change	Frequency (%) n=1571
W48X	143 G>A	0.0636
M89V	265 A>G	0.076
C196F	517G>T	0.191
G212R*	634G>A	0.0636
C226G	796T>G	0.0636
R230W	688C>T	0.0636
Q309P*	926A>C	0.0636
T312M	935C>T	0.1273
Y349C	1046A>G	0.0636
R393H*	1178G>A	0.0636
F404S	1211T>C	0.2546
R479C	1435C>T	0.0636

*SNPs not selected in my study

Table 2.5 Details of the primers for SIAE single nucleotide polymorphism (SNPs) genotyped by Sequenom iPLEX assay.

This was a 14-plex assay, with the remaining 5 SNPs consisting of other genes of interest being studied by other members of the research group. The multiplex was designed using the sequenom iPLEX assay design software programme.

SP ID	Primer 1	Primer 2	Extension primer	Amplicon length(bp)
W48X	ACGTTGGATGTGATATGGTGCTGCAGAAGG	ACGTTGGATGTCACTGTGGCTCCAGGTGTA	TGCTGGGGCAGTGATAT	91
M89V	ACGTTGGATGTCACTCTGATACGTGGATGG	ACGTTGGATGCCAAAGTCTGTTGTGCCATC	TACGTGGATGGTGGTACTGGATCCT	100
C196F	ACGTTGGATGCAGAAAACTTAGGCCATGGA	ACGTTGGATGGTGTCATAAAGGTGACGTCC	CAAGTACATGTCAGCAGTGT	96
C226G	ACGTTGGATGGGTACCATACTACCCCTTTC	ACGTTGGATGTCCCAGTAAGCACTCTGTTC	CTACCCCTTTCAGAGTCATATTGC	100
R230W	ACGTTGGATGTTGTTTAGGGACCCCACAGG	ACGTTGGATGACACCCATTGAAGCCTGGTC	CCACAGGCTTTCAGTGACC	80
T312M	ACGTTGGATGGGACAAGTCCAAATGGGAAG	ACGTTGGATGATCGAAGACTGGCGTGAAAC	agtcaAAATGGGAAGAAACGCTCC	96
Y349C	ACGTTGGATGCTCAGACGATGGATTTCCCC	ACGTTGGATGTATTGGGCATCTTTGGGTTG	CATCAAACAGCAGACTTCGGCT	100
F404S	ACGTTGGATGTGCTCTGGCTTATGGTGAGA	ACGTTGGATGTGTGAGCCAAGAGTTCTATC	ATGGTGAGAAGAATTTGACCT	94
R479C	ACGTTGGATGTTCTTGTCATGGCACTGTGG	ACGTTGGATGGGGACACTGCTTATATTCAC	GCACTGTGGTTGCTCTC	93

Sequenom PCR and primer extension reaction:

PCR was performed to amplify the target DNA prior to extension primer binding. A uniplex PCR for each set of primer pairs from a multiplex design was initially performed to ensure a satisfactory product of the correct size. PCR was performed in a 10-μl reaction volume using a QIAGEN (Crawley, UK) PCR kit, with the concentrations per reaction as outlined in table 2.6. PCR was then performed by initial denaturation for 15 min at 95°C, followed by 45 cycles of denaturation at 95 ^{o}C for 20 sec, annealing at 56 ^{o}C for 30 sec, and extension at 72 ^{o}C for 1 min, followed by a final extension at 72 ^{o}C for 3 min. The PCR products were then checked by running on a 2% gel as previously discussed to visualise PCR product bands and to ensure that there was no contamination. Multiplex PCR was carried out following successful uniplex assay for each SNPs. 5 μl of each PCR product was then loaded into a 384 well plate. Amplified products were genotyped using Sequenom iPLEX chemistry for MALDI-TOF characterization of primer extension products, at the Sequenom MassARRAY facility located at the Institute of Genetic Medicine (Newcastle University, UK). Ten to fifteen percent of all samples were re-genotyped blind for each assay to ensure fidelity of genotyping (>99% for each SNP). The data was analysed by the MassARRAY reader mass spectrometer. A read-out as shown in figure 2.2 was obtained for each sample of the multiplex.

Table 2.6 Example of the PCR mixture for Sequenom genotyping

Number of sample	1	52	Final concentration
Sterile water	4.70	244.4	-
10x buffer	1.25	65	1.25x
Magnesium chloride (25mM)	0.65	33.8	1.63mM
dNTPs (6.25mM each- total 25mM)	0.2	10.4	0.5mM(total)
Primer mix (0.5 µM each- forward/reverse primers)	2	104.0	0.1 µM
Hot Star Taq (5U/µl)	0.2	10.4	1 unit
Total (µl)	9	468	
DNA	1		20ng/µl

A

B
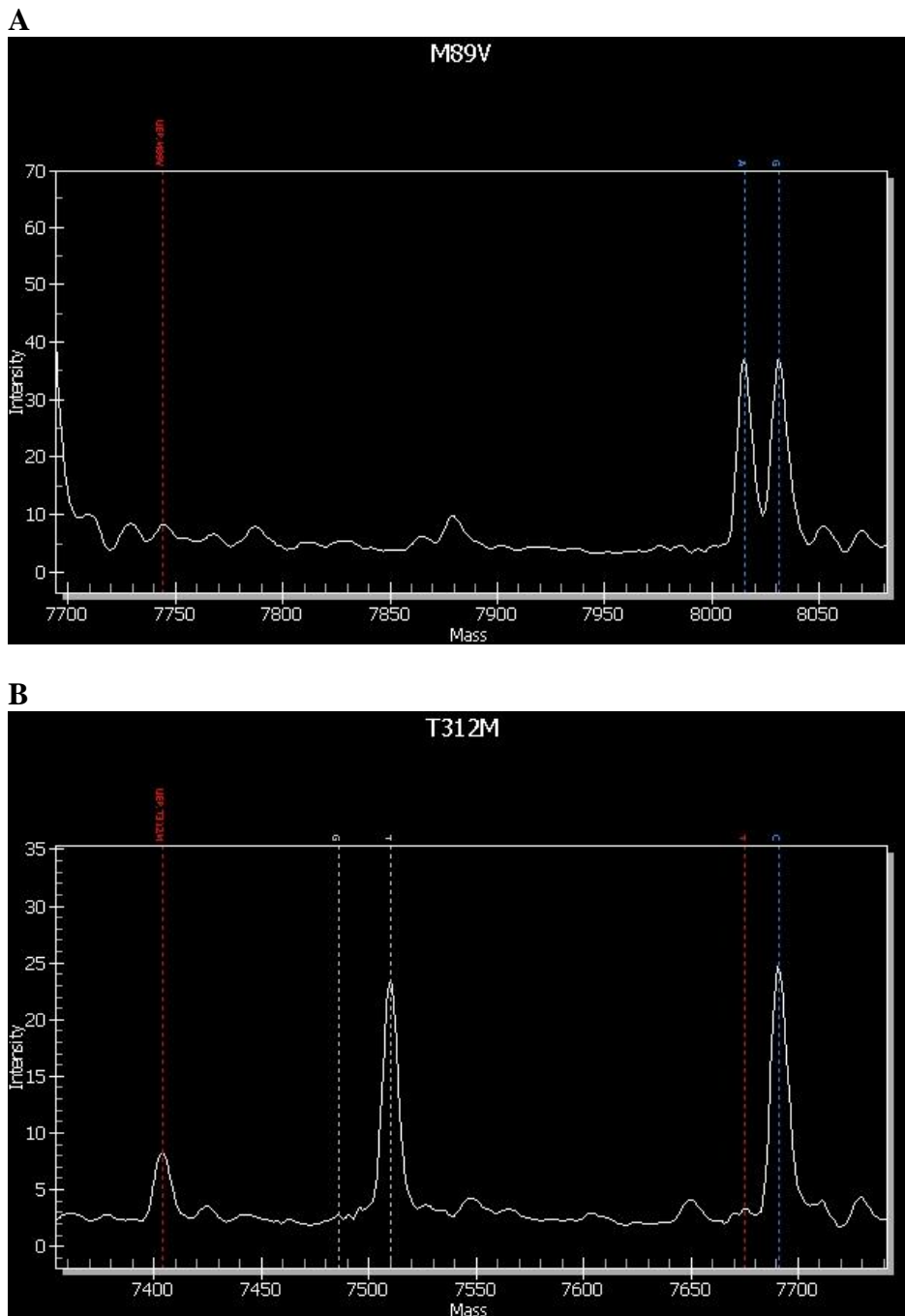
Figure 2.2 Representative of results obtained from a Sequenom assay for one sample.

This figure demonstrates the individual graphs for M89V(A) and T312M (B) genotyping. The coloured dotted lines indicate the expected position of peak if a particular allele is present. (A) showed a heterozygote (M89A) which gives a peak at both points with a GA call. (B) showed only one peak at the expected position for normal variant homozygote, a CC call for T312M.
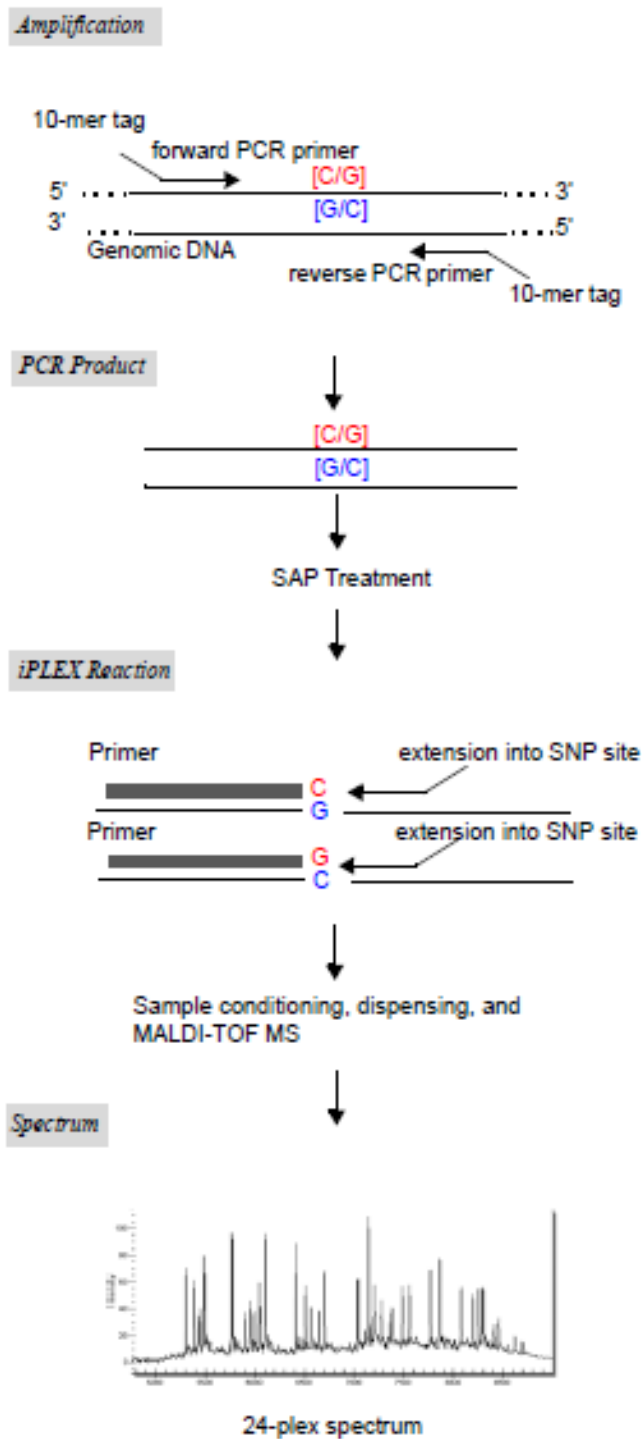
Figure 2.3 The basic principle of Sequenom IPLEX genotyping

Following PCR amplification, the addition of Shrimp Alkaline Phosphatase (SAP) deactivates the remaining non-incorporated nucleotides in the reaction. Following a brief incubation, the primer extension mixture is added and the assays are terminated after a single base extension, by incorporating mass-modified terminators that allow mass separation of the SBE products.

2.2.7 Restriction Enzyme Digestion (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction enzyme. I used this genotyping technique to verify Y349C results obtained from Sequenom genotyping, which showed a significant percentage of rare variants among AAD patient (0.26%).

In a typical case, the PCR products are digested with appropriate restriction endonuclease (RE), with the aid of Webcutter 2.0. Restriction enzymes that recognize and cut the sequence restrictions at the specific polymorphism/mutated point are selected. It was essential for the restriction site to be formed by one allele of the SNP but not on the other, so that there would be a difference in the length of PCR product between the wild type and the mutated PCR products. However, Webcutter failed to identify any suitable RE for the SNP in Y349C, so I resorted to designing a "mismatched" primer for the rare variant 349C as suggested by SNP cutter (http://bioapp.psych.uic.edu/SNP_cutter.htm), a tool for SNP PCR-RFLP assay design. The SNP Cutter is a web-based application that batches and automates assay design for SNP genotyping using PCR-RFLP. When the SNP does not affect any restriction sequences, a "mismatched PCR-RFLP" can be used by introducing an artificial restriction enzyme site. This is achieved using a primer containing additional mismatched bases adjacent to the SNP site. The introduced mismatch can construct a restriction site in one of the allele sequences with the SNP site, as shown in table 2.7.

Following PCR reaction, the PCR products were subjected to RE digestion. RE digestion was formed in 10µl digestion mixture containing 3µl of PCR product, 1µl of RE (PvuII) , 1µl NE buffer(1x) and 5µl sterile water. The digestion mixture was

incubated at 37°C for 1 hour. The RE digestion products were then visualized by electrophoresis on a 2% agarose gel under UV light.

2.2.8 Direct DNA sequencing

The appropriate PCR primers for direct DNA sequencing of Y349C SNP was designed using the Primer 3 programme (table 2.8). The PCR product (20µl) was first run on a 3% agarose gel to ensure that it was the appropriately-sized PCR product. The DNA band was excised from the gel using a sterile scalpel under UV light and placed in a clean microcentrifuge tube. The size of the gel was minimised by removing extra agarose. The DNA was then purified and extracted using QIAquick gel extraction kit.

Each sample was weighed and 3 volumes of buffer QG were added to 1 volume of gel (100mg/volume). The samples were incubated at 50°C for 10 minutes and each microcentrifuge tube was vortexed every 2-3 minutes during incubation until the gel slice had completely dissolved. The melted gel should be yellow in colour to indicate the correct pH (pH<7.5) to ensure good absorption of DNA to the QIAquick membrane. The yield of DNA fragment was then enhanced by adding 1 gel volume of isopropanol. The samples were then transferred to a QIAquick spin column with a collection tube and centrifuge for 1 minute to bind DNA. The flow-through was discarded and 0.5 ml of buffer QG was added to the spin column and centrifuge for another minute to remove all traces of agarose. The DNA was then washed with buffer PE, by incubating buffer PE with the DNA for 2-5 minutes before centrifugation. Once the flow-through was discarded, an additional centrifugation step at 10,000 x g for 1 minute was carried out. The QIAquick column was put in a clean microcentrifuge tube and 50ul of buffer EB

Table 2.7 demonstrates the oligonucleotide sequence and annealing temperature (Tm) used for the amplication of SNP sequence in Y349C.

The restriction enzyme used to confirm the base changes (A to G) identified through Sequenom assay was PvuII. The DNA base with low case indicates the mismatched base adjacent to the SNP site whereas the nucleotide sequence highlighted in bold and italic text indicates the SNP site. The underlined DNA bases indicate the forward and reverse primers

Forward primer (mismatch primer)	GCATCAAACAGCAGACTTCaGCT
Tm	63
Reverse primer	TTTATCTCGAGGGTGGATGC
Tm	60
Restriction Enzymes:	PvuII
Recognition sequence (forced restriction site)	CAGCT*G*
Fragment size	wild type (A): 125 bp rare variant (G): 101 bp & 24 bp
Base change	A/G
Allele sequences (SIAE_Y349C)	TCCGTTG*GCATCAAACAGCAGACTTCaGCT[A/G]*TGTCCCCAACCCAAAGATGCCCAATACTTTCATGGCTGTAGCTATGGATCTCTGTGATAGAGACTCGCCTTTTGGCA*GCATCCACCCTCGAGATAAA*C

(10mM Tris-Cl, pH 8.5) was added to the centre of the QIAquick membrane to elute DNA, and centrifuge for another minute at 10,000 x g. The DNA was then collected and the concentration was determined using Nanodrop spectrophotometer. The DNA was sent off to Eurofins MWG Operon's DNA sequencing service with the recommended DNA and primer concentration.

2.2.9 Statistical analysis

2.2.9.1 Case-control association analysis

Case control studies of SNPs were analysed using Fisher's exact and Chi-squared tests, by means of 2x2 and 2x3 contingency tables respectively. Genotypes were checked for Hardy-Weinberg equilibrium (threshold P >0.05). Hardy–Weinberg equilibrium is the basic principle of population genetics to provide a baseline for measuring genetic change. It assumes that both allele and genotype frequencies in a population remain constant, a state known as the Hardy–Weinberg equilibrium, unless specific disturbing influences are introduced such as mutation, migration or natural selection. Deviation from this equilibrium in a control population indicates potential problems with the population structure or genotyping. The Hardy–Weinberg equation was calculated using SHEsis software (Shi & He 2005). Odds ratios (OR) are used to compare the relative odds of the occurrence of rare alleles/genotypes. The 95% confidence interval (CI) is used to estimate the precision of the OR. A large CI indicates a low level of precision of the OR, whereas a small CI indicates a higher precision of the OR (Szumilas 2010).

2.2.9.2 Power calculation

The power to detect a relevant minor allelic OR for the SNP in a case control association study is based on the cohort size used and control allele frequency seen. I

calculate the study power according to pooled case and/or control allele frequencies from studies previously published, using a one-sided significance level of 0.05 ($\alpha = 0.05$) in a binomial model (Casagrande & Pike 1978). Power calculation was performed retrospectively using CaTS software (Skol et al. 2006). For CD226 variant association study, the study has 70% power to detect effects in the combined AAD cohorts, ($\alpha = 0.05$), with reference to the minor allele frequency established in the UK 1958 birth cohort controls (Hafler et al. 2009), by assuming the effect size (OR for allelic association) of 1.3. For the SIAE variant association study, a power estimation using the pooled case and control allele frequencies found by Surolia et al. (2.6% and 0.3%, respectively), showed that the study number used had 78% power to detect a similar sized effect ($\alpha=0.05$).

Table 2.8 demonstrated the oligonucleotide sequence and annealing temperature (Tm) used for the direct sequencing of SNP in Y349C

The underlined DNA base indicated the forward and reverse primer pairs where as nucleotide sequence highlighted in bold and small text indicate the SNP site.

Forward primer CATGGGATGAGAGGGACTGT Tm: 59.9^{o}C GC% 55	
Reverse primer CCCCAGCATACGTGACTCTT Tm: 60.1^{o}C GC% 55	
Product size	467 bp
Base change	A/G
Allele sequences (SIAE_Y349C)	<u>CATGGGATGAGAGGGACTGT</u>TGCAGCTGTCTTTGGAGA GACACTGCCACAACAGTAAGAAGCTCAGTAAATACGTG TTGAATGAGCATTTAAATGCAATGAGAGAAGCAGAAGC AGAGTCTGCCAGTGAAAAAGACTCCAGTGTGGGATTCT GATACAATAAAATTAGCAATGTCTCTGCTTTCTAGTTAT CTTCAGATTTGTCTAAGAAGAGCTCAGACGATGGATTTC CCCAGATCCGTTGGCATCAAACAGCAGACTTCGGCT*a/g*T GTCCCCAACCCAAAGATGCCCAATACTTTCATGGCTGTA GCTATGGATCTCTGTGATAGAGACTCGCCTTTTGGCAGG TATGATTGCTTCTTGGGCTTTGCATGAACAGTGTATGTTC TTTGTAGTCTTTCACTCTGATGTTCACCGGTGAGGGGCA ATTAGACTGAGGATTGGCACTT<u>AAGAGTCACGTATGCTG GGG</u>TGTGCT

2.3 Results

2.3.1 CD226 (rs763361) association analysis

Genotyping for *rs763361* was carried out in 326 patients with AAD and 311 healthy controls. All genotypes were in Hardy-Weinberg equilibrium (p>0.05). The T allele of *rs763361,* encoding the CD226 307*Ser variant, was found in 289/622 (46.5%) of healthy control alleles. This finding is nearly identical to that reported in the UK 1958 birth cohort controls of 47% (Hafler et al. 2009). In contrast, the T allele was found in 329/652 (50.5%) of AAD alleles, compared to 46.5% of healthy controls; p=0.161, NS (table 2.8).

Considering that CD226 307*Ser has been associated with many other autoimmune conditions, I re-analysed the AAD cohort according to whether the patient had isolated AAD or additional coexisting autoimmune conditions, namely autoimmune polyendocrinopathy type 2; APS2. In the subjects with APS2, the *rs763361* T allele was found in 197/366 (53.8%) of alleles, vs 46.5% of control alleles; p=0.030, Odds ratio 1.34 (5-95% CI 1.04-1.74). Individuals with isolated AAD showed no association at rs763361, with the T allele frequency being 132/286 (46.2%) compared to 46.5% in control alleles; p=0.94, NS.

2.3.2 SIAE rare variants association studies

Genotypes for the 9 *SIAE* SNPs were obtained for 378 patients with AAD and 387 healthy controls. Among the 761 subjects, I did not find any rare allele for the *SIAE* germline variants encoding W48X, C226G, R230W, Y349C, F404S and R479C (table 2.9). One AAD patient was a heterozygous carrier of the codon 312*M (T312M) variant, but all controls were found to have the wild-type allele. A single heterozygous carrier of

the SIAE 196*F (C196F) allele was found amongst both the case and control cohorts. Similar to previously published study (Surolia et al. 2010), the common variant of *SIAE*, codon 89*V allele was present heterozygously in 12.1% and 12.0% of the patient and control cohorts, respectively. However, no codon 89*V homozygotes were found amongst control patients, whereas 2 AAD patients were 89*V homozygotes (p=0.242).

All AAD patients who are either heterozygous carriers of the rare codon 312*M (T312M) and 196*F (C196F) variants, or the homozygous carriers of the codon 89*V(M89V), had other associated autoimmune diseases in the spectrum of type-2 autoimmune polyendocrinopathy syndrome (APS2). Among them, 3 had pernicious anaemia, 2 autoimmune hypothyroidism and 1 premature ovarian failure (table 2.10).

In summary, taking into account all 9 alleles examined, 4/378 (1.06%) AAD patients and 1/387 (0.25%) healthy controls inherited SIAE genotypes that would be expected to lead to functionally detrimental consequences; odds ratio of 4.13 (95% confidence interval 0.44- 97.45; two-tailed p-value 0.212. NS).

Verification of SIAE_Y349C results

Following genotyping with Sequenom –iPLEX chemistry, the rare allele for the *SIA*E germline variant encoding Y349C was strongly positive among patients with AAD, with an odds ratio of 5.35(1.1-35.5) and p- value of 0.019. Four patients were found to be homozygous carriers of the codon 349*C and 2 were heterozygous carriers. However, considering that allele 349*C is a rare allele and should have a MAF of <0.1, the rare 349*C polymorphism found in the AAD cohort is not in Hardy-Weinberg equilibrium. Hence , I repeated the genotyping using an alternative technique to verify the results: restriction enzyme digestion (RFLP) and direct DNA sequencing. However, both methods consistently showed the absence of the rare 349*C allele (figure 2.5 & figure

2.6) and refuted the positive association between Y349C and AAD demonstrated by

Sequenom-iPlex method. I repeated the genotyping of Y349C with the Sequenom

method, using all positive samples previously shown and a few random negative

samples. A completely different result was obtained showing 3 heterozygous 349*C

carriers from previously negative samples.

I explored the reasons for the false positive and inconsistent Y349C genotyping result

obtained from Sequenom iPLEX. When I re-examined the primer design and the

extensor primer seemed to have strong self- complementarity:

CATCAAAC*AGC*AGACTTCG*GCT*. The primer design tool indeed inserted a sign

signified strong hairpin warning (PWARN-H). Hence, the Y349C results from

Sequenom genotyping were disregarded in view of poor primer design.

Table 2.9 Genotype and allele data for CD226 Gly307Ser (rs763361)

Rs763361	Genotypes			P value	Alleles		P value	Odds ratio
	CC(%)	*TC(%)*	*TT(%)*	*(genotype)*	*C(%)*	*T(%)*	*(allele)*	*(5-95% CI)*
All AAD	82 (25.2)	159 (48.8)	85 (26.1)	0.36	323 (49.5)	329 (50.5)	0.16	1.17 (0.94-1.46)
APS2	41 (22.4)	87 (47.5)	55 (30.1)	0.08	169 (46.2)	197 (53.8)	**0.03**	**1.34 (1.04-1.74)**
Isolated AD	41 (28.7)	72 (50.3)	30 (21.0)	0.97	154 (53.8)	132 (46.2)	0.94	0.99 (0.75-1.31)
Control	90 (28.9)	153 (49.2)	68 (21.9)		333 (53.5)	289 (46.5)		

Genotype and allele data for CD226 Gly307Ser (*rs763361*) in the UK AAD cohort, together with case-control analyses of the genotypes and alleles. Probability values (P) are shown, with significant results highlighted in bold.
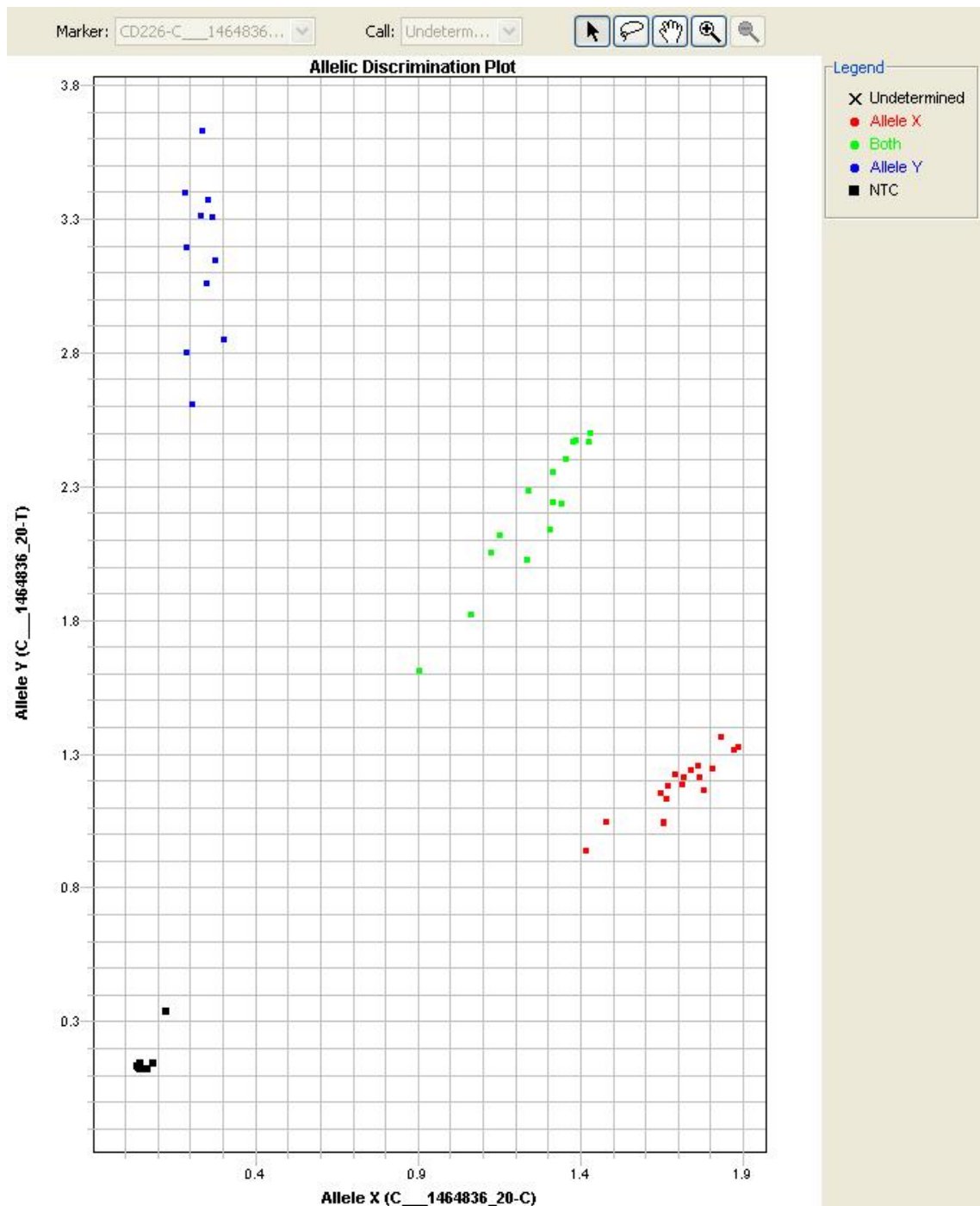
Figure 2.4. A representative allelic discrimination plot for CD226 (rs763361) genotyping using Taqman genotyping assay.

There is a clear separation between the signals derived from allele X (C variant) or allele Y (T variant). Allele T of *rs763361,* encoding the CD226 307*Ser variant is shown in blue whereas the commoner C allele is shown in red. Lime green represents a mixture of the two alleles.

Table 2.10 Genotypes for SIAE variants in autoimmune disease cases and controls

Amino acid change	Autoimmune Addison's disease		UK healthy controls		
	Genotype (*)	Rare allele/genotype frequency	Genotype (*)	Rare allele frequency	p value
W48X	0/0/370	0	0/0/373	0	-
M89V	2/42/303	0.0058†	0/43/315	0	0.242
C196F	0/1/374	0.0013	0/1/383	0.0013	-
C226G	0/0/371	0	0/0/383	0	-
R230W	0/0/375	0	0/0/386	0	-
T312M	0/1/372	0.0013	0/0/385	0	0.492
Y349C	0/0/360	0	0/0/381	0	-
F404S	0/0/372	0	0/0/382	0	-
R479C	0/0/358	0	0/0/362	0	-

*Data presented as numbers of rare homozygote/heterozygote/common homozygote genotypes

†Homozygous 89*V genotype

Table 2.11 Clinical details of AAD patients with rare SIAE variants

SIAE variants	Age at onset of AAD (yr)	Associated autoimmune conditions
M89V (homozygous)	29	Pernicious anaemia
M89V (homozygous)	60	Pernicious anaemia, autoimmune hypothyroidism, premature ovarian failure
C196F	83	Autoimmune hypothyroidism
T312M	59	Pernicious anaemia

Figure 2.5 RFLP result for Y349C polymorphism

The Y349C polymorphism was re-genotyped by PCR and digestion with the restriction enzyme PvuII. In the presence of 349*C allele, the enzyme will cut the 125 bp PCR product into 101 and 24 bp fragments. 100 bp DNA ladder was used. Land A-F showed single bands at around 125 bp, suggesting the absence of rare 349*C allele.
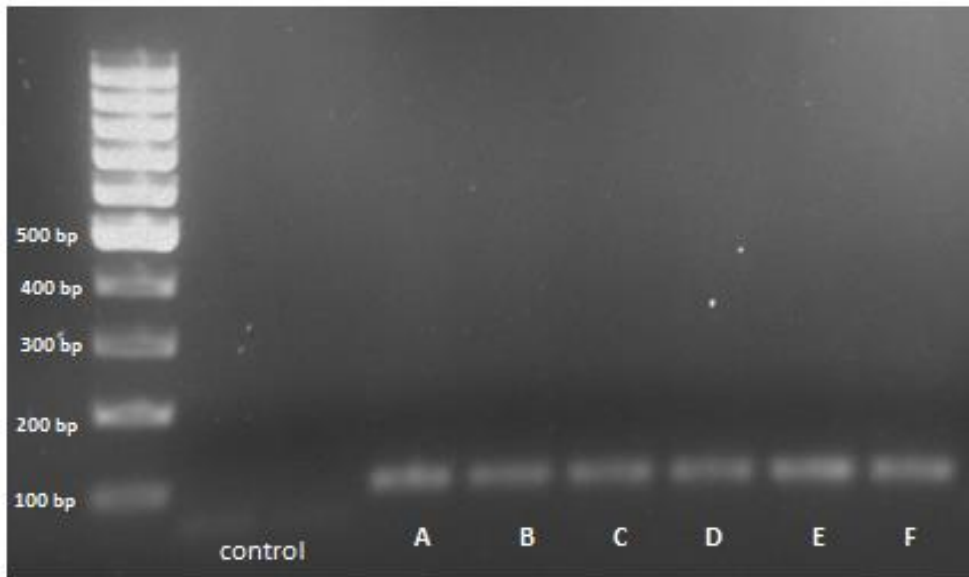
Figure 2.6 Direct sequencing of Y349C variant using reverse primer.

DNA sequence chromatograms (antisense strand) showing the T allel (A allel in the

sense sequence) at position 378, at the *Y349C* variant

280
G C A A A G C C C A A G A A G C A A T C A T A C C T G C C A A
290
300

360
A T C T T T G G G T T G G G G A C A T A G C C G A A G T C
370
380

Normal T variant (antisense strand)
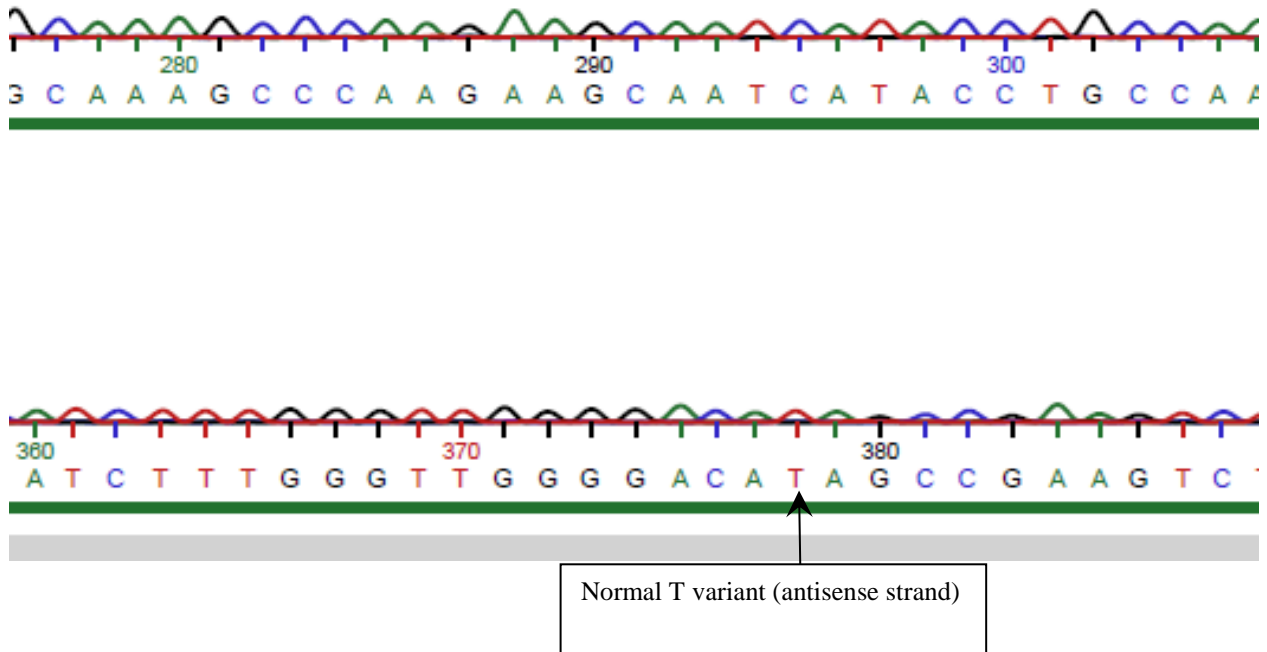
2.4 Discussion

Autoimmune Addison'disease is a non-Mendelian polygenic disease and numerous loci might underlie this disorder. Although most of the susceptibility alleles identified had modest disease effects, with Odds ratios ranging from 1.1 to 2.0 (Mitchell & Pearce 2012), the magnitude of their contributory risk could be significant as they appear quite frequently in the population. However, the genetic analysis of this rare condition has lagged behind that of other autoimmune diseases, largely due to the rarity of this condition, making it difficult to assemble a large patient cohort without international collaboration. The relatively small numbers of families with multiple affected AAD individuals also preclude linkage analysis which has good power. Hence, I carried out these candidate gene studies as part of my supervisor's team continuous effort to uncover more susceptibility loci for AAD in order to understand the disease pathogenesis better.

The 307*Ser allele of CD226 is a presumed aetiological variant that contributes to the susceptibility of numerous autoimmune conditions. A meta-analysis of 7876 cases and 8558 controls from 7 published studies established this CD226 variant as contributing to the aetiology of multiple autoimmune diseases, including type 1 diabetes and Graves'disease (Qiu et al.2013). In this study, I examined its role in AAD for the first time. While considering all patients with AAD, there was no association at CD226. However, when the subgroup with APS2 were analysed, there was modest evidence of association (p=0.030).

The association of this CD226 variant with APS2, but not in isolated AAD, is likely to suggest that the associated autoimmune conditions that define APS2 have the same genetic susceptibility as when found in isolation, such as autoimmune thyroid disease and type 1 diabetes (Hafler et al. 2009). Interestingly, the majority of our APS2 patients

actually have autoimmune hypothyroidism (AH) as the most frequent co-existing autoimmune disease (in 60% of our APS2 cohort), suggesting a probable association between AH and 307*Ser at CD226. This is in contrast with the study of Hafler et al. which demonstrated no association between this CD226 variant and a large cohort of Hashimoto's thyroiditis patients (Hafler et al. 2009).

I learned from the previous studies that the susceptibility genes contributing to AAD aetiology, including MHC, PTPN22, CTLA4, CLEC16A, CIITA, and PDL1 have predominantly a role in immune system functioning, particularly related to modulation of T cell receptor signalling, such as in PTPN22 , or at the cell-surface with a costimulatory role eg. CTLA4 and PDL1. Similarly, MHC, CIITA have roles in subserving antigen presentation to the T cell receptor, forming components in the afferent limb of the adaptive immune response. Thus, CD226, as a costimulatory T lymphocyte molecule was an excellent candidate as a contributor to many autoimmune diseases including AAD.

This is the first study demonstrating the role of CD226 variation in autoimmune Addison's disease. It showed that the 307*Ser allele contributes to APS2 susceptibility, but its effects in isolated AAD have yet to be confirmed. However, the main weakness of this study is the small cohort size, due to the rarity of this disease. In fact, the effective size of the association between CD226 variant in several other autoimmune conditions at *CD226 307*Ser* has been weak, with odds ratios for the allelic associations typically being between 1.1 and 1.3. Thus, given the relatively small collection of AAD patients, this study may have been underpowered to detect an effect, particularly if the actual contribution to AAD susceptibility is at the lower end of this range. Given the current cohort sizes, this study would have had 70% power to detect an effect of odds ratio 1.3 (at the 0.05 level), thus it is possible that the results of our study

for all-ADD (both isolated AAD and APS2) give a false-negative. Nevertheless, it is equally possible that there is simply no contribution of CD226 307*Ser to AAD susceptibility, given the almost identical allele frequencies between isolated AAD patients and healthy controls (46.2% vs 46.5%). Further studies with larger patient cohorts are required to solve this question.

While genome wide association studies have identified many common genetic variants that are linked to autoimmune disease, they would probably lack the power to detect rare variants that could be implicated in the disease. Rare variants are arbitrarily defined as having a frequency below 1% in the population (Cohen et al. 2004). It has not been the focus of genetic studies in autoimmune diseases, largely due to the requirement for labour-intensive deep sequencing and functional testing for each rare variant. I explored the hypothesis that rare SIAE variants would be associated with AAD, one of the uncommon autoimmune conditions via candidate gene approach. I demonstrated the presence of 2 codon 89*V homozygotes, two heterozygous carried of the 312*M and 196* F alleles, respectively in the AAD cohort. This is in keeping with the Surolia et al study, where they indicated T312M and C196M to be the most frequent rare variants found (frequency>0.1%; n= 1571), as shown in table 2.4. Nevertheless, these associations were not significantly different from healthy control genotypes. Thus, our findings extend the negative study of Hunt et al. who showed no differences for nine of the *SIAE* gene rare variants (including M89V but not T312M) in cohorts of the commoner autoimmune and inflammatory conditions, such as type 1 diabetes, atopic eczema, celiac disease, Graves' disease, and Hashimoto thyroiditis (Hunt et al. 2011). However, this large cohort of nearly 67,000 subjects did not include patients with the rarer condition of AAD. It is also noteworthy that the association analysis was calculated using the statistical method generally used for common genetic variants,

which might not be accurate and powerful in multiple rare variant model (Cooper & Shendure 2011).

While our study had good power to replicate findings of a similar magnitude to those previously seen in other autoimmune conditions (OR 8.6) (Surolia et al. 2010), our analysis has been underpowered to detect a more subtle genetic effect, such as is more frequently seen for commoner autoimmune disease susceptibility alleles. In fact, the same research team who pioneered works related to SIAE rare variants in autoimmune diseases has recently replicated their study and reported that the M89V common variant was erroneously considered to be catalytically defective (Chellappa et al. 2013). Following exclusion of M89V from the analysis, there was still a strong but relatively weaker association between the confirmed catalytically defective rare variants and various autoimmune diseases (OR of 3.51). Hence, we cannot exclude that a future analysis of an enlarged AAD cohort or a family-based genetic association study may cast further light on this question. However, the parameters for declaring significance in studies of rare genetic variants are currently not well defined, given the low frequency of rare variants (Cooper & Shendure 2011). More importantly, the key attribute of reproducibility has not been fulfilled for SIAE variants in autoimmune diseases to date.

Chapter 3.   Re-establishment of adrenocortical steroidogenesis in autoimmune Addison's disease with parenteral ACTH1-24 therapy

3.1 Background

Adrenocorticotropic hormone ($ACTH_{1-39}$) is the master regulator of adrenal proliferation and steroidogenesis. ACTH secretion exhibits diurnal variation and starts during sleep, at around 03.00 hours with a series of peaks of approximately 40 minutes periodicity, for 8-10 hours (Veldhuis et al.1990, Horrocks et al.1990). The circulating ACTH levels reach their nadir in the afternoon and evening in healthy people. This diurnal and pulsatile pattern of ACTH secretion is essential to maintaining normal adrenal cell mass and steroidogenic function.

Disruption to ACTH circulating levels and secretion patterns are implicated in many clinical conditions. Excessive ACTH secretion from the pituitary gland in Cushing's disease leads to adrenal gland hyperplasia and unwarranted glucocorticoid production. In a complementary fashion, ACTH is suppressed during chronic exogenous steroid therapy (i.e.long term oral prednisolone treatment) leading to adrenal atrophy and functional adrenal failure. Independent lines of evidence from animal studies also support the essential roles of ACTH. Mice homozygously deleted for the pro-opiomelanocortin gene (*Pomc*, containing the ACTH peptide) have small adrenals and reduced steroid production (Coll et al. 2004, Karpac et al. 2008). Interestingly, daily synthetic $ACTH_{1-24}$ (synacthen) injections for 10 days of these *Pomc*-/- mice was shown to result in the regeneration of the adrenal mass, as well as the steroidogenic function (Coll et al.2004).

As previously mentioned in chapter 1, adrenocortical tissue is highly plastic owing to the presence of adrenocortical progenitor or stem cells (ACSCs), which continuously repopulate the adrenal cortex in an ACTH-responsive fashion. In autoimmune Addison's disease (AAD), an autoimmune response targeted at the key steroidogenic enzymes, mainly 21-hydroxylase, results in the impairment of adrenal steroidogenesis.

This is associated with a mixed T and B cell infiltrate leading to destruction of differentiated adrenocortical cells. However, various studies using immunocytochemistry techniques have suggested that the adrenocortical progenitor cells do not express the steroidogenic enzymes, which are the primary target of the autoimmune process. Hence, the ACSCs are likely to be spared from the immune attack. The clinical diagnosis of AAD is commonly preceded by a 'pre-clinical' stage in which elevated ACTH is noted without adrenal hypofunction. A case report has shown that AAD can take up to 9 years to present (Torrejón et al. 2007). During the 'preclinical AAD' phase, the fall in circulating corticosteroid levels from immune-mediated steroidogenic failure is compensated by an increase in pituitary ACTH secretion, leading to increased ACTH-driven ACSC proliferation and differentiation, with compensatory adrenocortical regeneration (Betterle et al. 2002). Once the individual has been diagnosed with AAD, administration of exogenous steroid will lead to an iatrogenic suppression of ACTH with subsequent inhibition of ACTH-driven ACSC proliferation and differentiation. This results in a rapid shift of the balance towards adrenal destruction and steroidogenic failure. In view that ACSC is likely to be spared from the autoimmune attack, this group of cells might stay dormant following the suppression of their trophic hormone, the pituitary ACTH. Nonetheless, spontaneous recovery of adrenal function had been reported following a period of established AAD. A spontaneous partial adrenal recovery was reported in three men who suffered from AAD between 7 and 37 years (Smans & Zelissen 2008, Chakera & Vaidya 2012, Baxter et al. 2013). A full remission was also recorded in patients who had subclinical adrenal insufficiency with positive adrenal antibodies (Betterle et al. 2002).These published case reports unequivocally suggest that permanent destruction of ACSC or adrenal function is not universal in AAD. With this in mind, appropriate manipulation of ACSC

in vivo might have the potential to lead to adrenal regeneration in man, especially when the adrenocortical stem cells could be preserved in treated AAD patients.

The aim of this study is to investigate if a therapeutic regimen of parenteral subcutaneous tetracosactide ($ACTH_{1-24}$) therapy can revive adrenocortical stem cell function and allow re-establishment of adrenocortical steroidogenesis.

3.2 Methods

3.2.1 Study participants

Thirteen patients between the ages of 16 and 65 years who had autoimmune Addison's disease (AAD) for more than 1 year were recruited either from the endocrine clinics of the Newcastle upon Tyne NHS hospitals (n=12), or self-referred following an ethics committee agreed advert in the national Addison's disease self-help group (ADSHG) quarterly newsletter (n=1). A full list of eligibility criteria are depicted in table 3.1. We identified 16 patients for possible inclusion in the study. Three did not meet the inclusion criteria, with 2 not having autoimmune primary adrenal failure (two with pituitary disease), and 1 with asthma. The study was registered as NCT01371526 at clinicaltrials.gov. Ethical approval was granted by the Northeast Research Ethics Committee (reference 10/H0903/14).

3.2.2 Study design and treatment regimen

The study is an open-label trial of synthetic ACTH analogue ($ACTH_{1-24}$; tetracosactrin-'Synacthen') to stimulate adrenocortical function in adults with established AAD. Participants were recruited between September 2010 and April 2012. Written informed consent was obtained during the screening visit and participants underwent a seven-day run-in phase of the study, including a baseline assessment to confirm their eligibility

and 2 education sessions to learn how to self-administer subcutaneous synacthen. A refresher course on the indications for emergency parenteral glucocorticoid therapy was also given. The study ran for two parts, with each part lasting for 10 weeks (table 3.2). During part I of the study, participants self-administered 1mg depot synacthen (Synacthen® depot ampoule 1mg/ml; Alliance Pharma plc, Wiltshire UK) subcutaneously on alternate days. According to the product information sheet, the plasma concentrations of ACTH are maintained between 200 to 300pg/ml for 12 hours following 1 mg of intramuscular depot synacthen injection. The serum cortisol levels increase in a parallel fashion and the highest values are recorded during the first 8 to 12 hours after the injection. The increased cortisol levels are maintained for up to 24 hours and return to basal levels after around 36-48 hours. Hence, this will achieve a 24 to 36-hour sustained peak of ACTH with the aim of stimulating ACSCs back into active proliferation.

Patients were reviewed at 7 days and then 2 weekly during phase 1 for assessment of tolerability and wellbeing. Formal re-testing of adrenal function occurred at 5 and 10 weeks. If there was improvement in serum cortisol levels, participants would be maintained on the same regimen. However, if there was no improvement in adrenal function at 10 weeks, participants would be invited to take part in phase II of the study. In phase II, participants were randomly allocated to either continuous 24h subcutaneous infusion of synacthen (10µg/hr), or 12h synacthen with 30 minute 10µg boluses each hr, from midnight to midday. Intravenous bolus doses of synacthen above 5 µg are known to provide a close to maximal adrenal cortisol response (Dickstein et al. 1991).

The randomisation was executed by a research nurse using sequentially opened, pre-ordered sealed envelopes that contained randomly generated allocations, which was then verified by another research nurse. Allocation concealment was maintained throughout the study. I programmed the Infusion pumps (OmniPoD; Ypsomed UK) at

the start of the phase II study and participants replenished the reservoir with synacthen every third day.

Table 3.1 Full eligibility criteriaFull eligibility criteria

Inclusion criteria

- Aged between 16 and 65 years

- Established adrenocortical failure, as judged by a basal cortisol of less than 100nmol/l or peak ACTH-stimulated cortisol response of less than 300nmol/l that was established a minimum of 12 months previously and persists at run-in assessments

- Confirmation of primary origin of adrenal failure (basal ACTH level >55ng/L)

- Normal or atrophic adrenals on cross-sectional imaging (if performed): compatible with autoimmune Addison's disease

- If female, willing to take secure contraceptive measures for the duration of the study, and for 3 months following the last synacthen dose.

- Willingness to travel to Newcastle CRF for study

- Willingness to attend education sessions about indications for parenteral glucocorticoid administration and technique of administration

Exclusion criteria

- Significant cardio-respiratory, chronic renal or non-autoimmune liver disease; malignancy

- Asthma, current infectious disease, recent live vaccination, acute psychosis, peptic ulcer disease

- Pregnancy, breastfeeding or plan for pregnancy within 9 months

- Known non-autoimmune cause for adrenal failure (haemorrhage, adrenoleukodystrophy etc.)

- Known hypersensitivity or allergy to Synacthen (ACTH$_{1-24}$)

Figure 3.1 Study flowchart
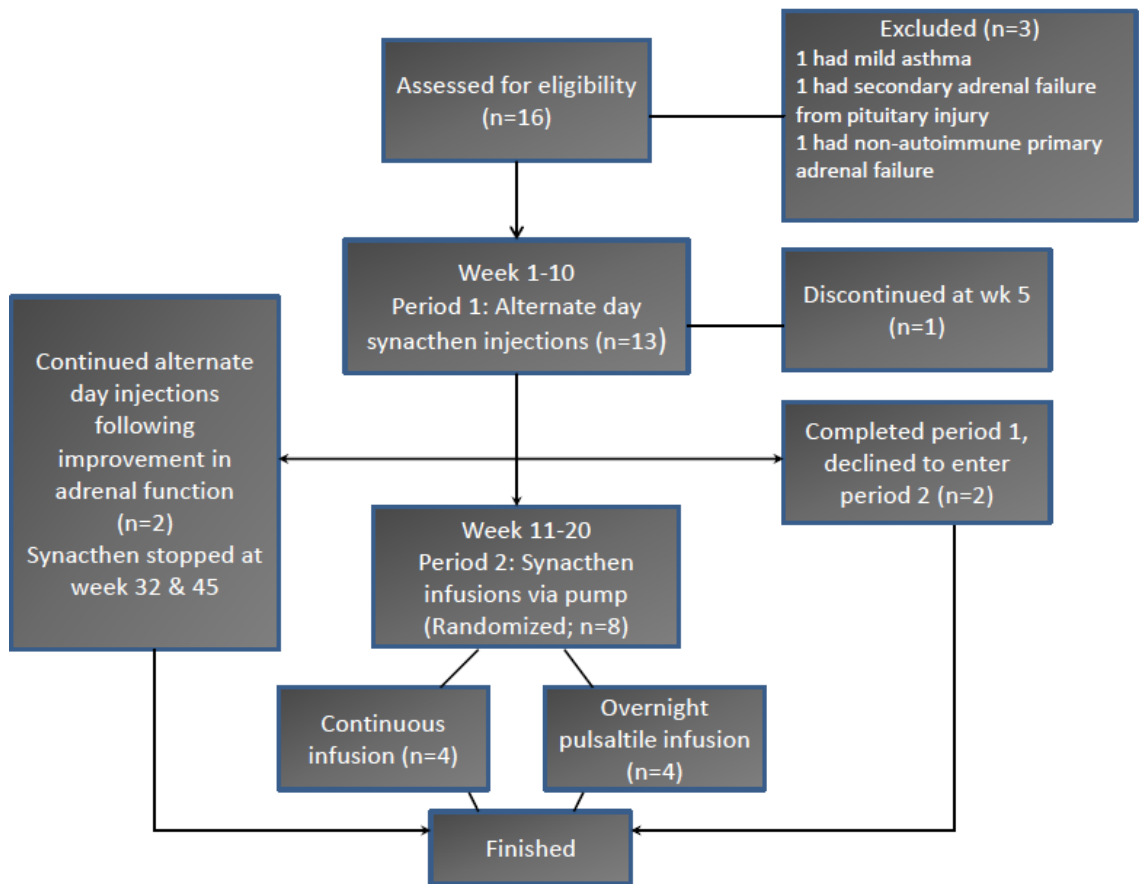
3.2.3 Objective outcome measures and assessments

Following baseline testing, outcome measurements were made at weeks 5, 10, 15 and 20. All of these assessments were made after 36-42 hrs of withdrawal from regular steroid replacement therapy during which participants were admitted to the Clinical Research Facility. These measurements included baseline aldosterone, cortisol, dehydroepiandrosterone sulphate (DHEAS), 17-hydroxyprogesterone, androstenedione, plasma ACTH, plasma renin activity, plasma metanephrine and normetanephrine, 21-hydroxylase antibodies, and overnight urinary steroids and metabolites. An ACTH-stimulation test (250µg $ACTH_{1-24}$, with cortisol measured at 30 and 60min) was then performed starting between 08.30 and 09.00hrs. All cortisol results in the text and figures are the peak concentration of the 3 timepoints during each test. Two of the outcome assessments were delayed by 1 and 3 weeks, due to participants having forgotten to omit steroid medication and unforeseen travel commitments, respectively.

For participants who achieved the primary end point (a peak cortisol >400 nmol/l), steroid replacement therapy was judiciously titrated down to produce maximal adrenal stimulation, without compromising patient wellbeing. Patients were reviewed every 4-6 weeks for optimisation and down-titration of their steroid dosage. Synacthen treatment was also weaned off over 8 to 10 weeks once glucocorticoid replacement was stopped with regular review of participant wellbeing. Throughout both phases of the study, patients were reviewed every 2 weeks to ensure their general wellbeing and tolerance to the treatment, including blood pressure and serum electrolytes measurements. Participants also have 24 hour access to the research team via mobile phone for advice or assessment in case of illness.

3.2.4 Methods for objective outcome measurements

3.2.4.1 Hormonal and biochemical assessments

Blood samples were taken and sent to the Biochemistry laboratories at the Royal Victoria Infirmary, Newcastle upon Tyne on the day of the assessment. Details of the assays used for each assessment were given by a biochemical scientist working at the laboratory. I entered the results into clinical record forms which were then verified by another clinical investigator.

Serum cortisol measurements were made by a competitive chemoluminescent immunoassay on a Centaur platform (Siemens, UK), with a limit of detection (LD) of 25nmol/l (0.91µg/dl). Serum aldosterone was measured using a solid-phase radioimmunoassay (DPC coat-a-count kit), with a limit of detection (LD) of 70 pmol/l. Serum DHEAS was assayed by solid-phase competitive chemoluminescence on an Immulite platform (Siemens) with a limit of detection (LD) of 0.1 µmol/l. Immunometric 'sandwich'assay using chemiluminescent detection technique (DPC Immulite) was used to measure ACTH. The normal reference range is 10-55 ng/L. Androstenedione was assayed by solid-phase competitive chemoluminescence on an DPC Immulite platform with a limit of detection (LD) of 1.05 nmol/L.

On the other hand, plasma renin activity was measured indirectly using the DiaSorin assay. This assay measured the rate of angiotensin-1 production from angiotensinogen by renin in patients' samples. Angiotensin-1 levels were subsequently measured by radioimmunoassay with values expressed as the amount of angiotensin-1 produced per hour, which was inversely related to the amount of renin present in the sample. Plasma metanephrines were first extracted using rapid, hydrophilic interaction liquid chromatography to isolate and concentrate the low femtomolar concentrations of free metanephrines in plasma into a purified low volume form.

Plasma metanephrine concentration was also measured as part of the biochemical assessment for adrenal steroidogenic function. Glucocorticoid is known to upregulate expression of the phenylethanolamine N-methyltransferase (PNMT) gene in adrenal chromaffin cells, which then catalyses the methylation of noradrenaline to adrenaline (Evinger et al. 1992). Hence, a patient with adrenal insufficiency usually has undetectable metanephrine level. In light of adrenal medulla being in close proximity to the adrenal cortex, which has a rich vascular supply, even a minimal rise in cortisol production could potentially result in a significant rise in plasma metanephrine levels. Hence, plasma metanephrine was measured as a potential marker to detect early steroidogenesis. The plasma metanephrine level was measured using a tandem quadrupole mass spectrometer with the limit of detection set at 40.

3.2.4.2 Serum 21-hydroxylase autoantibodies

Serum samples were saved and stored in a -80°C freezer at each outcome assessment. Aliquots of serum samples (5mls) were sent to the FIRS lab at Cardiff for assay of 21-hydroxylase antibodies within 2 years of collection. Serum 21-hydroxylase antibodies were measured by immunoprecipitation of radiolabelled (^{125}I) recombinant in vitro translated 21-hydroxylase protein (RSR ltd. Cardiff, UK), as described previously (Tanaka et al. 1997). The results are expressed as arbitrary units according to the kit manufacturer's instructions.

3.2.4.3 Urine metabolites

Urine samples were collected for 16-24 hours at each outcome assessment, following 36-42 hours of withdrawal from regular steroid replacement therapy. Urine samples were then stored at -80°C freezer before being sent to the Centre for Diabetes and

Metabolism (CEDAM) at Birmingham for analysis, which was carried out within 2 years of collection. Measurement of urinary steroid metabolite excretion was carried out by using gas chromatography/mass spectrometry (GC/MS). A detailed description of this methodology has been published previously by Arlt and colleagues (Arlt et al.2011). In summary, free and conjugated steroids were extracted from 1 ml urine by solid-phase extraction. GC/MS was carried out on an Agilent 5973 instrument operating in selected-ion-monitoring (SIM) mode to achieve sensitive and specific detection and quantification of steroid metabolites. There were 32 steroid metabolites selected by CEDAM, Birmingham to include important representatives of steroid groups such as androgen metabolites, glucocorticoid metabolites, mineralocorticoid metabolites, and 3β-hydroxy-Δ5 steroid precursors, as shown in figure 3.2.

A Adrenal Steroidogenesis

B Healthy Controls (n=88)

Androgens and androgen precursors

Mineralo-corticoids and MC precursors

Glucocorticoid precursors

Gluco-corticoids
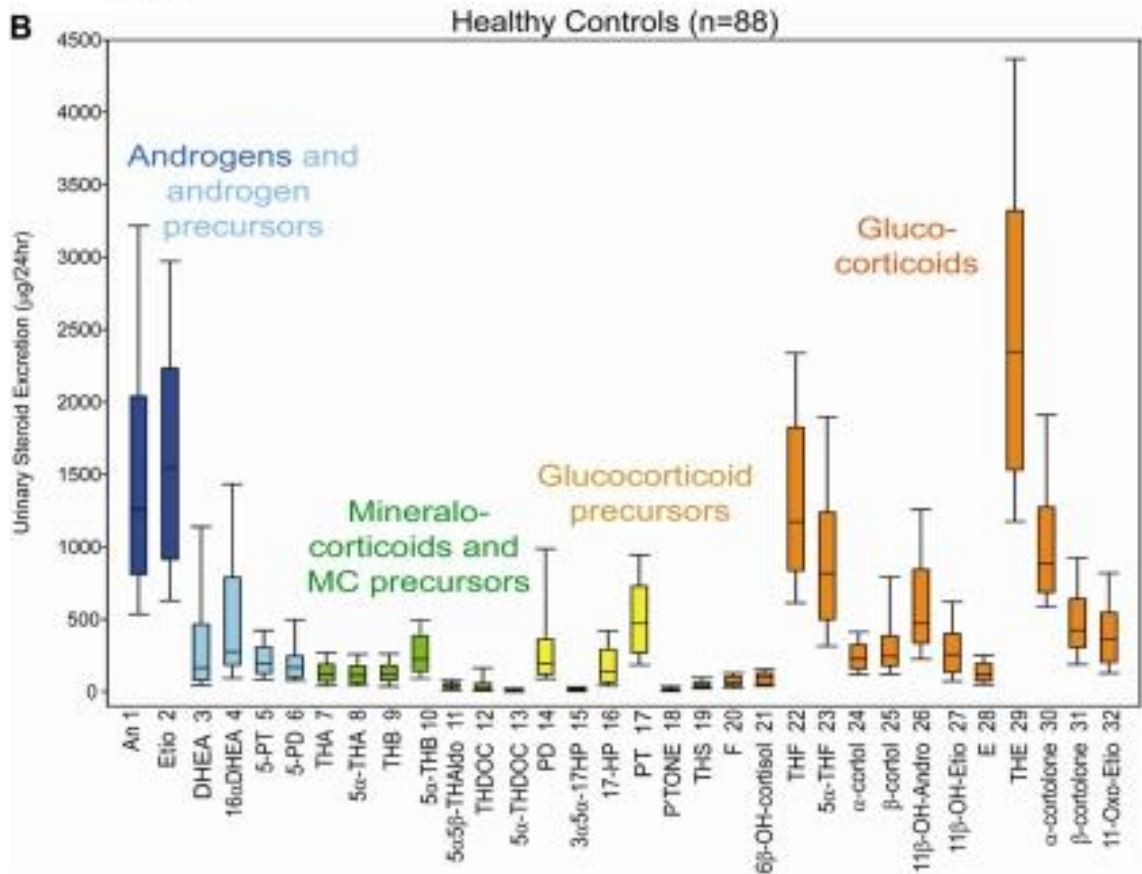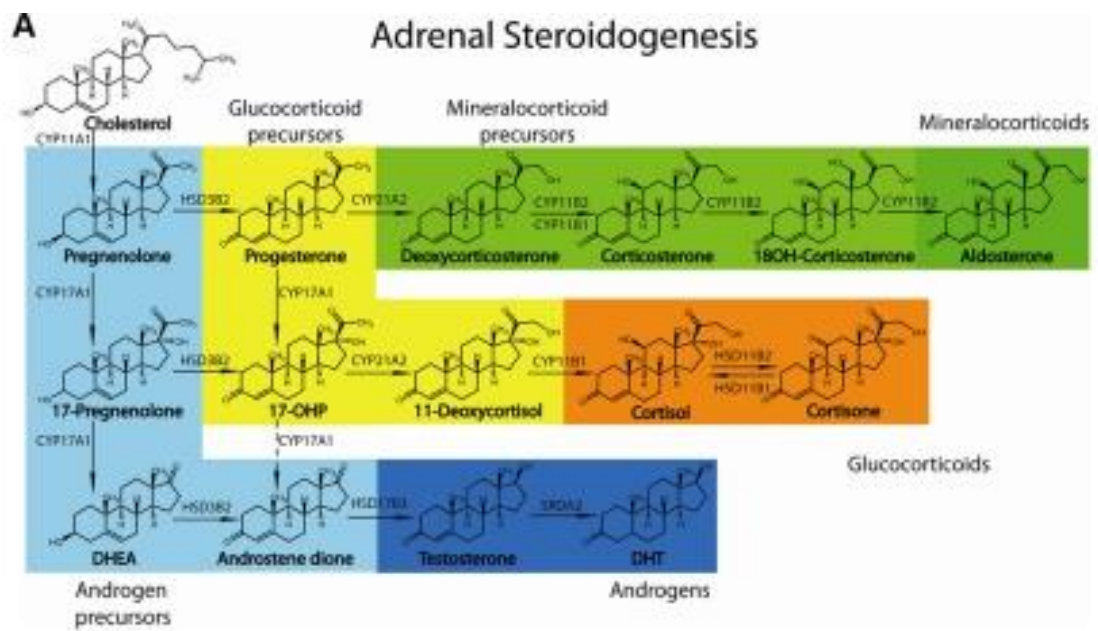
Figure 3.2 Schematic representation of adrenal steroidogenesis and 24 hour urinary steroid metabolite excretion in healthy people

(Extracted from Arlt et al. 2011)

A. Schematic representation of adrenal steroidogenesis depicting the major precursors of adrenocortical steroid synthesis, including the mineralocorticoid aldosterone (*dark green*) and its precursors (*light green*); glucocorticoid precursors (*yellow*), the active glucocorticoid cortisol (*orange*); its metabolite cortisone and the adrenal androgens and their precursors (*light blue*).

B. Representative of the 24-h urinary steroid metabolite excretion in healthy controls (n = 88) (Arlt et al. 2011). Color coding of steroid metabolites mirrors that were used for depicting the major adrenal corticosteroid classes in A.

[CYP, Cytochrome P450; HSD, hydroxysteroid dehydrogenase; DHT, 5α-dihydrotestosterone]

3.2.5 Subjective outcome measures- Quality of Life assessments

Subjective assessments of physical and emotional functioning were measured at baseline and at each outcome assessment. The short form 36 (SF-36) health survey questionnaire (version 2), the Addison's disease specific quality-of-life questionnaire, (AddiQoL; version2) and a visual analogue scale wellbeing questionnaire were used.

3.2.5.1 SF-36 health survey questionnaire (version 2)

The SF-36 health survey questionnaire (Ware & Sherbourne 1992) consists of eight domains: *physical functioning*, (*PF*), *role functioning physical*, (*RP*), *bodily pain* (*BP*), *general health perception* (*GH*), *vitality* (*energy* and *fatigue*, *VT*), *social functioning*, (*SF*), *role functioning emotional* (*RE*) and *mental health* (*MH*). Psychometrically based physical and mental health summary measures are also included in the analysis. The total domain scores range from 0 to 100, with higher values denoting better subjective health status. The scores were then converted using norm-based scoring, which employs a linear T-score transformation with mean = 50 and standard deviation = 10, to make it possible to meaningfully compare scores for the eight-scale profile and the physical and mental summary measures in the same graph.

3.2.5.2 Addison's disease specific quality-of-life questionnaire (AddiQoL)

AddQoL(version 2) (Oksnes et al. 2012) is an Addison's disease-specific questionnaire comprising 30 questions that quantify altered functional and emotional status in Addison's patients. The total score ranges from 30-120, with a higher score indicating better quality of life. The questionnaire is included in the appendices.

3.2.5.3 Visual analogue scale (VAS)

In addition, a concise questionnaire comprising 10 visual analogue scales (VAS) was developed to assess changes in the subjective functional and emotional health of all AAD participants' following treatment at each outcome assessments (figure 3.3). The VAS questionnaire was validated using the Pearson's coefficient correlation to compare the correlation between VAS wellbeing test with SF-36 and AddiQoL assessments.

3.2.6 Statistical analysis

The differences between baseline and outcome assessments (10 or 20 weeks) with respect to participants' quality-of-life and 21-hydroxylase antibody titre were assessed using paired-T test. Wilcoxon ranked sign test was used if normality was not demonstrated. All probability values shown are two-sided, except where stated.

One-tailed paired T test was used to examine the mean difference between stimulated and baseline serum cortisol concentration, performed at 30 and 60 minutes respectively following $ACTH_{1-24}$ administration. The data was gathered from both week-10 and week-20 outcome assessments. One-tailed paired T test was also used to assess the ability of various biochemical markers in detecting adrenal steroidogenesis. Statistical analysis was performed using Minitab 6.

RoSA study

WELLBEING QUESTIONNAIRE

•**How have you been feeling over the last week?**

Please make a cross on each line at the position that matches how you have been feeling

1. Daytime energy levels

No energy   Normal energy

Constant exhaustion   100% fully charged

2. Evening energy levels

I always go to   Normal bedtime

bed early

3. Stamina

I can't finish   I keep going

normal household jobs   as normal

4. Lightheadedness, dizzyness

I feel constantly   No episodes of

lightheaded   lightheadedness

5. Nausea, vomiting

I vomit every   No nausea or

day, constant nausea   vomiting

6. Appetite

I don't feel at   Normal appetite

all hungry

7. Salty foods

I have dreams   I don't feel the

about salty food   need to eat salt

8. Pain

I have constant   No aches or

aches & pains   pains

9. Thinking

My thinking   I think clearly

is always confused

10. Concentration

I can't focus   I have normal

                                         concentration

Figure 3.3 A representation of the visual analogue scale (VAS) wellbeing questionnaire used in this study

VAS questionnaire comprises 10 questions testing the psychometric domains commonly affected in AAD. It is expressed on a linear scale with a horizontal line, 100mm in length and anchored by word descriptors at each end. The participants marked the point that they felt represented their perception of their current state. The score was then measured in centimetres from the left side of the line to the point that was marked by the patient.

3.3   Results

3.3.1   Baseline characteristics

Thirteen subjects (11 females and 2 males), with a mean age of 39 years (range 16-65) were enrolled in the study. Twelve participants completed part I of the study (to 10 weeks), with 10 participants going on to complete the full protocol to 20 weeks. One male participant withdrew following the week 5 assessment owing to pain at injection sites. Baseline characteristics are shown in table 3.2 and the steroid replacement regimens of participants at study entry is depicted in table 3.3.

Table 3.2. Patient baseline characteristics

P ID	Sex; age (yr)	Time since diagnosis of AAD (yr)	Peak serum cortisol (nmol/l)[a]	Plasma ACTH (10-55 ng/L)	21-Hydroxylase antibody (<1 U/mL)	Associated autoimmunity	Oral steroid regimen (dosage)	Previous adrenal crisis
01	F; 65	12	<24	828	3.1	HT	HC,FC	Yes
02	F; 43	8	219	225	6.3	HT	PD, FC	No
03	F; 44	6	<24	>1250	169.8	HT	HC,FC	Yes
04	M; 46	19	<24	417	2.3	Nil	HC,FC	Yes
05	F; 23	8	<24	752	0.3	HT	HC,FC	No
06	F; 36	4	171	174	27.7	HT	PD,FC	No
07	F; 16	2	<24	1080	5.1	Nil	HC,FC	Yes
08	M; 45	3	<24	610	12.3	T1DM	HC,FC	No
09	F; 54	1.5	30	>1250	617.3	Nil	HC,FC	Yes
10	F; 24	6	<24	805	3072.7	T1DM	HC,FC	Yes
11	F; 48	6	32	645	1607	HT	HC,FC	No
12	F; 35	1	<24	550	73	Nil	HC,FC	Yes

13	F; 27	1	<24	1000	101	Nil	HC,FC	Yes

Table 3.2 legend

F, female; M, male; HC, hydrocortisone; PD, prednisolone; FC, fludrocortisone; HT, hypothyroidism; T1DM, type 1 diabetes mellitus

[a]Reference range for serum cortisol response after 250µg $ACTH_{1-24}$ is greater than 550 nmol/L. To convert from nmol/L to µg/dL divide by 27·6.

[b]Reference range for morning plasma ACTH is 10-55 ng/L.

[c]Reference range for serum 21-hydroxylase antibodies is less than 1·0 U/mL.

Table 3.3. Steroid replacement regimens of participants at study entry

Participant	Glucocorticoid (daily dosage, mg)	Fludrocortisone (daily dosage, µg)
01	Hydrocortisone (25)	100
02	Prednisolone (5)	200
03	Hydrocortisone (15)	100
04	Hydrocortisone (17.5)	100
05	Hydrocortisone (17.5)	150
06	Prednisolone (6)	150
07	Hydrocortisone (15)	100/200 alternate days
08	Hydrocortisone (20)	100
09	Hydrocortisone (17.5)	150
10	Hydrocortisone (17.5)	150/100 alternate days
11	Hydrocortisone (20)	100
12	Hydrocortisone (20)	100
13	Hydrocortisone (25)	150

3.3.2 Adrenal steroidogenic function

During the screening visit, nine of the 13 participants had undetectable ACTH-stimulated peak serum cortisol concentration. Four of them had detectable cortisol levels (≥25nmol/l) with 2 participants (subject 02 and 06) demonstrated concentrations of 219 nmol/l and 171 nmol/l, respectively (table 3.2). After 10 and 20 weeks of treatment with synacthen, ten of the twelve participants had a peak serum cortisol concentration that remained below 100 nmol/l (figure 3.4A). Similarly, the same group of participants had serum aldosterone levels below the threshold for detection (figure 3.4B). In contrast, participants 02 and 06 showed an increase in serum cortisol concentration by week 5. Nevertheless, serum aldosterone only started to rise above the threshold for detection at week 20 and week 40 for patient 02 and 06, respectively.

The response of adrenal androgen towards synacthen therapy was quite variable. Serum 17-α hydroxyprogesterone (17-α OHP) was detectable in all male participants, ranging between 1.9-3.8 nmol/L (normal range: 1-14 nmol/L) but no significant changes were observed throughout the study. Four female patients (including one post-menopausal woman) had detectable 17-α OHP levels at baseline (range 1.1-6.5nmol/L, mean 1.7). Two of them (02 & 06) demonstrated peak serum17-α OHP concentration at 1.3X above the upper limit of the normal range following synacthen therapy (figure 3.5A). However, no significant changes were seen in the remaining seven female participants who had serum 17-α OHP below the limit of detection (<1 nmol/L).

Another adrenal androgen precursor, serum androstenedione was detectable in six participants at baseline. These include 2 males and 3 female participants who had

detectable cortisol level at baseline assessment. The serum androstenedione level rose progressively in patients 02 and 06, with more than a threefold rise at week -20 and week-60 respectively (figure 3.5B). Serum dehydroepiandrosterone sulphate (DHEAS) was detectable in the male participants but no significant changes were shown throughout the study. The rest of the participants had either undetectable DHEAS concentration or level below the reference range (1-7 μmol/l). However, the DHEAS concentration in participant 02 showed a gradual rise into the reference range (>1.0μmol/l) by week 104 (figure 3.6A).

Interestingly, participants 02 and 06 showed a gradual rise in plasma metanephrine concentration and achieved a 5-fold and 3-fold rise in the peak plasma metanephrine levels respectively (figure 3.6B). However, it was found to be undetectable or below the normal reference (80-510 pmol/L) in 10 of the 13 participants at baseline (including subject 02). No sustainable increment was seen in the three remaining participants who had normal plasma metanephrine levels at baseline (range 86-129). Statistical analysis using a paired T-test (one-tailed test with mean difference >0) was carried out to compare the sensitivity of each hormonal and biochemical marker for adrenal steroidogenesis. A short synacthen test is the standard assessment for adrenal function and the study protocol measured serum cortisol concentration at both 30 and 60 minutes post $ACTH_{1-24}$ stimulation. These results suggest that stimulated cortisol levels at 60 minutes is more sensitive (60 minutes vs 30 minutes: p-value 0.012 vs 0.018) in detecting a rise in serum cortisol concentration following 250ug $ACTH_{1-24}$ administration, compared to a 30-minute cortisol level (table 3.4). Using a similar analytical method, serum 17 α-OHP (p=0.03) was found to be a

better predictor for the recovery of adrenal function, as it is more sensitive in

detecting small increments in adrenal androgen concentration ($P<0.05$) (table 3.4) .

Figure 3.4 Longitudinal course of serum cortisol and aldosterone concentrations in autoimmune Addison's disease patients during and after tetracosactide (ACTH1-24) therapy.
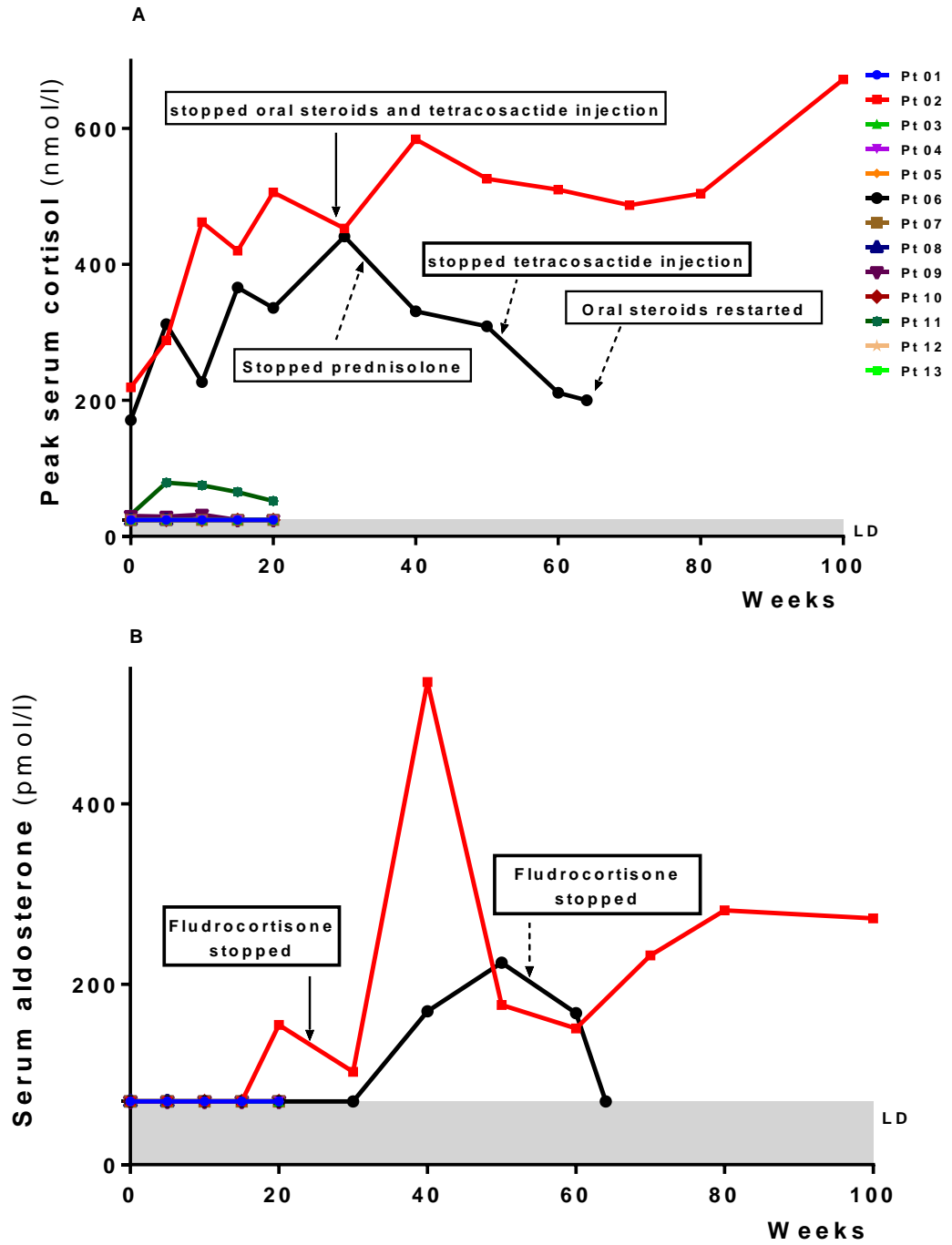
Figure 3.4 Panel A. Serum cortisol and all other steroid hormone measurements were made following 36 to 42 hours of withdrawal from regular glucocorticoid and fludrocortisone replacement therapy. For each timepoint we recorded the highest serum cortisol observed during a short tetracosactide test (250 μg i.m., with blood sampling at baseline, 30 and 60 min after injection). The normal response to this stimulus is a peak cortisol concentration over 550nmol/L. The limit of assay detection (LD) was 25nmol/L shown as the shaded horizontal area.

Panel B. Serum aldosterone concentrations. The reference range for recumbent serum aldosterone is 100-450 pmol/L, with a limit of detection (LD) of 70 pmol/l, shown as the shaded horizontal area. Several patients' data are superimposed on this limit of detection line.

Figure 3.5 Longitudinal course of serum 17-α hydroxyprogesterone and androstenedione concentrations in autoimmune Addison's disease patients during and after tetracosactide (ACTH1-24) therapy.
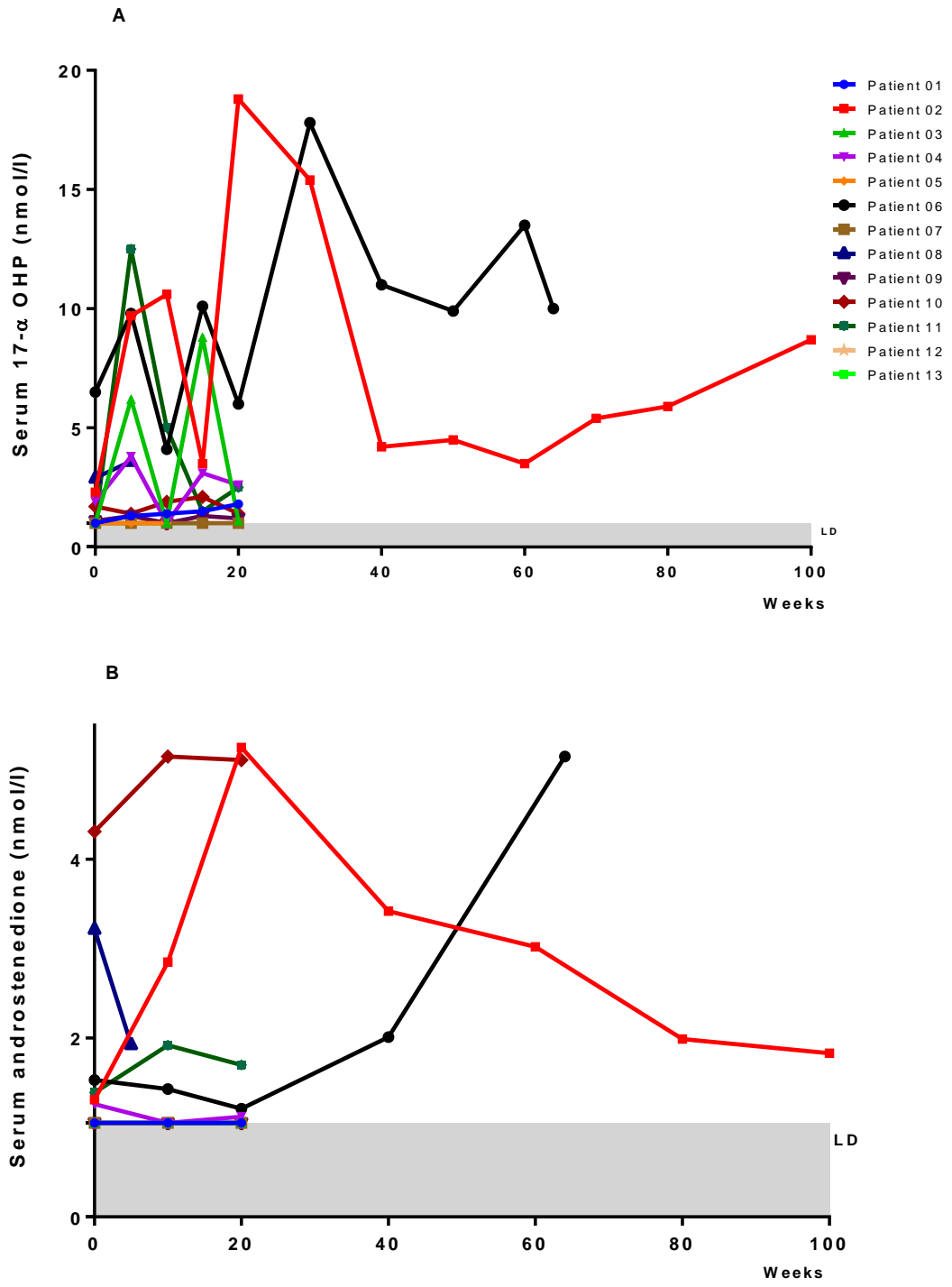
Figure 3.5 Panel A. Serum 17-α hydroxyprogesterone (17-α OHP) concentrations. The reference range for recumbent serum aldosterone is 0-14nmol/L, with a limit of detection (LD) of 1 nmol/L, shown as the shaded horizontal area. Several patients' data are superimposed on this limit of detection line.

Panel B. Serum androstenedione concentrations. The female reference range is 1·2 to 14·3 nmol/L and the male reference range is 1·4 to 9·1 nmol/L. The limit of detection (LD) of 1.05 nmol/L, shown as the shaded horizontal area. Several patients' data are superimposed on this limit of detection line.

Figure 3.6 Longitudinal course of serum DHEAS and plasma metanephrine concentrations in autoimmune Addison's disease patients during and after tetracosactide (ACTH1-24) therapy.
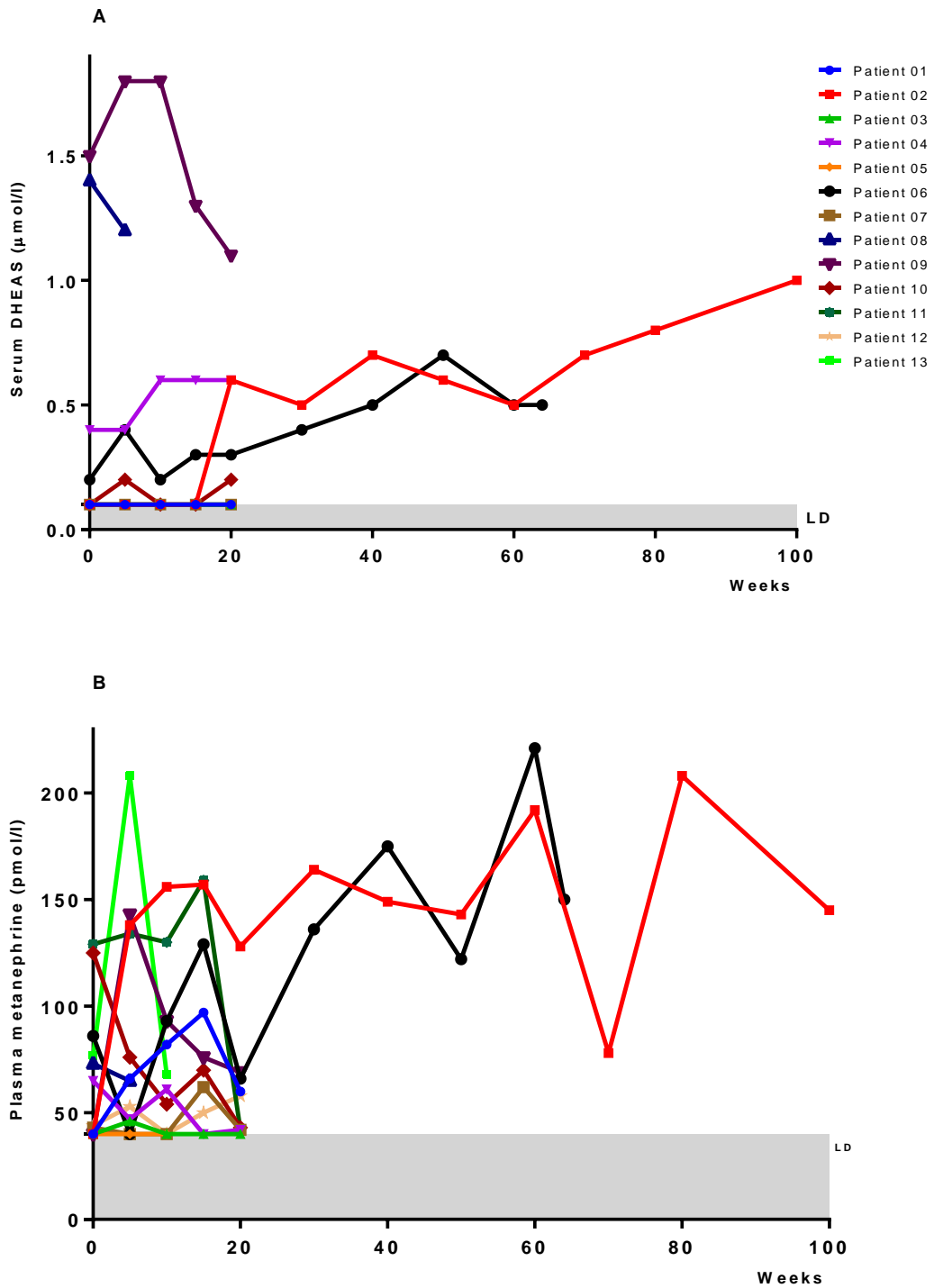
Figure 3.6. Panel A. Serum dehydroepiandrosterone sulfate (DHEAS) concentrations. The female reference interval is 1·0 to 9·2 µmol/L and the male reference interval is 2·4 to 11·6 µmol/L, with a limit of detection (LD) of 0·1 µmol/L (shown as the shaded horizontal area). Several patients' data are superimposed on this limit of detection line.

Panel B. Plasma metanephrine concentrations. The reference range for plasma metanephrine is 80-510pmol/L, with a limit of detection (LD) of 40pmol/L, shown as the shaded horizontal area. Several patients' data are superimposed on this limit of detection line.

Table 3.4. Serum cortisol concentrations at 30 and 60 minutes post ACTH 1-24 (250μg/ml) stimulation.

Changes in serum cortisol concentration (n=12)	Mean difference (standard deviation)	p-value (95% lower bound for mean difference)
Serum cortisol (30-minutes vs baseline)	15.42 (22.40)	0.018 (3.80)
Serum cortisol (60-minutes vs baseline)	22.33 (29.68)	0.012 (6.95)

Positive changes (increment) in serum cortisol concentrations at 30 and 60 minutes during $ACTH_{1-24}$ stimulation tests were examined using one-tailed paired t-test ($\mu > 0$). Short synacthen tests taking place at baseline, week-10 and week-20 for the 4 participants with detectable serum cortisol concentrations in this study were included (n=12).

*n= number of short synacthen tests

Table 3.5. Hormonal changes during synacthen treatment

	Baseline vs. week-10 (n=12)		Baseline vs. week-20 (n=10)	
	Mean difference (S.D)	p-value (95% lower bound for mean difference)	Mean difference (S.D)	p-value (95% lower bound for mean difference)
17-α OHP	1.73 (2.86)	0.030* (0.240)	2.56 (5.43)	0.066 (1.63)
Androstendione	0.26 (0.51)	0.050 (0.003)	0.71 (1.46)	0.059 (0.04)
DHEAS	0.042 (0.1)	0.088 (0.010)	0.00 (0.15)	0.500 (0.07)
Plasma metanephrine	10.90 (44.5)	0.207 (12.20)	47.8 (205.80)	0.219 (58.9)

One-tailed paired-T test (to detect mean difference, $\mu > 0$) was used to examine the sensitivity of various hormonal tests in detecting adrenal steroidogenesis (increment in hormonal levels) during synacthen treatment at week 10 and week 20.

* statistically significant

3.3.3 Participant 02 and 06

Two out of the 13 participants (participant 02 and 06) in this study achieved the primary endpoint during the study. The detailed clinical and biochemical characteristics when they were first diagnosed with AAD are summarised in Table 3.6 3.6. At week 10, participant 02 achieved the primary endpoint. She had a peak serum cortisol concentration, which improved further to 506 nmol/L at week-20. Her oral steroid replacement was judiciously weaned down, with close monitoring to ensure well-being. Her oral prednisolone therapy was stopped at week 13, fludrocortisone at week 23 and synacthen injections at week 32 without any deleterious consequences. Since the cessation medication, she has experienced a few episodes of cold-like symptoms and one significant episode of viral gastroenteritis with diarrhoea, but has neither needed to take steroid medication nor hospital treatment during these illnesses. Both her peak serum cortisol and aldosterone concentrations were normal at 672 nmol/L and 273 pmol/L, seventy two weeks after weaning off all therapies other than levothyroxine. At present, she has remained well for 42 months without steroid replacement.

At week 29, participant 06 achieved the primary endpoint of the study. She demonstrated biochemical evidence of steroidogenesis with peak serum cortisol concentration of 441nmol/L. Her oral steroid replacement therapy was then progressively reduced, ceasing oral prednisolone at week-36, synacthen injections at week-45 and fludrocortisone at week-57. She remained well with the staged reduction of steroid and synacthen therapy, but her peak serum cortisol concentrations decreased progressively once synacthen injection was stopped. Oral

steroid replacement therapy was restarted at week-64, when she became

symptomatic with a peak cortisol level of 211 nmol/L.

Table 3.6. Biochemical and clinical characteristics for participant 02 & 06 at diagnosis

Biochemical tests (reference range)	Participant 02	Participant 06
Sodium (133-146 mmol/L)	131	130
Potassium (3.5-5.3 mmol/L)	5.7	4.7
Urea (2.5-7.8 mmol/L)	9.4	5.7
Creatinine (55-95 umol/L)	112	92
Baseline cortisol	195	273
Peak cortisol after stimulation with $250\mu g$ $ACTH_{1-24}$ (>550 nmol/L)	212	279
ACTH (10-55ng/L)	498	55
Renin (0.5-2.1 pmol/L/h)	8.7	7.5
Aldosterone (100-450 pmol/L)	72	<70

Clinical symptoms	Duration of symptoms before diagnosis (months)	
Pigmentation	5	12
Weight loss	6	18
Lethargy/fatigue	6	24
Nausea/ vomiting	6	12
Postural lightheadedness	6	12
Salt craving	6	6

3.3.4   21-hydroxylase antibodies

All patients except participant 05 had positive 21-hydroxylase antibody titre (>1.0 U/ml) at baseline. None of them demonstrated significant changes in the 21-hydroxylase antibody titre at either 10 or 20 weeks of the study (p>0.05, paired t-test). Similarly, participants 02 and 06 who were followed up for 65 and 80 weeks respectively did not show any significant increment in the antibody concentration (figure 3.7).

Figure 3.7 Serum 21-hydroxylase autoantibodies
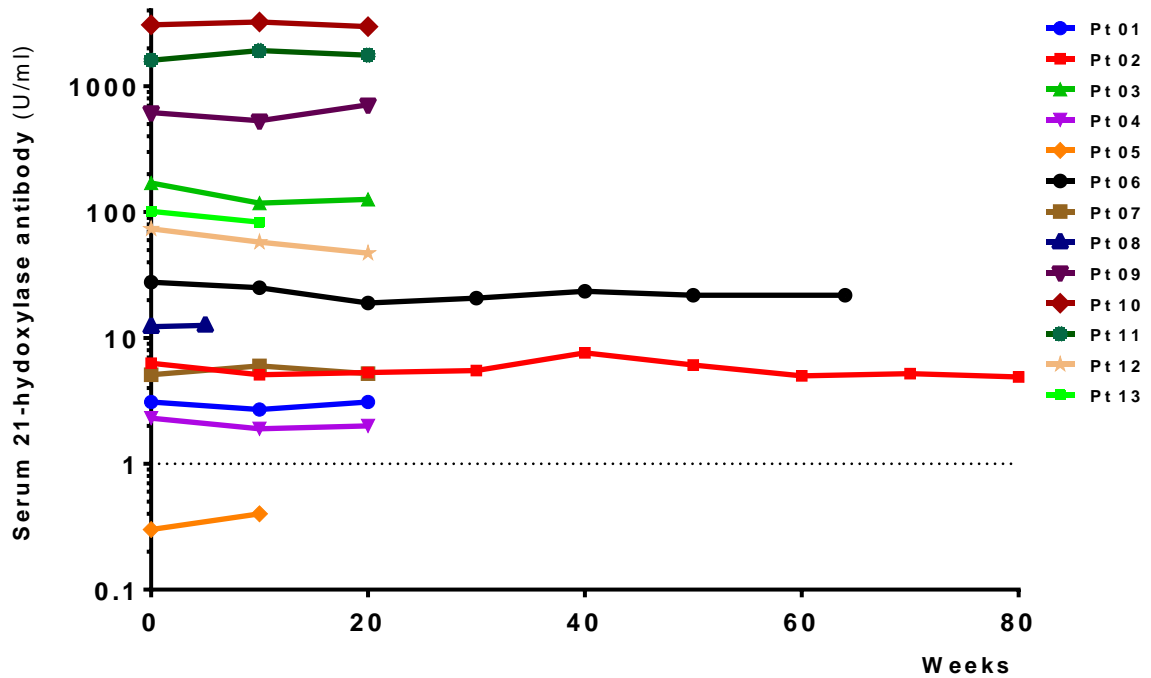
The concentration of serum 21-hydroxylase autoantibodies over time is shown (note the logarithmic Y-axis). The reference range is <1·0 U/ml. Measurements were made in duplicate by immunoprecipitation of radiolabelled recombinant 21-hydroxylase expressed in yeast (*S. cerevisiae*) (RSR ltd. Cardiff, UK) (Tanaka et al.1997). Samples from participants 02 and 06 were run in two separate batches (weeks 1-40 and weeks 40 onwards) and measurements corrected using duplicate samples run in both assays.

3.3.5 Urine steroid metabolites

Urinary glucocorticoid and glucocorticoid metabolite excretion increased in patients 02 and 06 from below the 5th centile to above the median of healthy female controls over 10 and 40 weeks, respectively (figure 3.8). This was paralleled by increases in urinary mineralocorticoid. However androgen precursor and androgen excretion were slower to increase into the reference range. Urinary steroid metabolites remained under the normal reference range for all male participants (n=2) (figure 3.9).

Figure 3.8 Longitudinal course of urinary steroid metabolite excretion in female participants during and after tetracosactide (ACTH1-24) therapy
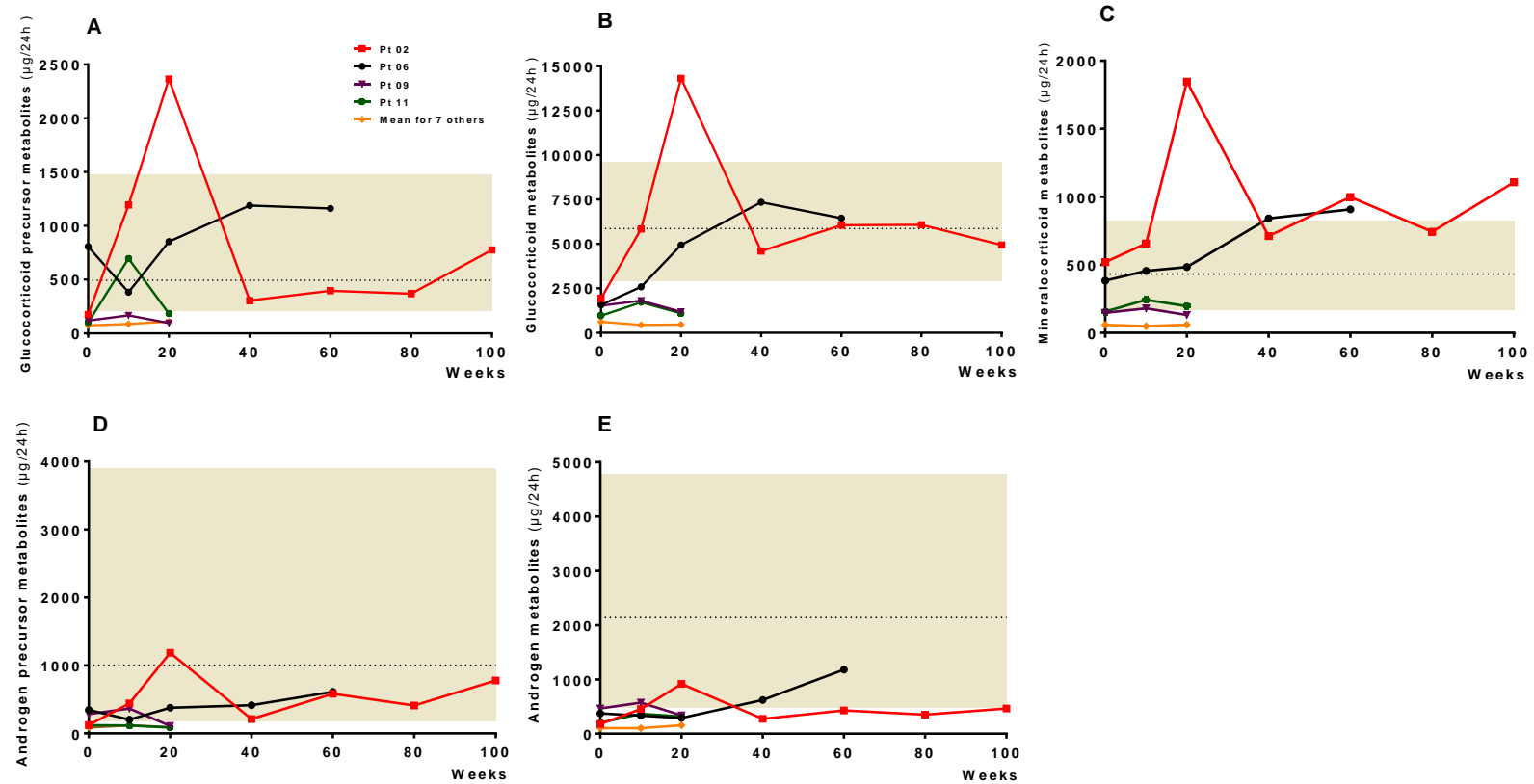
Error! Reference source not found.. Data from the 11 female participants are presented in comparison to results obtained in 55 healthy women ith the median of the normal cohort indicated as a dotted line and the shaded area representing the 5th-95th centile range.

Panel A: The sum of the metabolites of the glucocorticoid precursors 17-hydroxyprogesterone (17-hydroxy-pregnanolone, pregnanetriol) and 11-deoxycortisol (tetrahydro-11-deoxycortisol) are plotted against time (reference range median 493 [5th-95th centile 167-821] µg/24h).

Panel B: The sum of the active glucocorticoid metabolites (cortisol, tetrahydrocortisol, 5α- tetrahydrocortisol, α-cortol, β-cortol, cortisone, tetrahydrocortisone, α-cortolone, and β-cortolone) are plotted against time (reference range 5867 [2935-9632] µg/24h).

Panel C: The sum of the mineralocorticoid metabolites (3α,5β-tetrahydroaldosterone, tetrahydrocorticosterone, 5α-tetrahydrocorticosterone, tetrahydrodeoxycorticosterone, 5α-tetrahydrodeoxycorticosterone, tetrahydro-11-dehydrocorticosterone, and 5α-tetrahydro-11-dehydrocorticosterone) are plotted against time (reference range 432 [167-821] µg/24h).

Panel D: The sum of the androgen precursor metabolites dehydroepiandrosterone, 16α- dehydroepiandrosterone, 5-pregnanediol and 5-pregnanetriol are plotted against time (reference range 1003 [177-3897] µg/24h).

Panel E: The sum of the major active androgen metabolites androsterone and etiocholanolone are plotted against time (reference range 2139 [484-4790] µg/24h).

Figure 3.9 Longitudinal measurements of 24-h urinary steroid excretion (µg/24h) in the two male participants (patients 4 and 8) at study baseline, during tetracosactide treatment, and after tetracosactide (ACTH1-24) therapy
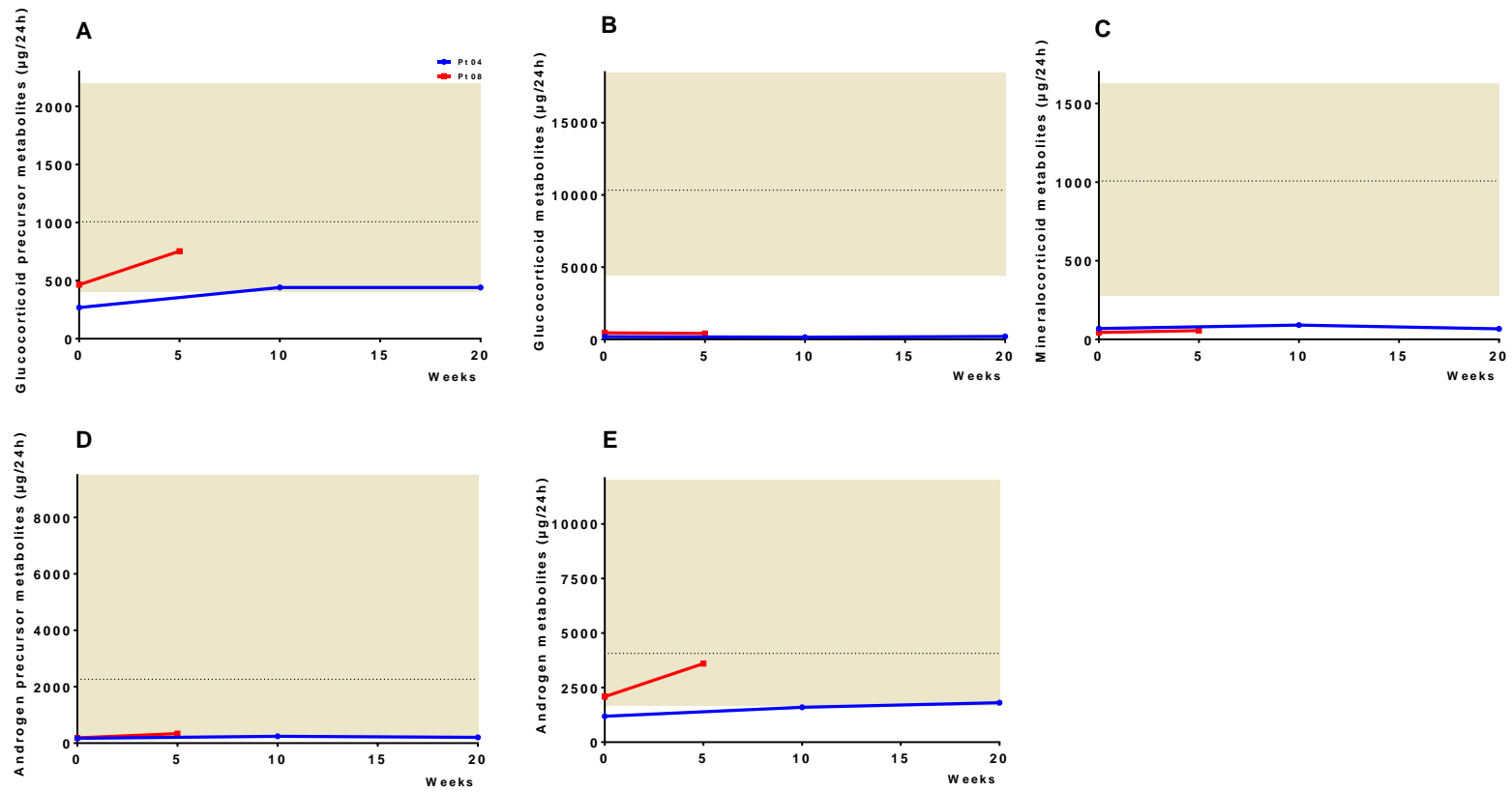
Figure 3.9. Normal reference ranges derived from healthy male controls (n=27; 20-60 years) are represented by the median (indicated by the dotted line) and the 5th to 95th centile range (represented by the shaded areas).

Panel A: The sum of the metabolites of the glucocorticoid precursors 17-hydroxyprogesterone (17-hydroxy-pregnanolone, pregnanetriol) and 11-deoxycortisol (tetrahydro-11-deoxycortisol) are plotted against time (normal range median 1007 [5th-95th centile 405-2196] µg/24h).

Panel B: The sum of the active glucocorticoid metabolites (cortisol, tetrahydrocortisol, 5α- tetrahydrocortisol, α-cortol, β-cortol, cortisone, tetrahydrocortisone, α-cortolone, β-cortolone) are plotted against time (normal range 10329 [4447-18496] µg/24h).

Panel C: The sum of mineralocorticoid metabolites (3α,5β-tetrahydroaldosterone, tetrahydrocorticosterone, 5α-tetrahydrocorticosterone, tetrahydrodeoxycorticosterone, 5α-tetrahydrodeoxycorticosterone, tetrahydro-11-dehydrocorticosterone, 5α-tetrahydro-11-dehydrocorticosterone) are plotted against time (normal range 628 [279-1631] µg/24h).

Panel D: The sum of the androgen precursor metabolites, dehydroepiandrosterone, 16α- dehydroepiandrosterone, 5-pregnanediol and 5-pregnanetriol are plotted against time (normal range 2262 [237-10548] µg/24h).

Panel E: The sum of the major androgen metabolites, androsterone and etiocholanolone, are plotted against time (normal range 4067 [1660-12017] µg/24)

3.3.6   Quality of life assessments

Subjective assessment of patient's wellbeing was performed using 3 sets of questionnaires (SF-36, AddiQoL and wellbeing visual analogue score). These questionnaires were completed by all participants at baseline, week 10 and week 20. However, one patient did not complete the SF-36 questionnaires at week 10.

In the SF-36 test, participants showed significant improvements in mental health at both week-10 and week-20 ($p<0.01$). Subjective improvement was also demonstrated in social functioning, vitality and general health, although they were not statistically significant, except in the vitality domain (table 3.7).

Similarly, participants showed significant increases in AddiQoL scores with a mean of 84.1 +/-10.2 at baseline that increased to 93.0 +/-11.6 and 94 +/-13.7 at week-10 and week-20 respectively ($p<0.05$) (table 3.8).

For the Addison wellbeing visual analogue scale (VAS), participants achieved a significantly higher score at the end of week 10 ($p=0.038$) and week 20 ($p=0.008$ (table 3.9). The construct validity of wellbeing VAS compared to AddiQoL questionnaire is good, with a Pearson's r varying between 0.82 to 0.97 ($P<0.001$). All domains in SF36 were also correlated strongly with the VAS wellbeing score, except bodily-pain and physical functioning, as shown in table 3.10.

Table 3.7. SF-36 scores at week-10 and week-20 assessment, in comparison with baseline score. The scores are expressed in mean (standard deviation)

SF36 domains	Baseline (n=11)	Week-10 (=11)	P-value	Baseline (n=10)	Week-20 (n=10)	P-value
Mental Health	46.11 (9.46)	52.51 (8.04)	0.003*	47.04 (8.86)	52.73 (9.82)	0.013*
Role-Emotional	49.56(10.9)	47.65 (11.61)	0.593	52.15 (7.09)	50.04 (11.37)	1
Social Functioning	41.83 (13.47)	48.25 (9.77)	0.106	44.64 (10.24)	48.43 (13.04)	0.365
Vitality	42.19 (10.72)	52.06 (10.81)	0.004*	43.39 (10.06)	51.19 (13.77)	0.091
Role-Physical	51.97 (7.59)	51.71 (10.11)	0.371	51.97 (7.59)	50.56 (11.89)	0.593
General Health	39.85 (9.97)	42.31 (10.71)	0.265	40.39 (8.61)	44.19 (9.45)	0.137
Bodily Pain	53.95 (8.26)	53.66 (11.96)	0.941	52.08 (8.37)	52.99 (7.6)	0.576
Physical Functioning	51.80 (3.87)	51.60 (5.77)	0.889	52.10 (3.94)	52.30 (3.92)	0.854
PCS	48.23 (5.60)	48.65 (9.49)	0.863	48.06 (4.83)	48.28 (5.03)	0.865
MCS	43.63 (11.46)	49.79 (9.86)	0.021*	46.02 (7.97)	50.73 (12.47)	0.083

*Statistically significant

PCS- Physical component summary

MCS- Mental component summary

Table 3.7 Short –form 36 (SF36) comprise 8 physical and mental health variances that form 2 distinct higher-ordered components. Physical functioning (PF), bodily-pain (BP) and role-physical (RF) correlate most highly with the physical component and contribute to the scoring of the Physical Component Summary (PCS) measure (Ware et al., 1994). Mental health (MH), role-emotional (RE), and social functioning (SF) scales contribute most to the scoring of the Mental Component Summary (MCS) measure. Vitality (VT) and general health (GH) have correlations with both components.

The total domain scores from 0 to 100, with higher values denoting better subjective health status. The scores were then converted using norm-based scoring, which employs a linear T-score transformation with mean = 50 and standard deviation = 10, to make it possible to meaningfully compare scores for the eight-scale profile and the physical and mental summary measures in the same graph. The final MCS and PCS were calculated using an SF-36 heath survey scoring as published in the SF-36 physical and mental health summary scales. A manual for users of the second edition can be found on their website (http://www.sf-36.org).

Table 3.8. AddiQoL scores (means and standard deviations) for week-10 and week-20 in comparison with the baseline assessment

	Baseline (n=12)	Week-10 (=12)	P-value	Baseline (n=10)	Week-20 (n=10)	P-value
AddiQoL score	84.08 (10.18)	93.00 (11.62)	0.030*	84.50 (6.72)	94.00 (13.66)	0.022*

AddQoL(version 2) (Oksnes et al 2012) is an Addison's disease-specific questionnaire comprising 30 questions that quantify the altered functional and emotional status in Addison's patients. The total score ranges from 30-120 and the higher score indicates a better quality of life. A paired t-test was used to calculate the p-value.

Table 3.9. VAS for Addison wellbeing assessment (means and standard deviations) for week-10 and week-20 in comparison with the baseline assessment

	Baseline (n=12) mean (S.D)	Week-10 (=12) mean (S.D)	P-value	Baseline (n=10) mean (S.D)	Week-20 (n=10) mean (S.D)	P-value
VAS Wellbeing score	72.89 (15.25)	80.96 (17.92)	0.038*	74.11 (9.29)	88.92 (11.56)	0.008*

A 10-question visual analogue scale (VAS) was developed to assess the changes perceived by participants in terms of functional and emotional health. It is expressed on a linear scale with a horizontal line, 10cm in length and anchored by word descriptors at each end. The measurements in centimetres were converted to the same number of points ranging between 0 and 10 for each quetion, summed up to give a total score out of 100cm. The participants marked on the line the point that they felt represented their perception of their current state. The scores were then measured in millimetres from the left hand end of the line to the point where the patients marked. Pearson's coefficient correlation was used to validate the questionnaire.

Table 3.10. Correlation between Addison VAS vs. AddiQoL and SF 36

	Baseline		*Week-20*	
	Pearson's r	*p-value*	*Pearson's r*	*p-value*
AddiQoL	0.82	0.001	0.97	<0.0001
SF 36				
Mental health	0.68	0.015	0.97	<0.0001
Role-emotional	0.88	<0.0001	0.91	<0.0001
Social functioning	0.69	0.013	0.73	0.017
Vitality	0.54	0.072	0.95	<0.0001
Role-physical	0.82	0.001	0.93	<0.0001
General Health	0.58	0.050	0.89	<0.0011
Bodily-pain	0.162	0.615^	0.249	0.487^
Physical functioning	0.50	0.098^	0.50	0.14^
Physical component score	0.52	0.084^	0.82	0.004
Mental component score	0.83	0.001	0.97	<0.0001

^ Statistically non-significant

Construct validity of the wellbeing VAS was carried out by comparison with SF-36 and AddiQoL by means of Pearson's correlation analysis.

3.3.7 Safety and tolerability

The synacthen therapy was well tolerated by the large majority of participants, with only one participant withdrawing from the study due to pain at injection sites. The common side-effects from synacthen that were observed include the development of transient redness, itches and swelling at the injection sites. Areas of erythema and swelling of up to 4 cm in diameter were reported and resolved over a 48-72hr period following each injection. The size of the lump tended to be smaller with synacthen infusion, which has a lower cumulative dose compared to the alternate day injection. The adverse events reported by participants during the study are summarised in table 3.11.

Four of the eleven female participants developed menstrual disturbance, with disruptions to a previously regular menstrual cycle and lower abdominal cramps at some point during the study. In addition, one participant developed inter-menstrual spot bleeding for 24 hours after ACTH-stimulation test at week-20. One participant felt unwell at week-25 during an assessment, around 30 minutes after 250μg synacthen intramuscular injection. She experienced generalised weakness, heavy legs, nausea and abdominopelvic cramps, like menstrual pain. There was a drop in blood pressure to 76/50mmHg accompanied by sinus tachycardia (104bpm, regular) at the height of discomfort. There was complete resolution 70 minutes after the onset of the event.

Two participants developed allergy-like reactions during major outcome assessments at week-15 and week-20. They both developed wheals at previous abdominal synacthen infusion sites 5 to 10 minutes after 250μg synacthen had been administered intramuscularly. They also reported itchiness and redness over their palms and soles. One of them developed lower abdominal cramps and vomiting which were resolved within 30 minutes. They remained haemodynamically stable throughout the events,

which were resolved after an hour. The potential investigational medicinal product

(IMP)-related adverse events are summarised in table 3.12.

Table 3.11. Adverse events reported by participants during the study (unlikely casuality to IMP)

Adverse events	Severity grade*	Actions	Outcome	Number of participants affected
Cough, sore throat, feverish	1-2	Doubling steroid dose	Full recovery	10
Generally unwell, flu-like symptoms	1-2	Doubling steroid dose		5
Vaginal candidiasis	2	150mg oral fluconazole (single dose)		1
Migraine	1-2	Simple analgesics		5
Nausea/poor appetite	1	None		2
Acute tonsilitis	2	Penicillin V 500mg qds for 10 days		1
Diarrhoea and vomiting	2	Two patients doubled the steroid doses and 1 patient required IM steroid injection (not in crisis and no hospital admission required)		3

*Severity Grade according to the Common Terminology Criteria for Adverse Events (CTCAE)

- Grade 1: mild effect; asymptomatic or mild symptoms with clinical or diagnostic observations only; intervention not indicated.

- Grade 2: moderate effect; minimal, local or non-invasive intervention indicated

Table 3.12. Potential adverse drug reactions (IMP-related) reported by participants during ACTH1-24 therapy

Adverse events	Severity grade*	Actions	Outcome	Number of participants affected
Menstrual irregularity with menstrual-like abdominal cramps	1	None	Full recovery	4
Generally unwell with transient hypotension, tachycardia and lower abdominal cramps following short synacthen test at week-25. Normal tryptase level	2	100mg intravenous hydrocortisone, 200mg ibuprofen and 10mg buscopan		1
Wheal developed at previous synacthen injection sites following short synacthen test. Associated with itchiness and redness over palms and soles.	1	Piriton 4mg and ice packs applied over the wheals		2
Lower abdominal cramps and vomiting (one episode) following short synacthen test and spot intermenstrual bleeding	2	Cyclizine and ibuprofen		1

*Severity Grade according to the Common Terminology Criteria for Adverse Events (CTCAE)

- Grade 1: mild effect; asymptomatic or mild symptoms with clinical or diagnostic observations only; intervention not indicated.

- Grade 2: moderate effect; minimal, local or non-invasive intervention indicated

3.4 Discussion:

Autoimmune Addison's disease (AAD) has long been perceived as a chronic and incurable condition. Patients with AAD rely on lifelong steroid replacement therapy for survival. However, exogenous steroids failed to mimic physiological corticosteroid secretion and many patients have impaired quality of life or recurrent Addison crisis. I sought to find out if adrenal steroidogenic function is salvageable in established AAD by using the synthetic analogue of adrenocortical trophic hormone (synacthen, $ACTH_{1-24}$). Four out of the thirteen participants had detectable serum cortisol at baseline testing, despite having established AAD for periods ranging from 1.5 to 8 years. Two of them had progressive improvements in adrenal steroidogenic function during treatment with synacthen. In both of these patients, improvements in serum cortisol level were detected as early as 5 weeks following synacthen therapy. Daily steroid replacement medication was able to be withdrawn with maintenance of good health in both cases. However, one participant only achieved transient improvement with a progressive decrease in stimulated serum cortisol once synacthen injections stopped. A glucocorticoid replacement-free period of 28 weeks was achieved in this case. In the other participant, the treatment has led to a prolonged improvement in adrenal steroidogenesis, such that she has remained well and without steroid replacement medication for 42 months at the time of writing. She encountered a few acute illnesses such as gastroenteritis and cold-like symptoms but no steroid therapy was required. Nevertheless, despite a peak cortisol level of 672 nmol/l during stimulation testing, she does not currently have normal adrenal function, as her plasma ACTH (159ng/l, reference range 10-55) and renin (14pmol/l/h; reference range 1-5.5) remain elevated. Synacthen therapy has produced clinical remission in 2 AAD patients although one was transient. This is a paradigm shift for AAD from a chronic treatable condition to a potentially curable disease. Although clinical remission happened following boluses of 1mg synthetic $ACTH_{1-24}$

injections in an alternate day pattern, other regimens of subcutaneous synacthen therapy (continuous infusion or pulses via pump) were not tested in these 2 patients. The dosage of synacthen used is also supra-physiological and further studies are required to establish the best regimen and the optimal dosage required to stimulate endogenous adrenal steroidogenesis.

In terms of subjective assessment of general wellbeing, it is interesting to note that the majority of participants (mostly female) reported better quality of life following synacthen therapy, despite not having significant changes in serum cortisol concentration. The perceived improvement in mental health and social functioning remained statistically significant at both week-10 and week-20. It is well known that female patients with AAD suffer from severe androgen deficiency as adrenal dihyroepiandrosterone (DHEA) is the most important androgen source for them. Considering that most participants demonstrated significant fluctuation in serum androstenedione and 17-α hydroxyprogesterone concentration, with many showing short-lived increments in these adrenal androgen precursors following synacthen therapy, this potentially accounts for the improvement in quality of life. Indeed, a few studies have demonstrated improvements in mood and wellbeing following DHEA replacement in patients with primary adrenal insufficiency (Arlt W et al.1999, Gurnell et al.2008). Nevertheless, this positive effect could well be due to a placebo effect, as no control group was involved in this study.

Although synacthen therapy was tolerated well by the majority of participants, a few of them developed allergic cutaneous reaction, with 1 patient developing more severe side effects of transient hypotension and near-syncope. In light of these reactions occurring within minutes of $ACTH_{1-24}$ administration, an immune-mediated response involving antibody-antigen reaction might have taken place. Hence, I proceeded to investigate this relationship, with the next chapter explaining the details of my findings.

This study demonstrates that AAD is a heterogeneous condition, with 2 of the 13 participants having useful residual adrenal steroidogenic function, and a third patient having a small rise in serum cortisol. Contrary to expectations, these patients with residual adrenal functions were not those with the most recently diagnosed disease. Indeed, the two patients exhibiting signs of recovery had been treated for AAD for 4 and 8 years prior to the study respectively. In contrast, 4 participants who were diagnosed with AAD within two years before the baseline visit exhibited no signs of residual steroidogenesis. This suggests that certain AAD patients may have residual steroidogenic function that is durable over long periods of time. One previous study of AAD patients showed increases in serum cortisol in 10 of 27 patients during synacthen testing, despite prior dexamethasone administration (Smans & Zelissen 2011). Along with the data from this study, residual steroidogenic function may be present in around 30% of AAD patients. There are only three reports in the medical literature of spontaneous partial recovery from established Addison's disease (Smans & Zelissen 2008; Chakera & Vaidya 2012, Bater et al.2013). These cases showed partial and intermittent remission with peak cortisol concentrations above 200 nmol/l following $ACTH_{1-24}$ stimulation test, having have AAD diagnosed between 7 to 37 years. Nevertheless, their plasma ACTH and renin levels remained elevated. In light of the data from this study, these 3 patients are likely to have residual steroidogenic function that improved owing to endogenous ACTH stimulation, once the patients had decided to reduce or stop their steroid medication.

Residual islet beta-cell function (marked biochemically by C-peptide 'positivity') is well described in about 20% of patients with autoimmune type 1 diabetes, and the findings from this study could represent a parallel state of partial destruction of the target organ in an autoimmune disease process. In C-peptide positive type 1 diabetes patients, the residual insulin secretion is significant, and has been correlated with

improved glycaemic control and fewer microvascular complications (The DCCT group 1987 & 1998, Panero et al. 2009). In AAD, around 40% of patients have never been hospitalised with an adrenal crisis (White & Arlt 2010, Hahner et al. 2010), and this was the case for our 2 participants who exhibited ACTH-responsiveness with useful residual adrenal function. It is possible that long-term freedom from adrenal crises could be due to residual adrenal function in certain AAD patients.

In contrary to the conventional concept of the natural history of AAD, this study provides new and valuable information about the heterogeneity of the natural history of the disease, by demonstrating that certain AAD patients have residual steroidogenic function several years after diagnosis. The residual adrenal function is clinically important as it can be exploited to ameliorate the condition. This study has clearly demonstrated that clinical remission is achievable following administration of exogenous trophic ACTH stimulation in patients with useful residual adrenal function. An important ramification of our study is that at the time of the first treatment of AAD using standard replacement therapy, it was unknown as to whether the exogenous glucocorticoid and mineralocorticoid treatment which decreases trophic ACTH drive would compound the functional adrenal failure by producing an additional degree of steroid-induced adrenal suppression/atrophy.

In light of the curable potential for AAD patient with residual adrenal function, it is paramount to identify sensitive markers of residual adrenal function at the time of diagnosis to enable this patient group to be distinguished. Measurement of peak serum cortisol level following $ACTH_{1-24}$ stimulation testing has been the gold-standard dynamic assessment of adrenal function. However, residual adrenal function could be well below the conventional lower limit of assay detection. A more sensitive biochemical marker should be established to detect residual adrenal function in AAD. Interestingly, a few participants demonstrated improvements in serum 17-α

hydroxyprogesterone, androstenedione, urinary corticosteroid metabolites and plasma metadrenaline levels, without changes in basal or stimulated serum cortisol. Hence, these markers could be a highly sensitive indicator of endogenous adrenocortical cortisol production and a large population-based study is required to verify this hypothesis.

Despite a concern over the potential for reactivation of the autoimmune response, there has been no worsening of humoral anti-adrenal autoimmunity over time in patients 02 & 06, as judged by 21-hydroxylase antibody concentrations. This is consistent with the hypothesis that adrenocortical steroidogenesis may protect the adrenal against immune attacks because the high local glucocorticoid concentrations impair the functions of antigen-presenting dendritic cells and several other immune cells (Betterle et al 2002, Mitchell & Pearce 2012). Nevertheless, T lymphocyte reactivity against adrenal antigens was not studied and an ongoing or renewed T cell attack could have contributed to the early decline in steroidogenic function in participant 06.

This study established an important proof of principle that ACTH stimulation by means of synacthen therapy can lead to recovery of adrenal function following long-term adrenal insufficiency in AAD. The approach described in this study needs further exploration, including the optimal treatment duration and dosage regimen. However, it is likely that a proportion of AAD patients could have similar long-term benefits from such therapy, particularly those with useful residual adrenal function. Hence, identification of this group of AAD patients is crucial as they might benefit from lowered doses of exogenous steroid replacement and a trial of synacthen therapy. Future studies are also required to investigate if a second morphogenic signal is required to work synergistically with ACTH to promote adrenal steroidogenesis in AAD.

Chapter 4.   Spontaneous and tetracosactide-induced anti-ACTH antibodies

4.1 Background

As previously discussed, four of the 13 patients with autoimmune Addison's disease (AAD) developed allergic cutaneous reactions immediately after $ACTH_{1-24}$ (synacthen; tetracosactide) injections during short synacthen tests. This happened following a prolonged duration of high-dose synthetic adrenocorticotropic hormone (zinc tetracosactide; depot synacthen) therapy, in an attempt to stimulate adrenal regeneration (RoSA study). One participant (06) whose adrenal function initially improved during $ACTH_{1-24}$ therapy failed to sustain the clinical improvement. Despite ACTH being an endogenous peptide to which immune tolerance should be established, these phenomena made me wonder if there was an immunogical reaction generated against the depot synacthen therapy.

The immune system is programmed to discriminate between self and non-self peptides, with the aim of eliminating foreign organisms and proteins that are harmful, but without generating an immune response to endogenous self-peptides. Nevertheless, tolerance to endogenous self-proteins does break down to produce autoimmune disorders, in which immune responses may be directed against many different proteins, most typically enzymes (eg. thyroid peroxidase, steroid 21-hydroxylase, glutamic acid decarboxylase) or cell-surface receptors (eg. TSH-receptor, acetyl choline receptor, calcium-sensing receptor) (Kaufman & Tobin 1993, Song et al. 1996, Merbl et al. 2007, Li et al.1996, Gavalas et al. 2007). In addition, a small number of conditions are characterised by antibodies against secreted peptide hormones, the most common of which is type 1 diabetes in which anti-insulin antibodies are highly prevalent (Palmer et al. 1983, McEvoy et al.1986).

ACTH is a polypeptide composed of 39 amino acids and is produced from proopiomelanocortin (POMC), by endopeptidase activity yielding ACTH and other

peptide products, including β-melanocyte stimulating hormone (MSH) and β-endorphin (Lee et al. 1961). ACTH exerts its effects on the adrenal cortex by binding to a member of the melanocortin receptor family, melanocortin-2 receptor (MC2R). The amino N-terminal segment of the peptide (residues 1-24) is the biologically active region of ACTH and is highly conserved across vertebrate species whereas the carboxyl terminal is considered to be more antigenic (Costa et al. 2004). Tetracosactide, also known as cosyntropin or synacthen, is a synthetic polypeptide identical in structure to the amino-terminal 24 amino acids of the naturally existing self-peptide, corticotroph (ACTH $_{1-39}$), and contains the biological activity to stimulate adrenal steroidogenesis.

Depot tetracosactide used in the RoSA study has prolonged duration of action compared to soluble, unconjugated tetracosactide, as the peptides are absorbed onto a zinc phosphate substrate, delaying its absorption from the site of injection. The structural differences between depot synacthen and un-conjugated ACTH$_{1-24}$, as well as full length ACTH is depicted in figure 4.1.

In this study, I investigated the possibility that immunoreactivity towards synacthen therapy had developed in these trial patients. I extended the study to additional autoimmune patient cohorts, including unrelated AAD and Graves' disease patients with no history of prolonged exposure to synathen therapy. In view of a significant proportion of individuals with isolated ACTH deficiency having coexisting autoimmune diseases and the target autoantigen has yet to be defined, I also investigated the potential role of anti-ACTH autoantibodies in isolated ACTH deficiency.
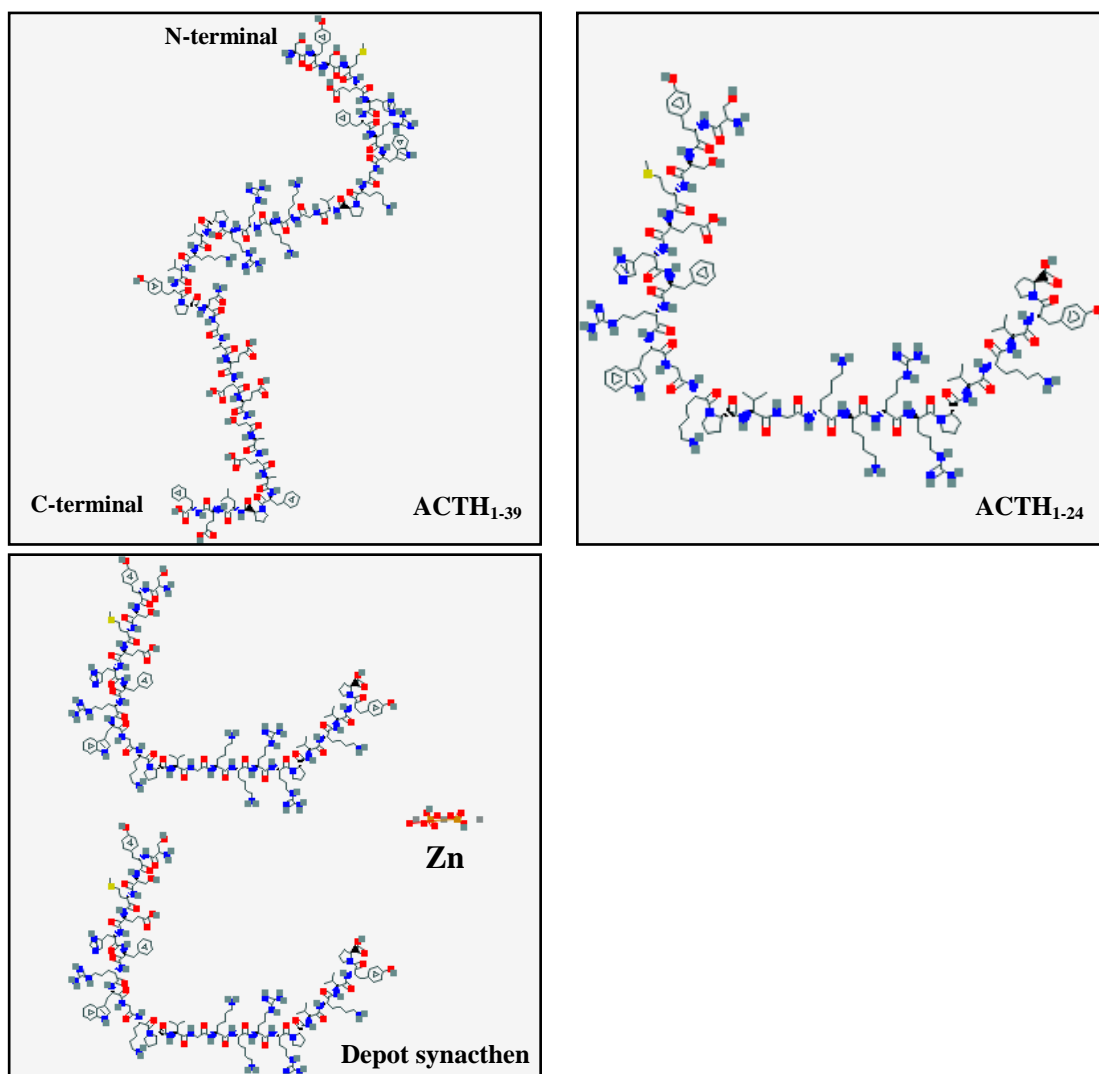
Name	ACTH$_{1-39}$	ACTH$_{1-24}$; tetracosactide; synacthen	Depot synacthen; ACTH$_{1-24}$ mixed with zinc phosphate(3:2)
Molecular formula	$C_{207}H_{308}N_{56}O_{58}S$	$C_{136}H_{210}N_{40}O_{31}S$	$C_{272}H_{422}N_{80}O_{70}P_2S_2Zn_3^{+2}$
Molecular weight	4541.06582	2933.437	6254.972604

Figure 4.1 Schematic representative of the 2-dimension structure of full length ACTH$_{1-39}$, ACTH$_{1-24}$ (synacthen or tetracosactide) and depot synacthen (tetracosactide mixed with zinc phosphate (3:2).

Synacthen is a synthetic peptide that is identical to the 24-amino acid segment at the N-terminal of (ACTH$_{1-39}$) and is similar among all species. (resource: PubChem Substance; http://www.ncbi.nlm.nih.gov).

4.2 Patients and methods

4.2.1 Patients

Thirteen Caucasian patients with established AAD were recruited for the RoSA study (revival of steroidogenic function in AAD study; NCT01371526), either from the endocrine clinics of the Newcastle upon Tyne NHS hospitals (n=12), or self-referred following an ethics committee agreed advertisement in the national Addison's disease self-help group (ADSHG) quarterly newsletter (n=1). Clinical details of these patients are provided in chapter 3.

The sera from 131 AAD and 92 Graves' disease patients were obtained via recruitment since 1996 through outpatient endocrinology services in the North East of England and the UK Addison's disease self-help group. Sera from 102 patients with negative autoimmune screening (rheumatoid factor, anti-nuclear antibody, anti-mitochondrial antibody, anti-gastric cell antibody) were obtained from the Clinical Pathology department at the Newcastle upon Tyne NHS hospitals trust. These sera were used as "non-autoimmune" controls. The thyroid and adrenal autoantibody status of the control group is unknown. Fifteen Caucasian patients with isolated ACTH deficiency were recruited either from the John Hunter Children's Hospital, Newcastle Australia (n=11) or Newcastle upon Tyne NHS hospitals (n=4). This study was carried out with the approval of the North East-Sunderland Research Ethics Committee, UK (ref. 12/NE/0101) and the Hunter New England Human Research Ethics Committee, Australia.

4.2.2 Methods

4.2.2.1 Tricine-SDS-PAGE

In this study, Tricine- sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoretic (PAGE) technique was used to separate proteins. SDS is an anionic

detergent that denatures the secondary and tertiary structures and imparts an even distribution of negative charge per unit mass. This results in the separation of protein by their approximate size during electrophoresis. Tricine-SDS PAGE is useful in separating low molecular mass protein (1-30kDa). Both $ACTH_{1-24}$ (~3kDa) and $ACTH_{1-39}$ (~ 5kDa) have molecular weight <30kDa.

Having reviewed the protocol published by Schägger H, a three-gel technique comprising stacking, spacer and separating gels was used to increase the resolution of the separating bands (Schägger 2006). A 16% acrylamide separating gel was selected as it covers protein with 1-70 kDa. In order to enhance the resolution for proteins <5 kDa, acrylamide-bisacrylamide(AB)-6 stock solution (49.5% T, 6% C mixture) that contains a higher percentage of crosslinker bisacrylamide (C) was used in the separating gel. A 10% 'spacer gel' inserted between the 4% stacking and 16% separating gels, considerably sharpens the bands for proteins of 1-5 kDa.

The following reagents were used for gel preparation and SDS-PAGE. The detailed protocols of making each solution were provided in the supplementary section.

- AB-3 stock solution (49.5% T, 3% C mixture)
- AB-6 stock solution (49.5% T, 6% C mixture)
- 3x Gel Buffer
- 10% Ammonium Persulphate (APS)
- Glycerol
- Tetramethylethylenediamine (TEMED)
- Loading buffer
- 1x Cathode Buffer
- 5x Anode Buffer
- 1x Transfer Buffer
- 1x TBST (Tris buffered saline with 1% Tween 20)

4.2.2.2 Acrylamide gel preparation

The three-layered acrylamide gels were prepared as following:

16% separating gel:

	16% Gel
Acrylamide-bisacrylamide (AB)-6	10ml
Gel buffer (3x)	10ml
Glycerol	3g
Add water to final volume	30ml
Polymerise by adding:	
APS (10%)	100ul
TEMED	10ul

10% Spacer Gel:

	10% Gel
AB-3	6ml
Gel buffer (3x)	10ml
Add water to final volume	30ml
Polymerise by adding:	
APS (10%)	150ul
TEMED	15ul

4% Stacking Gel:

	4% Gel
AB-3	1ml
Gel buffer (3x)	3ml
Add water to final volume	12ml
Polymerise by adding:	
APS (10%)	90ul
TEMED	9ul

The plates and combs were cleaned with 70% ethanol and all dust or left-over

acrylamide gel were removed. The plates were locked with a holder and set up on the

rack. The bottom edges of the 2 plates were flushed to ensure that there was no leakage.

The 16% separating gel solution was then pipetted carefully onto the plates, to avoid

generation of bubbles which could inhibit polymerisation. Once the gel was formed, 10% spacer and 4% stacking gel solutions were pipetted in sequence.

4.2.2.3 SDS-PAGE Electrophoresis

Tetracosactide (synacthen) or full length $ACTH_{1-39}$ was mixed with 1x SDS loading buffer and boiled at $95^{o}C$ for 5 minutes. Samples were immediately loaded onto the16% polyacrylamide tricine gel (2.5μg of tetracosactide or $ACTH_{1-39}$ per well) and run at 90V for an hour, with a pre-chilled running buffer (1x cathode and 1x anode buffers). The synacthen or $ACTH_{1-39}$ polypeptide was then electroblotted onto Hybond C membrane (Amersham), with ice-cold transfer buffer at 350mA for 45 minutes. Ponceau stain was used to locate the transferred protein. Ponceau stain has no deleterious effect on the blotted polypeptides and is easily reversible with water washes to facilitate subsequent immunoblotting.

4.2.2.4 Detection of anti-synacthen antibodies by immunoblotting

The blotted synacthen membranes were incubated with patient and control sera (1:250 dilution) for 75 min at room temperature, following blocking with 5% non-fat milk powder. Following washing with TBST (3 x 5 minutes), goat anti-human IgGγ-chain-horseradish peroxidase (HRP)(A6029, Sigma-Aldrich) was used to oxidise diaminobenzidine (DAB) substrate (Sigma-Aldrich, Poole, Dorset). Additional blots made using full length $ACTH_{1-39}$ (Sigma-Aldrich, UK) were probed and a monoclonal $antiACTH_{1-24}$ antibody (1B55, Santa Cruz, CA) was used as a positive control (detected with goat antimouse IgG (A4416, Sigma-Aldrich).

4.2.2.5 Detection of anti-tetracosactide antibodies by enzyme-linked immunosorbent assay (ELISA)(figure 4.2)

Tetracosactide ($ACTH_{1-24}$) was bound to the solid phase of ELISA plates (NUNC maxisorp) in a concentration of 1μg/ml overnight at 4^oC in 1x carbonate coating buffer. Plates were washed three times in phosphate-buffered saline tween 20 (PBST; PBS, 1% tween 20) and blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature. Serum diluted to 1:250 in 2mg/ml BSA were incubated in plates at room temp for 75 min, followed by washing and secondary antibody (goat anti-human IgG γ-chain-HRP) diluted 1:500 in 2mg/ml BSA for 1 hour at room temperature. Following washing and detection with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, Poole, Dorset T0440) for 3 minutes, reactions were stopped by 1M hydrochloric acid, and absorbance measured at A_{450} on an Ascent Multiskan (Thermo). The anti-tetracosactide binding activity was measured in triplicates for each participant sera, and each result reproduced on at least 2 different days. Results are expressed as arbitrary units of absorbance, having subtracted the background absorbance (mean of 3 wells without serum). A positive control was run on each occasion to check for interassay variation, which was within 10% on successive days. To check for non-specific serum binding to peptides, all positive samples were run with an identical ELISA protocol, with plates coated with octreotide (somatostatin 1-8) 1μg/ml instead of tetracosactide.
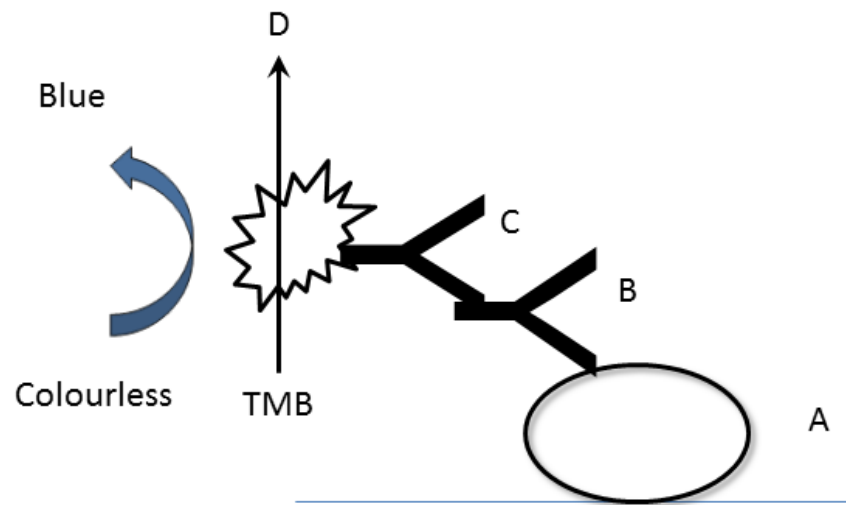
Figure 4.2 Schematic of direct binding ELISA to detect anti-syacnthen activity

A. Synacthen is bound to solid phase

B. Anti-synacthen antibody from patient's sera recognise and binds to synacthen

C. Bound sera are detected with anti-human IgG γ-chain-conjugated to horseradish peroxidase (HRP)

D. HRP catalyses the oxidation of TMB (3,3',5,5'-tetramethylbenzidine) chromogen, results in a blue colour change. The colour then changes to yellow with the addition of sulphuric acid with maximum absorbance at 450nm.

4.2.2.6 Immunohistochemistry study of pituitary tissues

Sections of human pituitary tissue were kindly provided by the Cellular Pathology department at the Royal Victoria Infirmary, Newcastle upon Tyne. These tissue sections were embedded in paraffin wax blocks and then sectioned at a thickness of 4μm by the department. I carried out immunohistochemistry study on these pituitary tissue sections, using the indirect HRP-labelled antibody method. Sections underwent deparaffinization and rehydration as delineated in table 4.1. Antigens were retrieved by boiling the sections in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 10 minutes in a microwave (850W). The sections were allowed to cool for 20 minutes followed by 2 washes with PBS plus 0.025% Triton X-100. The sections were then blocked with 10% goat serum in 1% BSA for an hour. This was followed by 4°C overnight incubation with pooled patient sera (from 3 positive subjects) diluted 1:100. Following 2 washes with PBS plus 0.025% Triton X-100, sections were incubated in 1% hydrogen peroxide for 15 minutes to block peroxidase activity. The slides were then washed twice prior to incubation with HRP conjugated goat anti-human IgGγ-chain antibodies (A6029, Sigma-Aldrich) for 1 hour. Following 3 washes with PBS, Antibody-binding was visualised with the chromogen diaminobenzidine (DAB) (Sigma-Aldrich, Poole, Dorset) and counterstained with haematoxylin. The slides then underwent the rehydration protocol using Histoclear and ethanol as stated in table 1. Monoclonal anti $ACTH_{1-24}$ antibody (1B55, Santa Cruz, CA) was used as a positive control (detected with goat antimouse IgG (A4416, Sigma-Aldrich). The slides were mounted with DPX (Sigma Aldrich) and glass-coverslip added. Microscopy was performed using Axioplan 2 widefield microscope with Axiovision software (Carl Zeiss)

The immunocytochemistry staining was optimised by different antibody (primary and secondary) dilution and blocking system. The antibody concentration titration was

carried out using positive controls (monoclonal antibody towards ACTH). A 4-point

scoring system was employed to assess the intensity of staining (Adams et al. 1999):

- (no stain); + (weak staining); ++(moderate staining); +++ (strong staining), with the

positive control slide taken as +++.

Deparaffinise and rehydrate		Dehydrate and clear	
Histoclear	2x 3min	**50% ethanol**	3 min
Histoclear:100% ethanol (1:1)	3 min	**70% ethanol**	3 min
100% ethanol	2x3 min	**95% ethanol**	3 min
95% ethanol	3 min	**100% ethanol**	3 min
70% ethanol	3 min	**Histoclear:100% ethanol (1:1)**	3 min
50% ethanol	3 min	**Histoclear**	2x 3min
Running cold tap water to rinse		**Clear and mount**	

Table 4.1 Protocols for depraffinisation/rehydration and dehydration in immunocytochemistry study

Paraffin embedded sections require thorough depraffinisation and rehydration as incomplete removal of paraffin will result in poor staining of the section. The slides are to be kept in the tap water following the deparaffinasation steps to avoid dehydration, that could cause non-specific binding and high background staining.

4.3 Results

4.3.1 Anti-tetracosactide immunoreactivity among patients receiving high-dose depot tetracosactide

Using immunoblotting of immobilised $ACTH_{1-24}$, protein bands with an apparent weight of 3-4kDa were recognised in patients' sera. Sera from all of the patients who had an adverse reaction to tetracosactide identified a ~4kDa band on immunoblotting against the tetracosactide ($ACTH_{1-24}$) peptide (figure 4.3 panel A). This band was found in 10/13 (77%) of patients who had received multiple doses of tetracosactide depot as part of the clinical study. In eight patients, the immunoreactivity was absent in baseline samples but noted to develop over time during exposure to $ACTH_{1-24}$. However, there was similar immunoreactivity in 2 participants at baseline, before sustained peptide exposure.

Immunoblotting against full-length $ACTH_{1-39}$ peptide showed that all of the patient sera which reacted with the tetracosactide ($ACTH_{1-24}$) peptide, also showed specific reactivity at ~6kDa with full-length ACTH peptide (figure 4.3 panel B). This is in contrast to results from 3 healthy control sera, which showed no binding to either the $ACTH_{1-24}$ or $ACTH_{1-39}$ peptides. A commercial anti-ACTH amino-terminal (corresponding to the tetracosactide peptide) monoclonal antibody was used to probe blots and showed bands corresponding to the expected size with both the tetracosactide and the full-length ACTH peptides (figure 4.3 panel B). An ELISA was then designed, using tetracosactide peptide captured onto solid phase, to quantify the anti-synacthen binding activity. The ascertainment of a threshold for normality was carried out using a larger sample cohort as stated in the next section. Sera from five of the 13 patients (38%; all female) demonstrated relative absorbance above 0.21 units at baseline (the threshold for positivity; see below) with 2 of them having relative absorbance of more than 2 units (figure 4.3 panel C). Upon completion of either 10 or 20 weeks of tetracosactide therapy,

11/13 (84.6%) patients showed immunoreactivity towards tetracosactide, with relative absorbance scores above 0.21 units. Nine of the 11 female participants demonstrated a 1.5 to 12-fold increase in the ELISA absorbance score following tetracosactide peptide treatment but sera from both male patients were negative throughout the study.

Panel A
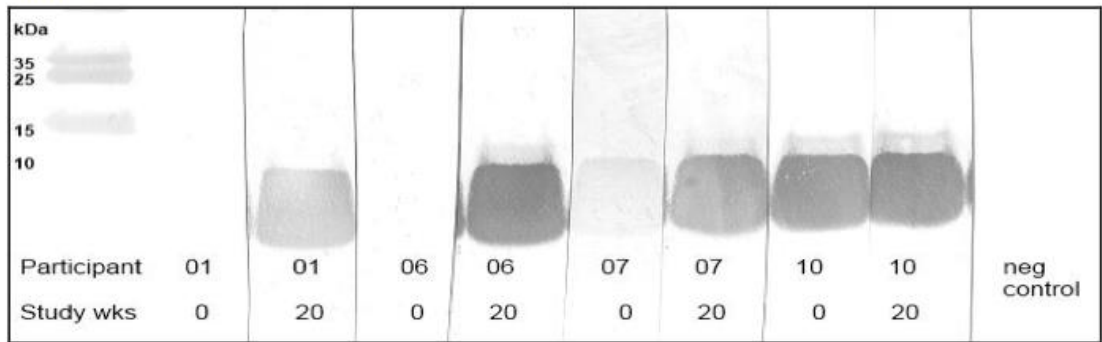
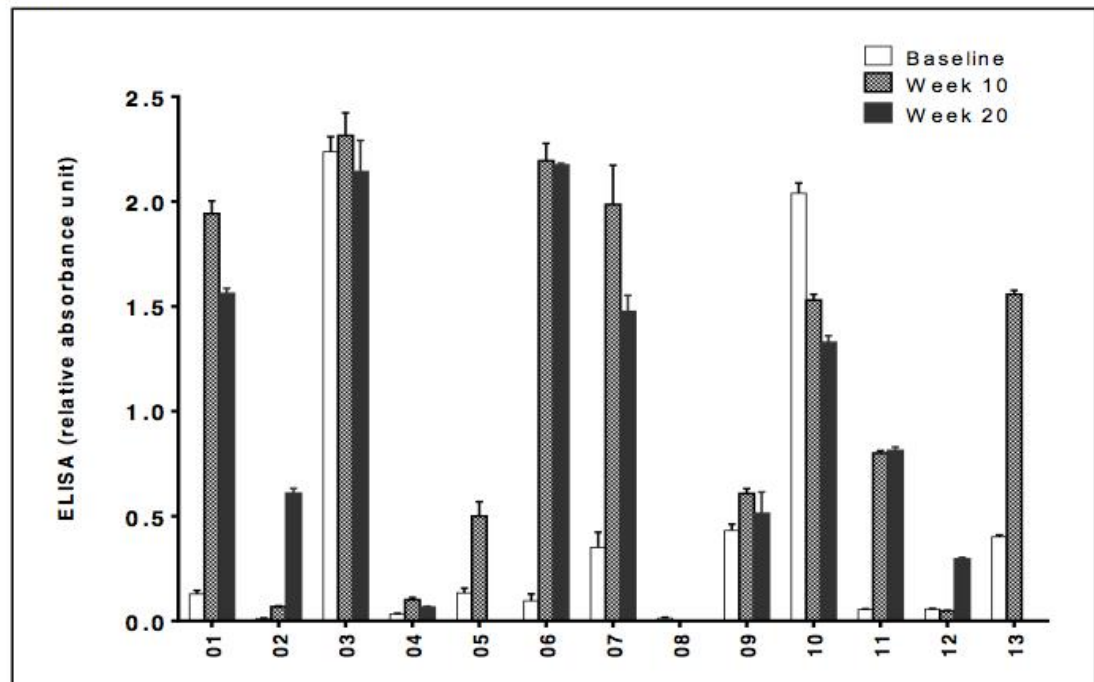

Panel B
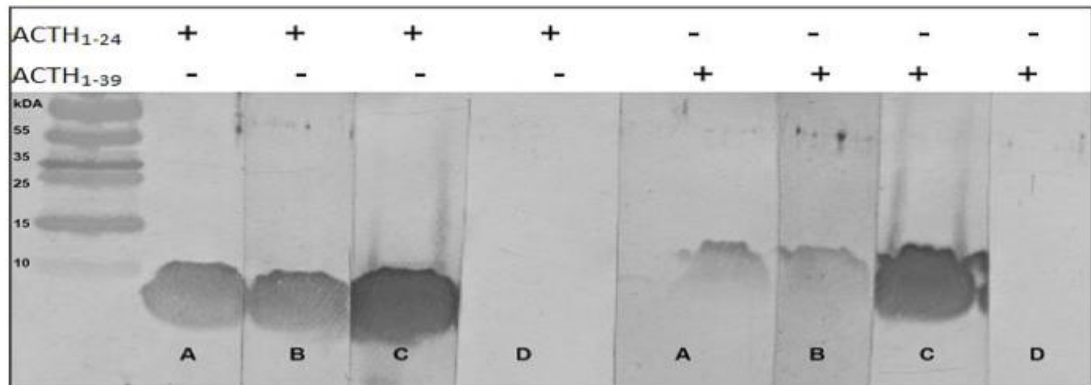
Panel C

Figure 4.3 Anti-tetracosactide immunoreactivity detected with immunoblotting & ELISA.

Figure legend (4.3)

Panel A: Detection of serum binding activity to immunoblotted $ACTH_{1-24}$, at baseline and week 20 for participants 1, 6, 7 and 10, as assessed by the band of ~4kDa, the expected molecular weight of $ACTH_{1-24}$. Sera from healthy subjects were used as a negative control.

Panel B: Immunoblotting was performed with blots made using both tetracosactide ($ACTH_{1-24}$) peptide and full-length human $ACTH_{1-39}$, both 2.5µg per well. Representative positive sera from 2 participants (sera A & B), a monoclonal anti-$ACTH_{1-24}$ antibody (C) and serum from a healthy control (serum D) are shown. The monoclonal anti-ACTH antibody recognizes bands of the same molecular mass as the positive participant sera for both $ACTH_{1-24}$ and $ACTH_{1-39}$ peptides, which was not seen with control sera.

Panel C. Anti-tetracosactide binding activity on immunoblotting from all the patients of RoSA study was quantified using ELISA. Results (±SEM) are expressed as arbitrary units of absorbance at 450nM, having subtracted the background absorbance (mean of 3 wells without serum). All except 5 patients had relative absorbance units of less than 0.21 at baseline. A positive control was run on each occasion to check for interassay variation, which was within 10% on successive plates.

4.3.2 Anti- tetracosactide immunoreactivity among controls vs. patients with autoimmune diseases (autoimmune Addison's disease, Graves' disease and isolated ACTH deficiency)

The study of autoimmunity towards $ACTH_{1-24}$ peptide was extended to larger groups involving healthy controls, patients with Graves' disease, autoimmune Addison's disease and isolated ACTH deficiency. Initially, 102 control sera from anonymous hospital attendees with negative autoantibody status were investigated, along with a patient serum that had proven positive on immunoblotting. Only one of the 102 control sera exceeded 0.21 absorbance units in the ELISA (figure 4.4). This value was then taken as an arbitrary threshold of positivity. Of the sera from 131 patients with autoimmune Addison's disease, 28 (21%) were positive (>0.21 U) for anti-tetracosactide binding on the ELISA assay. Similarly, sera from 92 patients with Graves' disease and 15 with isolated ACTH deficiency were run: 13 (14%) and 1 (7%) were positive, respectively.

To investigate whether the anti-synacthen peptide binding in the ELISA could be owing to non-specific binding, all positive sera were tested in an identical assay, except that the plates were coated with octreotide (somatostatin octapeptide) instead of tetracosactide. None of the sera gave a positive signal in the assay with octreotide (figure 4.5, panel A). Similarly, all positive patient sera in the ELISA were tested against the tetracosactide peptide on immunoblotting, and all 41 showed specific 4kDa binding (figure 4.5, panel B).
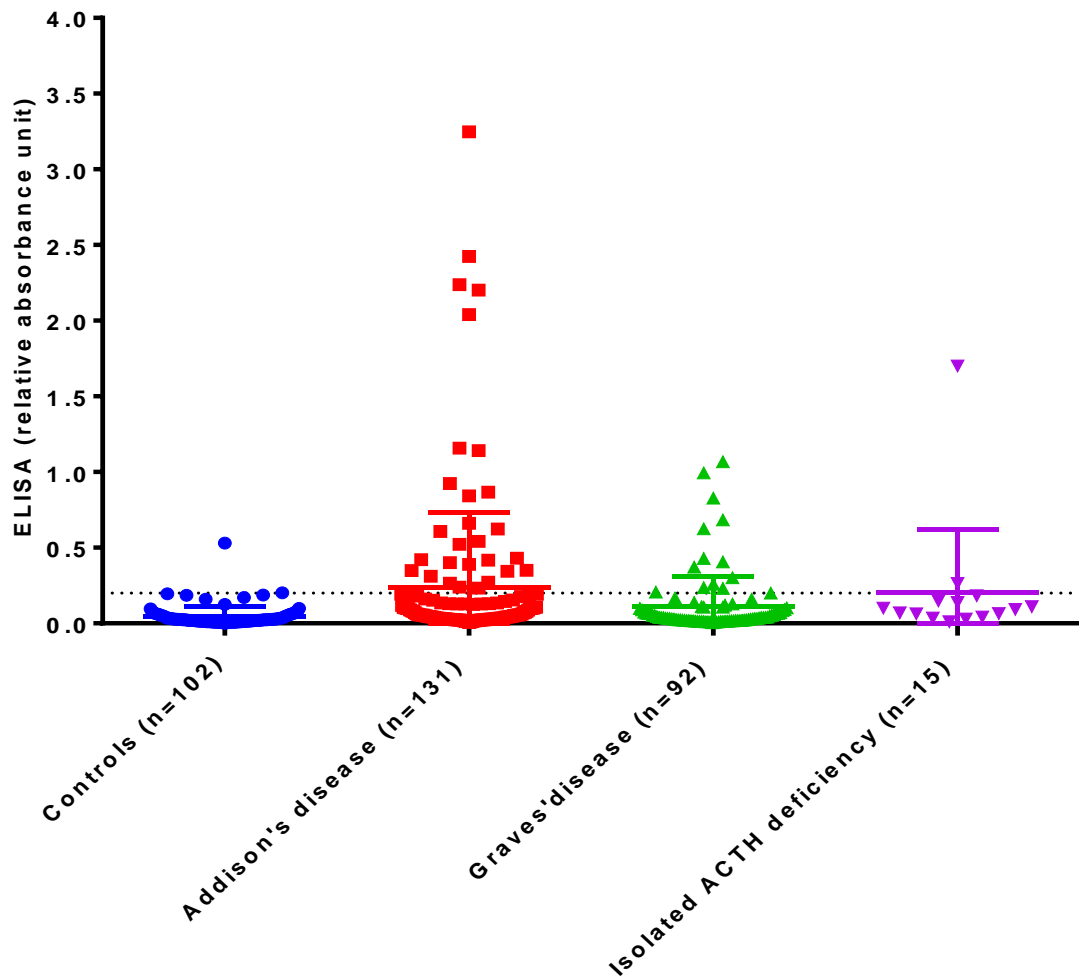
Figure 4.4 Anti-synacthen binding activity detected by ELISA

Relative quantitation of anti-tetracosactide binding activity using ELISA with sera from 102 controls, 131 unrelated patients with AAD, 92 patients with Graves' disease and 15 patients with isolated ACTH deficiency. Tetracosactide ($ACTH_{1-24}$) 1μg/ml was bound to solid phase ELISA plates (NUNC maxisorp). Results (±SEM) are expressed as arbitrary units of absorbance at 450nM, having subtracted the background absorbance (mean of 3 wells without serum). A positive control was run on each occasion to check for interassay variation, which was within 10% on successive plates. ELISA of the 102 control sera (from anonymous hospital attendees with negative autoantibody status) showed absorbance readings of ≤0.2 units in 101 sera, with 1 serum showing an absorbance of 0.6 units.
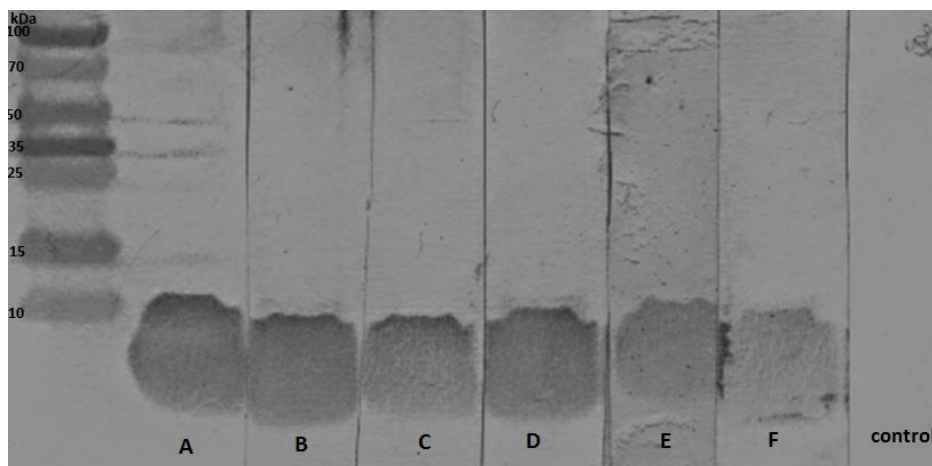
Panel A
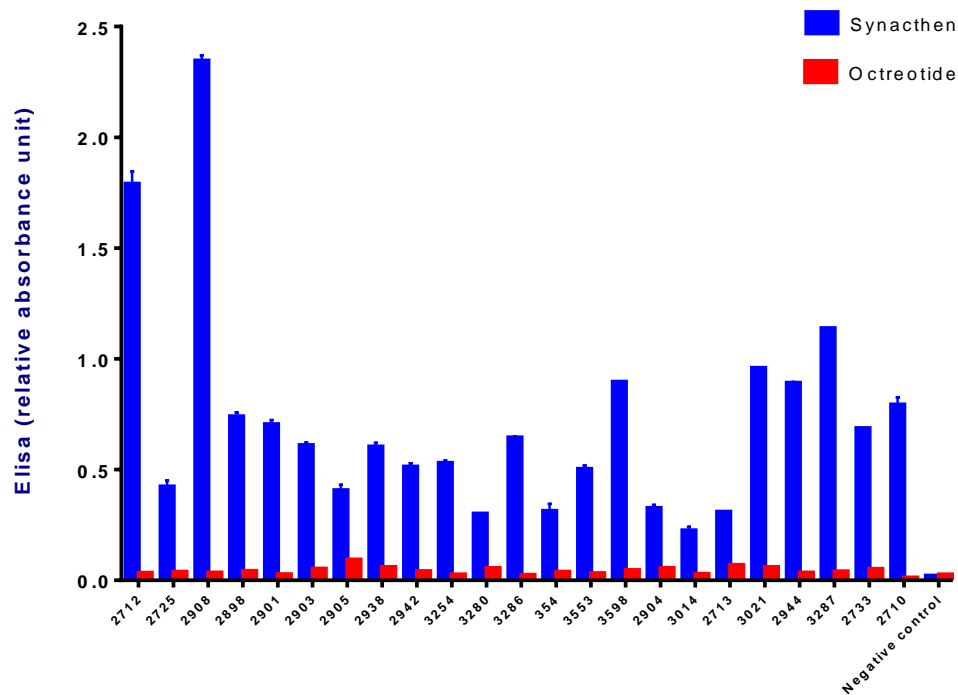
Panel B

Figure 4.5 Verification of ACTH immunoreactivity in samples' sera

Panel A. All positive samples were run against an identical ELISA protocol, with plates coated with octreotide (somatostatin$_{1-8}$) 1μg/ml. None of the sera gave a positive signal in the assay with octreotide.

Panel B. All positive patient sera in the ELISA were tested against the tetracosactide peptide on immunoblotting, and all 41 showed specific 4kDa binding. Representative positive sera from 6 patients (A-F) are shown.

4.3.3 Immunohistochemistry study

Lastly, I examined normal human pituitary sections by immunohistochemistry to determine whether the patient sera with anti-synacthen binding activity would recognise native ACTH in tissue sections. Figure 4.6 shows that these positive sera produce moderate (++) cytoplasmic staining in a similar pattern to that of a commercial anti-ACTH antibody (+++).
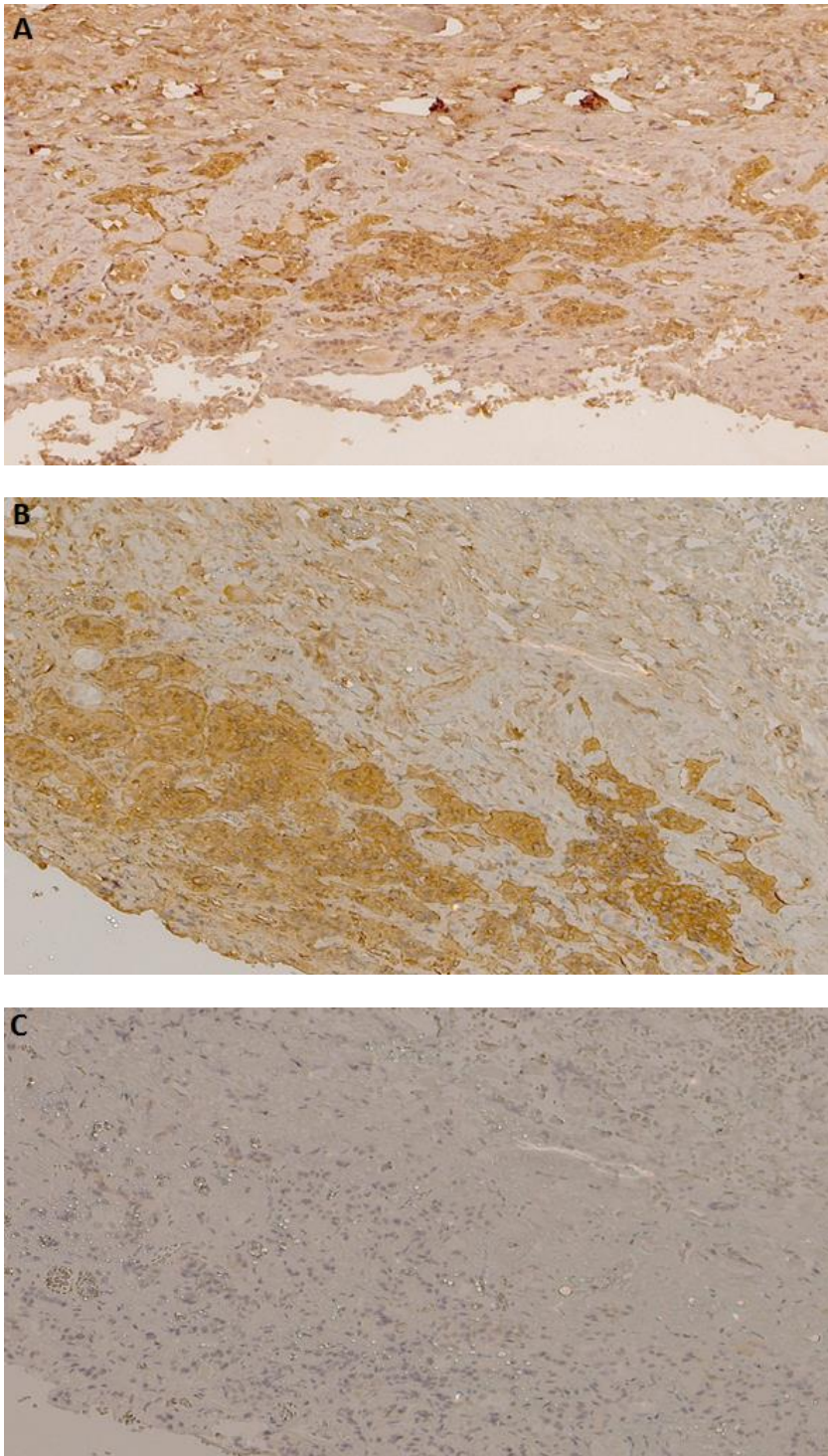
Figure 4.6 Immunohistochemistry results

Immunohistochemistry was performed to identify whether sera with anti-tetracosactide binding activity would recognise native ACTH in contiguous tissue sections. Representative positive patient sera (panel A) and negative patient sera (panel C) were used. Monoclonal antiACTH$_{1-24}$ antibody (1B55, Santa Cruz, CA) was used as positive control (panel B). The positive sera produced a cytoplasmic staining in a similar pattern to that of the commercial anti-ACTH antibody. (Magnification x100)

4.4 Discussion

The development of serum anti-ACTH antibodies following long-term porcine or synthetic full-length ACTH peptide therapies was reported in the 1960s &70s (Felber et al. 1966, Glass et al. 1971). Glass and colleagues detected anti-ACTH antibodies using haemagglutination and antigen displacement methods in 32% of 38 rheumatoid arthritis subjects treated with long-term depot tetracosactide (zinc tetraocosactide)(Glass & Daly 1971). In line with these early observations, this study demonstrated the development of anti-tetracosactide and full-length ACTH binding activities using immunoblotting among 77% of 13 patients with autoimmune Addison's disease, following multiple doses of depot tetracosactide therapy.

Although antibodies to self-peptide should not generally occur, the development of antibodies to various peptide therapies has been extensively reported. Anti-insulin antibodies were observed in patients with diabetes treated with exogenous insulin leading to immunological resistance and poor glycemic control (Berson et al. 1956, Berson &Yalow 1959). A similar experience was shared among patients treated with growth hormone therapy, and to enzyme replacement in Gaucher's disease (Okada et al. 1987, Ponce et al. 1997, Ahangari et al. 2004). Hence, my findings are consistent with this phenomenon and may explain some of the side effects seen in the RoSA study. In addition, modification of circulating tetracosactide concentrations by sequestering or increased clearance mediated by circulating anti-ACTH antibody could account for treatment failure in some of the patients and 'treatment resistance' observed in one participant (patient 06), who lacked a sustained improvement during tetracosactide therapy. Indeed, Felber and colleagues described inactivation of corticotropin following formation of antibody towards porcine ACTH, using a radioimmunoassay assay (Felber et al. 1966). It was also demonstrated that antibodies generated in guinea pigs

immunised with synthetic full-length ACTH were responsible for a loss of ACTH

bioactivity *in vitro*. Furthermore, as antibodies against tetracosactide would be directed

against the biologically active amino acid sequence of ACTH, the 1-24 segment, this

clearly has the potential to reduce the therapeutic potency and effectiveness of depot

tetracosactide therapy. This might also explain the well described phenomena of

acquired resistance to chronic adrenocorticotropic hormone (ACTH) therapy (Goslings

et al. 1951, West 1962).

Interestingly, the same autoreactivity has not been reported with soluble synthetic

tetracosactide. Indeed, animal studies showed that conjugates of the $ACTH_{1-24}$ peptide

were antigenic, but that unconjugated $ACTH_{1-24}$ peptide failed to induce an antibody

response in rabbits or guinea pigs (Felber et al.1966, Gelzer 1968). The N-terminal

segment (1-24) of ACTH peptide is highly conserved among all species and is believed

to have low antigenicity (Lee et al. 1961, Kertesz et al. 1998, Costa et al. 2004). Hence,

it seems likely that the complex of $ACTH_{1-24}$ and zinc in the depot tetracosactide

formulation preparation may be important in the generation of the humoral immune

response. However, I was intrigued to find out that five of the 13 participants from the

RoSA study, who were never exposed to depot tetracosactide, demonstrated positive

serum anti-ACTH binding activity at baseline. Similar findings were made in the larger

cohort of Addison's patients. These latter patients had all received one or two soluble

$ACTH_{1-24}$ peptide injections during diagnostic testing for Addison's disease. Hence, I

speculate that the anti-ACTH antibody response could potentially have been generated

either from previous exposure to soluble tetracosactide, or more likely towards native,

full-length endogenous corticotropin. This group of patients may be more susceptible to

the breakdown of immune self-tolerance, by dint of their autoimmune Addison's

disease, but may also be susceptible owing to the very high levels of endogenous

plasma ACTH circulating in the months before their diagnosis of AAD. Indeed, several previous studies have revealed activity in the sera of AAD patients that appeared to attenuate the *in vitro* steroidogenic response of cultured adrenal tissue to ACTH (Wulffraat et al. 1989, Wardle et al. 1993, Kendall-Taylor et al. 1988). Although this was believed to be due to patient immunoglobulins that bound to and blocked stimulation of the ACTH receptor, several of the effects described would be consistent with antibodies that neutralized ACTH action *in vitro*. With this in mind, could these ACTH antibodies that were found in 21% of the unrelated AAD patients in our cohort contribute to the pathogenesis of autoimmune Addison's disease? If these antibodies had functional consequences in terms of binding or clearing endogenous ACTH, then a state of relative "corticotropin resistance" could potentially lead to an accelerated decline in adrenal function during the natural history of evolving Addison's disease. Plasma ACTH may be elevated for many years prior to the diagnosis of Addison's disease (Torrejón et al. 2007) and this ACTH drives small islands of adrenal hyperplasia and regeneration (Guttman 1930, Saphir & Binswanger 1930), slowing the ultimate onset of adrenal steroidogenic failure. Thus early adrenal unresponsiveness to ACTH stimulation in evolving Addison's disease may have the potential to tilt the balance from subclinical or partial adrenal insufficiency to clinically apparent adrenal insufficiency. This hypothesis would need to be confirmed in a future study.

The significance of anti-ACTH antibodies in a small number of patients with Graves' disease is also unknown, especially in the absence of clinically apparent adrenal insufficiency. Nevertheless, several studies have documented a blunted cortisol response to tetracosactide in hyperthyroid Graves' disease, which would be consistent with the effects of anti-ACTH antibodies in these patients (Karl et al. 2007, Price et al. 2012). It is also well known that patients suffering from one autoimmune disease are

susceptible to development of a second autoimmune condition, either manifesting as an overt disease following pathological destruction of self-tissue, or as the presence of circulating autoantibodies alone. Outside the context of autoimmune conditions, ACTH autoantibodies have been detected in patients suffering from anorexia nervosa and bulimia nervosa (Fetissov et al.2002). Patients suffered from major depression were also found to have impaired hypothalamic-pituitary-adrenal axis (Demitrack et al.1991, Scott et al.1998). Hence,it has been suggested that the presence of ACTH antibodies could potentially lead to a subclinical hypocortisolemia state or subnormal ultradian/circadian rhythm of ACTH secretion. This might result in chronic lethargy, anorexia, depression and even maladaptive behaviour due to poor stress response (Wheatland et al. 2005). The origin of these autoantibodies is unknown although some suggested that past or persistent infection could bring on this autoreactivity, potentially from molecular mimicry where some microorganism share common sequence fragment with ACTH, such as H.pylori and staph aureus infections (Oldstone et al. 1998). Hence, the significance of auto-ACTH antihbody in human health warrants further investigation.

With the demonstration that sera with anti-ACTH antibodies recognise native ACTH in human pituitary sections, I also wondered whether anti-ACTH antibodies could have a role in isolated ACTH deficiency. I was able to obtain sera from just a small number of individuals due to the rarity of this condition, and only one serum showed the anti-ACTH binding activity. While isolated ACTH deficiency is a heterogeneous condition, a significant proportion of individuals have coexisting autoimmune thyroid disease or manifest other features of autoimmunity, and the target autoantigen has yet to be defined. Nevertheless, the presence of anti-ACTH antibodies in just one (7%) of these patient groups suggests that although these antibodies would be a logical mediator of isolated ACTH-deficiency, they are not a major cause of this condition. Larger patient

cohorts of this rare condition would needed to be examined in the future to confirm our preliminary findings.

In summary, this study demonstrates that repeated administration of depot tetracosactide can lead to anti-ACTH autoreactivity. In addition, a significant number of untreated AAD and GD patients also had similar autoreactivity (P<0.001 vs controls). It is possible that these anti-ACTH antibodies mediated some of the adverse effects seen in participants of the RoSA study and that their propagation in individuals following multiple ACTH injections could explain the well-described phenomenon of resistance to chronic ACTH therapy. In future, further investigation is necessary to better define the role of these anti-ACTH antibodies in the pathogenesis of autoimmune Addison's disease, isolated ACTH deficiency and other autoimmune conditions.

Chapter 5. Characterisation of adrenocortical stem cell phenotype: Isolation of mesenchymal stem cell-like cell populations from human adrenal cortex

5.1 Background

The plasticity of the adrenal cortex suggests the presence of adrenocortical stem cells (ACSC) but the exact *in vivo* identity of ACSC remains elusive. In the past few decades, mesenchymal stromal or stem cells (MSCs) has emerged as a popular topic of interest in the field of research, owing to their multipotent differentiation capacity, low tumorigenicity and low immunogenic nature. They were initially isolated from the adult bone marrow and have subsequently been harvested from several other tissues, including the adipose tissue (Zuk et al. 2001), pancreas (Hu et al. 2003), umbilical cord (Wang et al. 2004), synovium (De Bari et al. 2001), dental pulp (Pierdomenico et al. 2005), trabeculae bone (Tuli et al. 2003) and skeletal muscle (Young et al. 1995). MSCs are conceptually the postnatal progenitor cells of most derivatives of mesoderm (Bianco et al. 2008). The adrenal gland is thought to have originated from the intermediate mesoderm embryonically and hence MSC could potentially be the progenitor/stem cell source. In recent years, it has been shown that mouse and human MSC (adipose tissue or bone marrow-derived) could be induced to steroidogenic cells that produce a variety of steroids, via steroidogenic factor 1 (Gondo et al.2004, Yazawa et al. 2006, Tanaka et al. 2007, Gondo et al. 2008, Yazawa et al. 2011). With this in mind, I investigated the direct isolation of MSC from human adrenal cortex.

MSCs lack a unique surface antigen that could be used for positive selection. However, they are characterised by their ability to be enriched and adhered to plastic dishes in a low-serum medium, growing as a homogenous population of adherent cells and express a set of marker proteins on their surface, including CD44, CD90, 105 and CD166 (Pittenger et al. 1999, Dominici et al. 2006). However, these markers are not specific to MSCs and they are mainly characterized by their capacity to differentiate along

mesodermal lineages into osteocytes, chondrocytes, adipocytes, and skeletal muscle cells (Pittenger et al. 1999). The *in vitro* culture condition for the multilineage differentiation of human MSCs is driven by signalling cues directed by both cell density and supplementation with exogenous soluble factor (Barbero et al. 2003).

On the other hand, studies attempting to define adrenocortical stem cells with murine models have suggested that adrenocortical progenitor cells reside at the capsule or subcapsular region of the adrenal cortex. The adrenal cells residing at the subcapsular region were found to co-express SF1 and DAX, with SHH responsive cells localized to the capsular region. Interestingly, DAX1 expression was found to be limited to the subcapsular region of the adrenal cortex and inhibits the ability of SF1 to transactivate steroidogenic enzymes. This prevents the differentiation of adrenal cells into the 3-zonal adrenocortical lineages. Hence, it has been postulated that subcapsular adrenal cell population that express SF1, SHH and DAX1 is a good candidate for adrenocortical progenitor cells.

The aim of this study was to investigate if the human adrenal cortex harbours multipotent MSCs which were capable of differentiation into multiple mesodermal lineages, as well as their role as adrenocortical progenitor cells. This was carried out via cell culture, immunocytochemistry and flow cytometry studies.

5.2 Methods

5.2.1 Preparation of transport medium, growth medium and digestion solution

The following transport and growth media were prepared:

- Transport medium comprising Dulbecco's Modified Eagle Medium/Nutrient mixture F-12 (DMEM: Ham F12) medium (Invitrogen) with 1% penicillin/streptomycin (Invitrogen).

- Complete growth medium (CM) comprising DMEM: Ham F12 medium with supplement mix as stated in table 5.1 and 1% penicillin/streptomycin.

- Mesenchymal stem cell growth promotion medium (MGPM) comprising alpha modified Eagle's medium (alpha MEM) (Invitrogen), 10% (vol/vol) fetal bovine serum and 5ng/ml fibroblast growth factor 2 (FGF2) (Sigma Aldrich).

- Enzymatic digestion solution comprising 0.2% collagenase (2mg/ml) (Sigma Aldrich) and 0.01% deoxyribonuclease I (DNAse I) (Sigma Aldrich) (0.1mg/ml) dissolved in 30mls DMEM:Ham F12 medium.

Table 5.1 Components of complete growth medium (CM)

Component	Concentration
Insulin-Transferrin-Selenium (ITS) (Invitrogen)	0.5%
Ascorbic acid (Sigma Aldrich)	0.25%
Horse serum (Invitrogen)	5%
Fetal bovine serum (FBS) (Invitrogen)	10%

5.2.2 Preparation of adrenal tissue and cell culture

Full ethical approval was obtained from the local ethics committee (12/NE/0101).

Adrenal tissue was obtained from consented patients undergoing either radical nephrectomy or partial nephrectomy for upper pole tumours, whereby their adrenal glands would be removed at the same time. The removed adrenal tissue was transferred within 24 hours to the laboratory on ice in a transport medium. A small part of the adrenal tissue was sliced sagittally to 3mm width and fixed in 4% paraformaldehyde solution overnight at room temperature, before being subjected to a further histological process as described in the next section. The remaining adrenal tissue was dissected and separated from fat and the adrenal medulla by removing tissue adjacent to the central vein. Instruments used include a round tipped pair of sterile scissors, forceps and blades. All dissection work was carried out in a sterile laminar flow hood and tissues were placed in a 10cm diameter Petri dish containing 10mls growth medium. The remaining adrenal cortex with capsule attached was then minced with scissors into approximately $1mm^3$ and washed 3 times with DMEM/Ham 12 (1:1) medium, before being enzymatically dispersed in a digestive solution for 30 minutes at 37^oC in a shaker table. Small tissue pieces are essential for a better final cell yield.

The digested tissues were then disaggregated by gentle aspiration through a sterile 18 gauge cannula before being filtered through a 70 µm nylon cell strainer (BD Falcon) into a 50ml centrifuge tube. The undigested tissue fragments were resubmitted to the same digestion procedure until all tissues were fully digested. The filtered cells were centrifuged at 437x g (Heraeus Labofuge 400R) for 5 minutes. The supernatant was carefully removed leaving approximately 0.5 ml liquid and the cell pellet at the bottom of the tube. The cell pellet was then resuspended gently in 2ml of growth medium to avoid bubble generation. 1ml of the cells were seeded in a complete growth medium

(CM) at a density of 1.0×10^6 per T-75 cell culture flask. The remaining 1ml of the cells were seeded in mesenchymal stem cell growth promotion medium (MGPM) at a density of 1.0×10^6 per T-75 cell culture flask. Cells grown under complete growth medium were incubated at $37^{o}C$ under an atmospheric oxygen tension of 20% and 5% CO_2, whereas cells seeded in MGPM were incubated at a low oxygen tension of 5% O_2, using a hypoxia incubator. Non-adherent cells were removed after 48 hours and monolayer cells were washed twice with 1% phosphate buffered saline (PBS) (Invitrogen) before being replaced with a new medium. Medium changes were carried out every 3 days thereafter. Colony forming units-fibroblasts (CFU-Fs) were counted in each culture every 2 days. All cell culture work was carried out under sterile condition in a class II laminar flow hood.

5.2.3 Maintenance of adrenocortical cell culture

Established cultures of adrenal cells seeded in either complete medium or MGPM were passaged when 80-90%% confluent by enzymatic detachment using TrpLE^{TM} Express (Invitrogen) and seeded at a density of 1×10^6 /flask density. TrpLE^{TM} Express is a recombinant fungal trypsin-like protease with similar dissociation kinetics to trypsin but exhibits lower cellular toxicity. The medium was changed every 3 days.

5.2.4 Detachment of monolayer cells from tissue culture plastics

Monolayer cells were washed twice with 1% PBS before incubating with 3ml of TrpLE^{TM} Express at $37\ ^{o}C$ for 5 minutes. When adequate cellular detachment was achieved, as visualised under a light-phase microscope, the cells were pipetted up and down gently to form single cell suspension and transferred to a sterile polypropylene

conical tube (BD Falcon). The cell suspension was then centrifuged at 437 x g for 5 minutes. The cell pellet was resuspended carefully in 3ml of growth medium.

5.2.5 Cell count

Detached cells were resuspended in 3mls of complete medium. Cell count was performed by placing 10µl of fluid on a cell counter slide (haemocytometer) and counting the number of cells visible in 1 big square. The average count between the 3 large squares was made and the total number of cells within the cell suspension was calculated.

Viable cell counts were carried out with trypan blue (an acid dye which binds to intracellular proteins and would have only taken up when membrane integrity is lost), an exclusion dye that is only taken up by dead cells (Strober 2001). 90µl of cell suspension following detachment of monolayer cells was mixed with 10µl of 0.4% (v/v) trypan blue. 10µl of this mixture was then transferred to each chamber of a cell counter slide (haemocytometer) and viable cells were counted. The average cell count between the 3 chambers was made and the total number of cells within the cell suspension was calculated with the following formula:

Total number of cells = Total cell counted x Dilution factor x 10^4 cells/ ml

5.2.6 Population doubling time

Following primary cell culture, adrenal cortex-derived cells from passage 1 were plated in a 6-well culture plate at 10×10^4 cells/well. They were cultured in both complete

growth medium and MGPM, respectively. The cells from each well were detached with TrpLE^{TM} Express and counted in triplicate with a hemocytometer every day until day 6. The population doubling time, or the time required for a culture to double in number, was calculated with the formula previously described (Xu et al. 2010).

Doubling Time = T x log2/ log(N_t/N_0)

T = time interval in any units

N_0 = cell count at the beginning of the incubation

N_t = cell number at the end of the incubation time

5.2.7 *Freezing and thawing of cells*

Monolayer of cells were detached with TrpLE^{TM} Express and resuspended in a freezing medium (Gibco) at $1x10^6$ cells/ml. The cells were frozen down in 1 ml aliquots in cryogenic vials at the rate of a 1^oC decrease in temperature per minute using isopropanol chamber and stored at -80 ^{o}C in a freezer.

5.2.8 *Osteogenic, chondrogenic and adipogenic differentiation of human adrenocortical cells*

The monolayer adrenal cells grew in MGPM and complete media were detached with TrpLE^{TM} Express. The adrenal cells were then centrifuged at 630 x g for 4 minutes at room temperature. The supernatant was removed and the adrenal cells were resuspended in 4 ml of DMEM:Ham F12 medium with 10% FBS. Cells were then counted and collected as per the optimal quantity required for adipogenenic, osteogenic and chondrogenic differentiation, as stated below.

Chondrogenesis	500,000 cells/pellet
Adipogenesis	100,000 cells/ml
Osteogenesis	25,000 cells/ml

5.2.8.1 Chondrogenic differentiation of human adrenocortical cells

2×10^6 adrenocortical cells were resuspended in 4 ml of StemPro chondrogenic differentiation medium (Invitrogen) to a final cell density of 500000 cells/pellet/ml. 1ml aliquots were transferred to sterile 15ml polypropylene conical tubes. The cells were then centrifuged at 630 x g for 4 minutes at room temperature to the bottom. The tubes were loosely capped to allow for gas exchange. The cell pellets were incubated in a low oxygen tension of 5% O_2, hypoxia incubator. The chondrogenic medium was changed once every 3 days.

5.2.8.2 Osteogenic differentiation of human adrenocortical cells

The counted cells (100,000 cells) were resuspended in 4 ml DMEM:Ham F12 medium enriched with 10% FBS and seeded in a 12-well dish to a final cell density of 25,000 cells/ml/well. The cells were incubated overnight until settled. Twenty-four hours post seeding, the DMEM +10% FBS medium were replaced with StemPro osteogenesis differentiation medium (Invitrogen). The medium was changed once every 3 days.

5.2.8.3 Adipogenic differentiation of human adrenocortical cells

The counted cells (400,000 cells) were resuspended in 4 ml DMEM:Ham F12 medium enriched with 10% FBS and seeded in a 12-well dish to a final cell density of 100,000 cells/ml/well. The cells were incubated overnight until settled. The DMEM +10% FBS

medium was then replaced with StemPro adipogenesis differentiation medium (Invitrogen) the next day. The medium was changed once every 3 days.

5.2.9 Histological examination of differentiated osteogenic, chondrogenic and adipogenic cells from human adrenocortical cells

<u>Safranin O staining</u>

Safranin O staining was used to characterise the formation or deposition of glycoaminoglycans (GAG) within cell pellet (Rosenberg 1971). The cell pellet was first processed and dehydrated through the machine processor and embedded in wax as described in the section below. Cell pellet sections were then deparaffinised through Histoclear (4x10 minutes), rehydrated through 100% ethanol (4x10 minutes) and rinsed in deionised water. The sections were then stained in Harris'haematoxylin for 4 minutes, destained in acidic alcohol for 10 seconds and rinsed in deionised water. This was followed by staining with 0.02% aqueous fast green (FCF) (Sigma Aldrich) for 3 minutes, rinsing in 1% acetic acid for 30 seconds then staining with 0.1% aqueous Safranin O (Sigma Aldrich) for 5 minutes. The slides were rinsed thoroughly in deionised water and dehydrated with 100% ethanol (4x 30seconds) and Histoclear (4x1 minutes) before being mounted using DePEX mouting medium (Leica Biosystem) and visualised using a phase contrast microscope.

<u>Alizarin Red S staining</u>

Alizarin Red S (ARS) is an anthraquinone derivative and was used to characterise calcium-rich deposits by cells in culture, in which calcium will form a complex with ARS in the chelation process (Lievremont et al. 1982, Gregory et al. 2004). Alizarin Red working solution (40µM ARS, pH 4.2) was prepared by dissolving 0.7g of Alizarin

Red S (Sigma Aldrich) in 25mls double distilled water (ddH$_2$O). The pH of the solution was adjusted to 4.2 with 0.5% ammonium hydroxide. DdH$_2$O was added to bring the final volume to 50ml. The final solution was then filtered through a 100μm cell strainer.

The monolayer of adrenal cells were washed with 1x PBS and fixed in 2% (v/v) paraformaldehyde (Santa Cruz) for 10 minutes at room temperature. Paraformaldehyde was removed and the monolayers were washed twice with ddH$_2$O. 1ml of 40μM ARS was then added to each well and incubated at room temperature for 30 minutes with gentle shaking. The dye solution was then pipetted out carefully and wells were washed 4 times (4x5 minutes) with 3 ml of ddH$_2$O. The plate was then left at an angle for 2 minutes to facilitate removal of excess water. The stained monolayer cells were then examined under a phase contrast microscope.

Oil Red O staining

Oil Red O comprised of lysochrome (fat soluble dye) used to demonstrate the presence of triglyceride deposition. The monolayer of adrenal cells were washed with 1x PBS and fixed in 2% (v/v) paraformaldehyde (Santa Cruz) at room temperature for 10 minutes. Paraformaldehyde was removed and the monolayers were washed twice with ddH$_2$O. The cells were then washed with 2ml of 60% isopropanol for 5 minutes at room temperature before letting them dry completely at room temperature. 1ml of Oil Red O (Sigma Aldrich) solution was added to each well and incubated at room temperature for 15 minutes. The dye solution was then pipetted out carefully and ddH$_2$O was added immediately to the wells. The cells were washed 4 times (4x5 minutes) with 3 ml of ddH$_2$O. The plate was kept wet to keep the lipid vacuoles from disrupting. The stained monolayer cells were then examined under a phase contrast microscope.

5.2.10 Immunocytochemistry study of adrenal cells

5.2.10.1   Fixation of monolayer cells

Cells were harvested in either coverslips (sterilised with 70% ethanol) which had been

plated in 12-well dishes or Lab-Tek^{TM}II chamber slides (BD Bioscience), at cell

densities between 10 x10^4 to 30 x10^4 cells per wells. Cells were cultured for 24-48

hours.

5.2.10.2   Immunofluorescent staining

Cell culture media was removed and the monolayer cells were washed twice with

1%PBS. The cells were then fixed in 2% paraformaldehyde for 10 minutes at room

temperature and rinsed with 1%PBS (2x5 minutes). Autofluorescence quenching was

enhanced by incubation with 1M ammonia chloride diluted with 1% PBS for 5 minutes

followed by 2 washes (2x 5 minutes) with 1% PBS. Antigen retrieval was carried out

where appropriate by incubating the cells in 0.1% triton X-100 for 3 minutes at room

temperature followed by 3 washes with PBS (3x5 minutes). The cells were blocked with

1% bovine serum albumin (BSA) and 5% goat serum for 30 minutes at room

temperature. This was followed by incubation with the primary antibodies which were

diluted with 1% BSA, either in a humidified chamber overnight at 4°C or at room

temperature for 1 hour. One well was incubated with only 1% BSA, to use as a negative

control. The isotype-matched species antibodies (Santa Cruz Biotechnology) were also

used as IgG controls (with the same protein concentration as the primary antibodies

used). Following on from primary antibody incubation, the cells were washed with 1%

PBS (3X5 minutes). The appropriate secondary antibodies were then added for 1 hour at

room temperature in the dark. Cells were then washed with 1% PBS (3X 5 minutes) and

mounted with Vectashield containing 4', 6-diamidino-2-pheny-lindole (DAPI) (Vector

Laboratories), an anti-fade water-based mounting agent with integral nuclear stain and

coverslips. Slides were kept at 4°C in the dark for up to one week. The slides were

visualised using AxioImager Z1 microscope (Zeiss fluorescent microscope with

Apotome attachment). The dilution and manufacture of the primary and secondary

antibodies used are detailed below (table 5.2 & table 5.3).

Table 5.2. Primary antibodies used for immunofluorescence

Primary antibody	Source	Dilution (final concentration)	Catalogue number
SHH goat IgG	Santa Cruz Biotechnology	1:100 (2µg/ml)	SC1194
GLI rabbit IgG	Santa Cruz Biotechnology	1:100 (2µg/ml)	SC20687
SF1 goat IgG	Santa Cruz Biotechnology	1:100 (2µg/ml)	SC10976
DAX1 rabbit IgG	Abcam	1:200 (2µg/ml)	Ab97369
MSC antibody panel including CD19, CD44, CD45, CD90, CD105, CD106, CD146, CD166	R&D system	1:40 (2.5µg/ml)	SC017

Table 5.3. Secondary antibodies used for immunofluorescence

Secondary antibody	Source/manufacture	Dilution	Catalogue number
Bovine anti-goat IgG-FITC	Santa Cruz Biotechnology	1:1000 (0.4 µg/ml)	S2348
Sheep anti-rabbit IgG- Texas Red	Abcam	1:2000 (1 µg/ml)	Ab6793
Goat anti-mouse IgG-AF488	Invitrogen	1:2000 (1 µg/ml)	A-10667
Donkey anti-mouse IgG-AF 594	Invitrogen	1:2000 (1 µg/ml)	A-21203

5.2.11 Flow cytometry analysis

Flow cytometry was used to assess the immunophenotype and MSC immunoprofile of adrenal cells, as described by the position paper of the International Society for Cellular Therapy (ISCT) (Dominici et al 2006). Human adrenal cells were detached with TriplExpress and washed once in PBS followed by centrifugation at 1750 x g for 3 minutes. The cell pellet was resuspended in fluorescence-activated cell sorting (FACS) buffer (1%PBS with 0.1% BSA) at a concentration of 5×10^5 cells per 100 μL. These cells were filtered through a 100 μm nylon cell strainer (Falcon BD) and transferred to sterile 1.5ml microfuge tubes ($1x10^6$ cells/ 200μl/tube). For indirect flow cytometry, the single-cell suspensions were incubated at 4°C for 1 hour with appropriate unconjugated primary antibodies or isotype-match control antibodies (Santa Cruz Biotechnology), as shown in table 5.4. The cells were washed once and centrifuged at 1750 x g for 3 minutes. The supernatant was aspirated and the cell pellets were resuspended in a 200μl cold FACS buffer. The cells were incubated with appropriate secondary antibodies on ice in the dark for 40 minutes. They were then washed twice with cold PBS and resuspended in 700ml 1% PBS for immediate analysis. For direct flow cytometry, single-cell suspensions were incubated with fluorochrome-conjugated primary antibodies (table 5.4), on ice and in the dark for 40 minutes. They were washed with cold 1%PBS twice and resuspended in 700ml of 1% PBS for flow cytometry analysis.

For intracellular staining, 100μl of fixation medium (reagent A) from the Fix and Perm cell permeabilization kit (Invitrogen) were added to 100μl of single cell suspension following detachment of monolayer cells. These cells were incubated at room temperature for 15 minutes and washed once with 1ml of PBS. The supernatant was aspirated and cell pellets were resuspended in 100μl of FACS buffer. 100μl of reagent B (permeabilization medium) and the appropriate unconjugated antibody or the

corresponding isotype control were added. Cells were incubated for 20 minutes at room temperature and washed once with 1ml PBS. The cell suspensions were incubated with secondary antibodies on ice and in the dark for 40 minutes. Cells were then washed twice and resuspended in 1% PBS for immediate analysis. The controls comprised unstained cells, cells incubated with secondary antibodies only and isotype-matched species antibodies as IgG control (same protein concentration as primary antibodies used). The dilution and manufacture of the antibodies and fluorochromes used are detailed in table 5.5. I used mesenchymal progenitor/stromal cells harvested from knee aspirate as the positive control. These knee-aspirate derived MSCs were a gift from the musculoskeletal stem cell group at the Institute of Cellular Medicine, Newcastle University.

Flow cytometry analysis was carried out using FACS Canto II Colour flow cytometer (BD Biosciences) with the following parameters (table 5.6). Electronic gating on the basis of forward scatter (FCS) and side scatter (SSC) was used to eliminate cell debris and non-viable cells. Data was analysed using FACS Diva version 8 and Venturi One version 2 softwares. Figure 5.1 and figure 5.2 depicted the details of FACS analysis; 20,000 events were recorded for each sample.

The flow cytometry analysis was performed at passage 1, 3, 5, and 7 for MPGM and passage 1, 2 and 3 for cells harvested in CM.

Table 5.4. Unconjugated and conjugated primary antibodies used in flow cytometry

Primary antibody	Source	Dilution	Catalogue number
Unconjugated primary antibody			
DAX1 rabbit IgG	Abcam	1:10 (40 µg/ml)	Ab97369
GLI rabbit IgG	Santa Cruz Biotechnology	1:10 (20 µg/ml)	SC20687
MSC antibody panel (mouse antibodies)	R&D system	1:1 (100 µg/ml)	SC017
Conjugated primary antibody			
MHC I HLA-ABC Antibody, FITC conjugate	Thermo Scientific	1:1 (100ug/ml)	MA1-80454
MHC II HLA-DR Antibody, FITC conjugate	Thermo Scientific	1:1 (100ug/ml)	MA1-19620

Table 5.5. Secondary antibodies and fluorochrome used in flow cytometry

Secondary antibody	Source/manufacture	Dilution	Catalogue number
APC-conjugated goat anti-rabbit IgG *(400 µg/ml)*	Santa Cruz Biotechnology	1:100	SC3846
FITC-conjufated goat anti-mouse IgG	Sigma Aldrich	1:100	F2653

Table 5.6. Flow cytometry set up and parameters

Parameters/fluorochrome	Emission filters	Voltage
FSC	-	280
SCC	-	300
FITC	488/530/30	310
APC	633/660/20	420
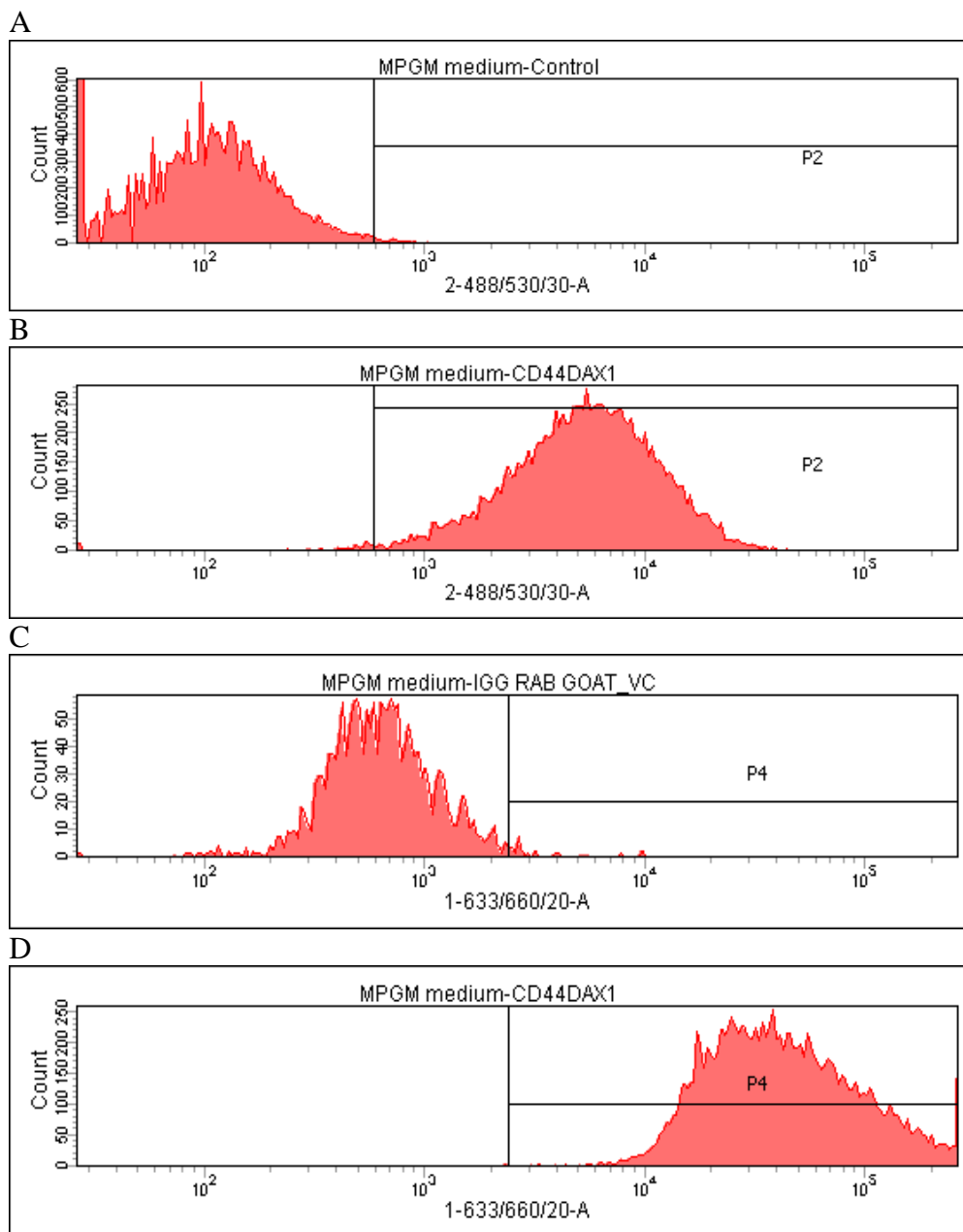
Figure 5.1 An example of a FACS analysis with one of the MSC marker–CD44 (FITC labelled) and DAX1 marker (APC-labelled) in the study

Figure legend 5.1

A. The negative control graph for FITC labelled antibodies as shown by the 488/530/30 band pass filter (X-axis) against cell counts (Y-axis). The histogram is shifted to the left as the sample was not incubated with primary antibodies but only FITC-conjugated anti-mouse antibodies. The P2 section was configured in a way that allowed 98% of the negative control counts to fall outside this area.

B. Histogram for sample labelled with primary antibody (CD44) and FITC-conjugated secondary antibody, showing nearly 100% cell counts falling in the P2 section, suggesting positivity of CD44 in the sample.

C. The IgG isotype control graph for APC labelled antibody as shown by 633/660/20 band pass filter (X-axis) against cell counts (Y-axis). The P4 section is set so that 98% of the isotype control counts fall outside this area.

D. Histogram for sample labelled with primary antibody (DAX1) and APC-conjugated secondary antibody, showing nearly 100% cell counts falling in the P4 section with strong positive staining of DAX1 marker.
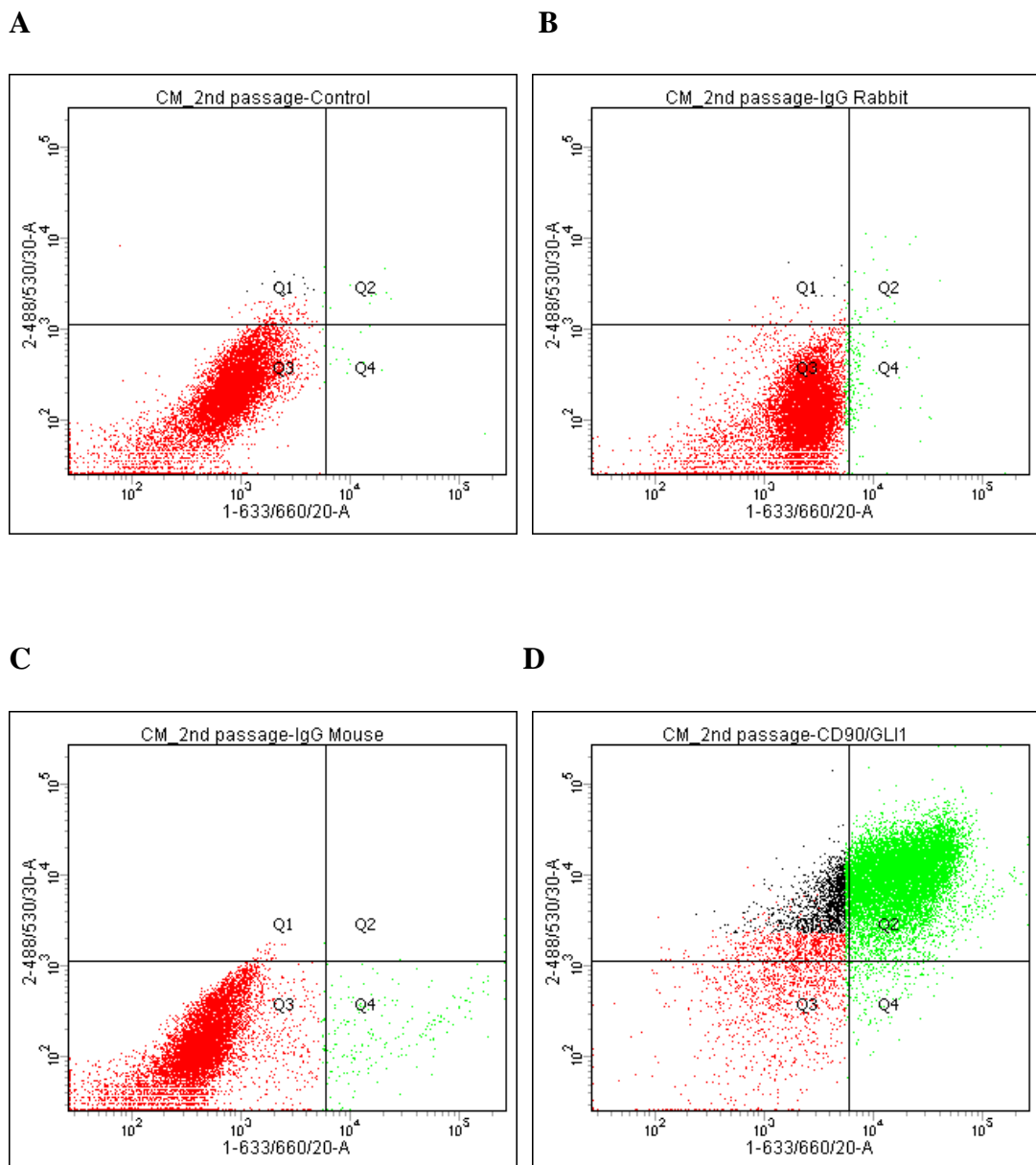
Figure 5.2 A-D demonstrated the dot-plot for the double-immunostaining with CD90 (FITC labelled- 488/530/30) and GLI (APC-633/660/20) markers in the study

APC-labelled GLI1+ count (X-axis) was plotted against the FITC labelled CD90+ count (Y-axis). Positivity with dual-staining was indicated by the Q2 region, in which >98% of the control and species-specific isotype controls fall outside this area.

5.2.12 Tissue embedding and paraffin wax blocks.

A small section of adrenal tissue (cut sagittally) or cell pellet from chondrogenic culture medium was washed in 1% PBS before being fixed in 4% paraformaldehyde for at least 3 hours at room temperature. The tissue was then transferred to a machine processor programmed to carry out the following dehydration steps. The tissue was first dehydrated in 50% ethanol for a minimum of 2 hours at room temperautre in the shaker and then overnight in 70% ethanol. Dehydration was completed in 3 changes of 100% ethanol (total 6 hours) at room temperature. Tissue was then cleared overnight in a glass container in Histoclear (National Diagnostics) and stored in a fume hood before being equilibrated in 3 changes of paraffin over 6 hours at 60°C. The glass tube was swirled occasionally to ensure adequate penetration of the wax. The adrenal tissue or cell pellet was then orientated in a plastic mould so that the cut surface accurately represented the tissue. The block was then left to set at room temperature for at least 1 hour and cooled on ice before removing the paraffin block from the mould.

5.2.13 Microtome sectioning

Paraffin blocks of adrenal tissues or cell pellets were sectioned at a thickness of 4 μm using a Leica RM2135 rotary microtome with disposable microtome blades. The cut sections were floated onto a water bath at 37°C. This allowed the sections to expand and removed any creases. The sections were then floated onto Superfrost slides (Thermo Scientific). The slides were dried for 1 hour on a heating plate (37°C), to promote adhesion of the section onto the glass slides, followed by heating in an oven at 60°C for 1 hour. These slides were then stored in plastic slide boxes at room temperature until staining was required.

5.3   Statistical analysis

To ascertain if the mean values of a measurement on one sample was significantly different from another un-paired sample, the two sample Student's T test was carried out on experimental data which was normally distributed. For experimental data that could not be transformed to exhibit a normal distribution, either due to out-lying data points or if the distributions curve of the data was skewed, the Kruskal-Wallis H test was carried out as a non-parametric equivalent to the student's T test. Data was analysed using Microsoft Excel, GraphPad version 6 and Minitab version 16. All the data was expressed as mean±SEM (standard error of the mean).

5.4 Results

In order to determine if mesenchymal stem cells could be isolated from human adrenal cortex, I performed cell culture using 3 independent adrenal tissues and performed the experiments in triplicates of the same culture condition, using normal human adrenal tissues obtained from nephrectomy for upper pole tumours. For each culture, adrenocortical cells were seeded in a complete medium (CM) and mesenchymal stem cell growth promotion medium (MGPM).

5.4.1 *Morphological characterisation of monolayer adrenocytes in the CM vs MGPM*

The initial cultures of both CM and MGPM were relatively similar, demonstrating a highly heterogenous population of cells, which are sparsely distributed (figure 5.3 & 5.4A). The cultures were predominantly comprised of larger adrenocotical cells, with a polygonal appearance and a lipid core. Within the same culture, a number of smaller cells that had a long, thin and spindle-like shape appearance were observed. There are characteristics of the bone-marrow derived mesenchymal stem cell (MSC) fibroblasts (Friedenstein et al. 1976, Pittenger et al. 1999). With increasing passage numbers, morphological differences were observed between the CM versus MGPM treated adrenal cell populations (figure 5.3 & 5.4E). The control adrenal cells grown in CM continued to exhibit a morphologically heterogenous cell population which were more spread out. On the contrary, the adrenal cells grown in MPGM became more homogenous by demonstrating a uniform population of spindle-shape cells, consistent with MSC-like cell population. These cells were smaller, elongated and aligned in parallel to each other.

The colony forming-unit (CFU) fibroblast-like cells were seen in as early as day 5 and

day 2 of seeding in CM and MGPM respectively. Cells seeded in MGPM exhibited a

higher number of CFUs. Another distinctive feature was the presence of a number of

phase-bright specks (indicated by black open-head arrows) in MGPM that are largely

absent from the complete medium. These are thought to represent mitotic bodies,

indicating increased proliferative ability caused by fibroblast growth factor 2 (FGF2) in

MSC-promoting medium. The differences between cells seeded in CM and MGPM are

summarised in table 5.7.

Table 5.7. Differences between adrenal cortex-derived cells harvested in CM vs. MGPM

Differences	CM	MGPM
Cell population	Heterogeneous	Homogenous
Morphology	Large polygonal	Elongated spindle-shape
Lipid core/secretion	Yes	None of first passage
Number of CFUs/T-75 flask (prior first passage)	2-3	8-10
Presence of mitotic bodies	Few	Abundant
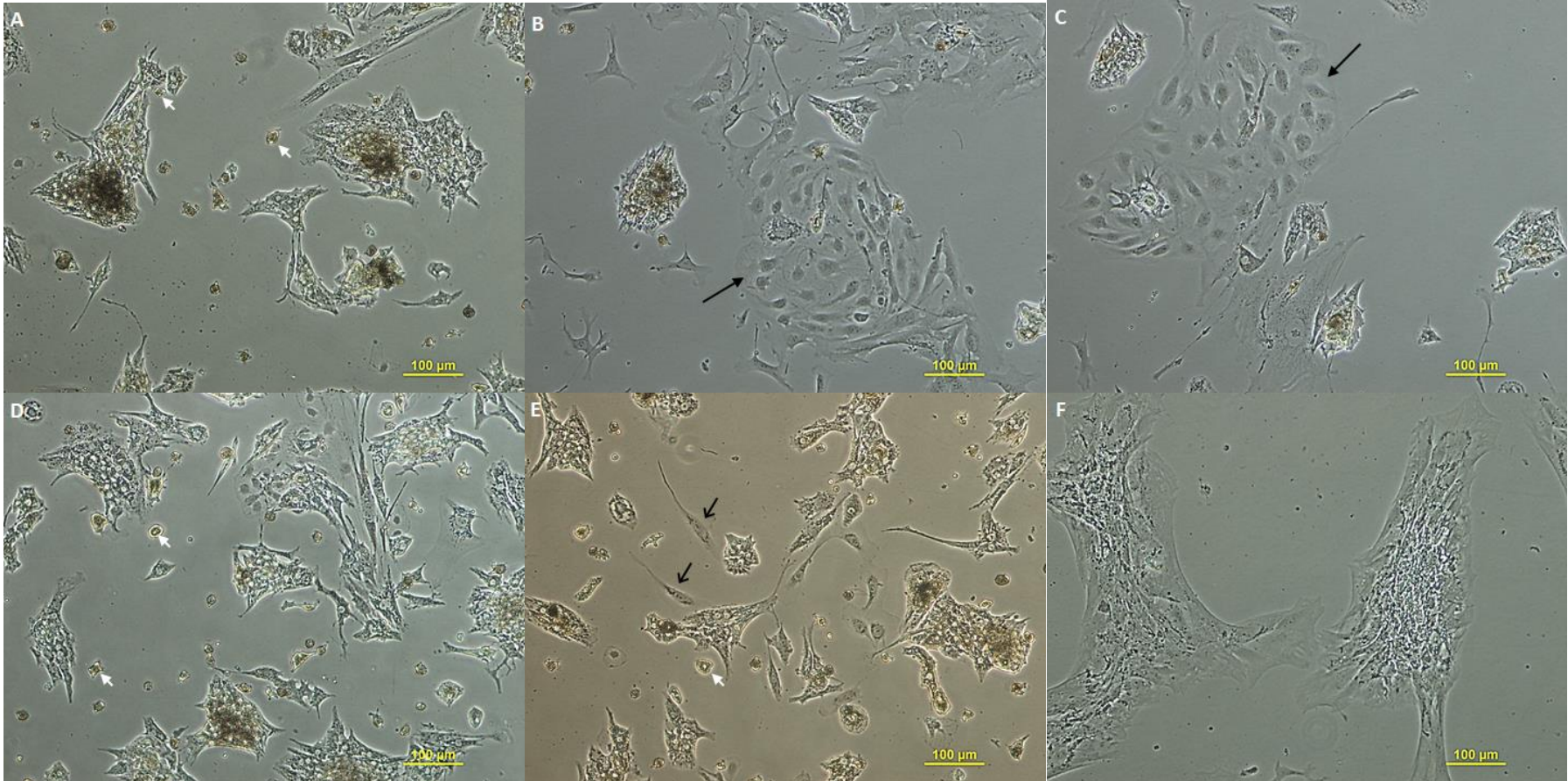
Figure 5.3. Morphological characterisation of monolayer adrenocortical cells in complete growth medium (CM).

Human adrenal cells isolated from patients undergoing nephrectomy for upper pole tumours were expanded by plastic adherence in T-75 flasks. Representative images were taken of culture at day 3(A), day 5(B), day 7(C), day 10(D), passage 1 (E), passage 3(F). Most of the cells are comprised of adrenocortical cells that are larger with polygonal appearance and light refractive lipid (steroid) core. Black closed-head arrows indicate colony-forming units with fibroblast-like cells (B&C). White closed-head arrow show lipid fragments protruded or elongated from steroid-producing adrenocortical cells (A,D&E). Black open-head arrows indicate long, thin, spindle-shape fibroblast-like cells (E). (Magnification x100)
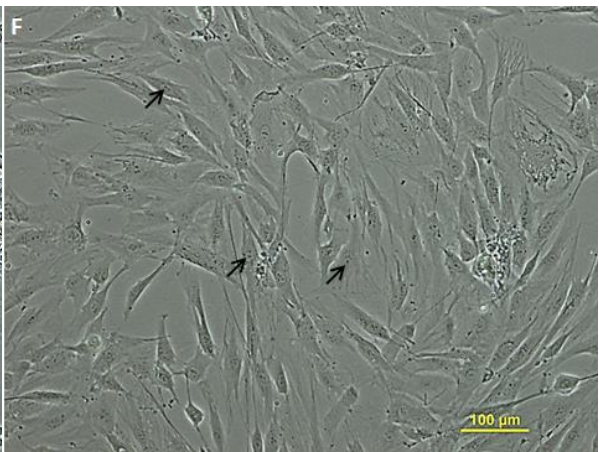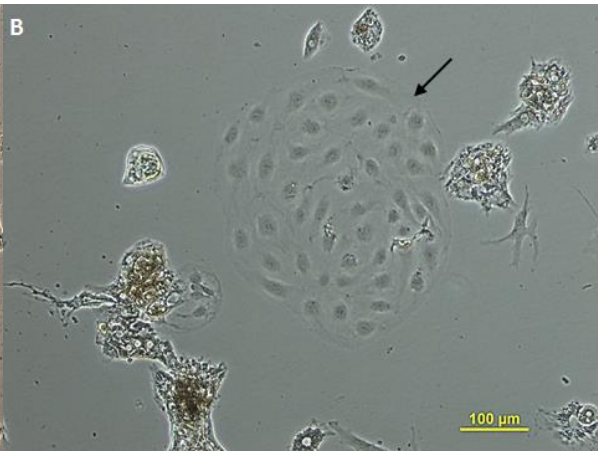
Figure 5.4. Morphological characterisation of monolayer adrenal cells in mesenchymal stem cell growth promotion medium (MGPM)

Human adrenal cells isolated from patients undergoing nephrectomy for upper pole tumour were expanded by plastic adherence. Representative images were taken of culture on day 2(A), day 3(B), day 4(C), day 5(D), day 6(E) and passage 1(F). Cells on day 2 largely comprised of adrenocortical cells that were larger with polygonal appearance and light refractive lipid (steroid) core, similar to those seeded in CM. However, cells seeded in MGPM were gradually replaced by spindle-shape fibroblast-like cells. Black closed-head arrows indicate colony-forming unit fibroblast-like cells (A&B). White closed-head arrow show lipid fragments protruded or elongated from steroid-producing adrenocortical cells (A). Black open-head arrows indicate the presence of phase-bright mitotic bodies (F). (Magnification x100)

5.4.2 *The proliferative capacity of monolayer adrenal cells in CM vs MPGM*

Adrenal cortex-derived cells isolated and expanded in MSC promoting media were noted to have an increased proliferative capacity compared to those cultured in normal media (figure 5.5). The doubling time for cells cultured in MGPM and CM was 38 and 69 hours respectively. The adrenal cells harvested and maintained in MGPM had been maintained for up to 10 passages without changing their morphology and might be capable of long-term culture. In contrast, adrenal cells cultured in CM showed replicative senescence following passage 3, as suggested by growth arrest despite renewal of the culture medium and sub-culturing. For adrenal cells maintained in MGPM but switched to CM at passage 5 and 7, replicative senescence was observed in the same passage when the medium was changed. The cells in CM were grown to confluence approximately once every 10-14 days whereas cells in MGPM had reached confluence by day 5. Adrenal cells cultured in both CM and MGPM achieved more than 90% viability upon each passage of cell culture, as determined by the trypan blue dye.
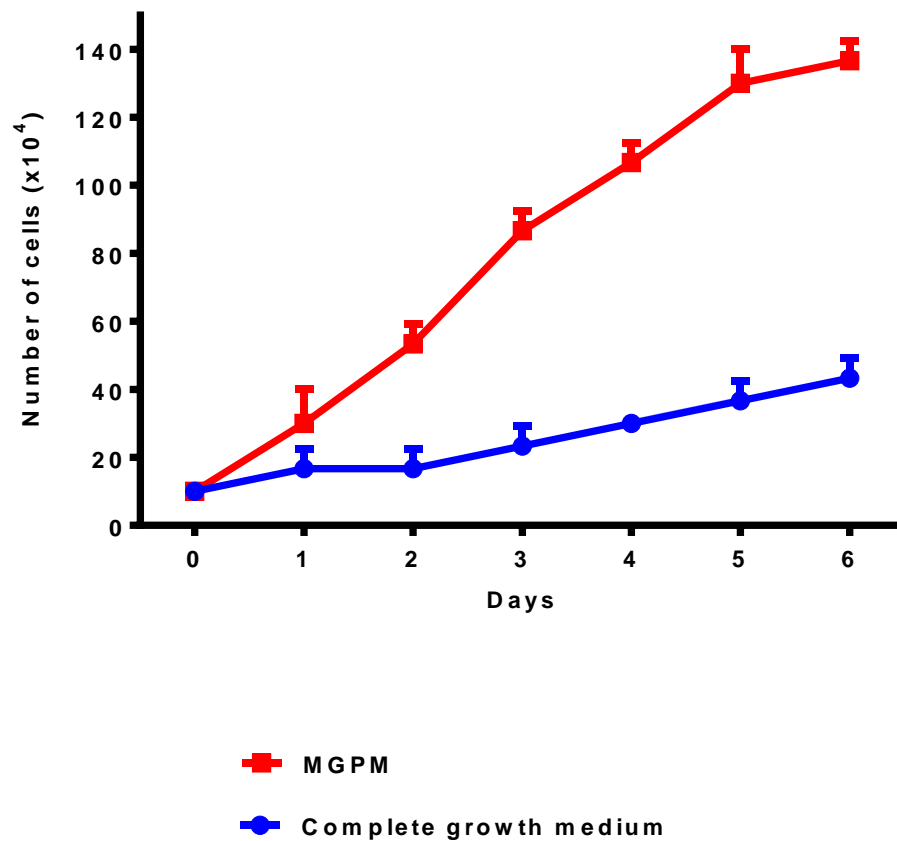
Figure 5.5. Comparison proliferation rate in MGPM vs. CM

Adrenal cortex-derived from passage 1 showed a higher growth rate in medium
with mesenchymal stem cell growth promotion medium (MGPM) as compared to
the proliferation rate observed in complete growth medium (CM). The values are
expressed as means $\pm$ SD of 3 independent measurements.

5.4.3 *In-vitro differentiation capacity of adrenal cortex-derived MSCs-like cells*

I evaluated the mesenchymal lineage differentiation potential of adrenal cortex-derived cells, which is the hallmark of MSCs. Cells from passage 2 (CM) and passage 3 (MGPM) were cultured under conditions favourable for osteogenic, adipogenic and chondrogenic differentiation, as detailed in the method section.

Osteogenic differentiation was induced in cells cultured in both CM and MGPM although the staining for Alizarin red S was significantly weaker in cells harvested in CM (figure 5.6). In contrast, cells harvested from CM failed to survive both chondrogenic and adipogenic medium.

For adrenal cells cultured in MGPM, morphologic changes consistent with adipogenesis were observed as early as day-14 (figure 5.7A). Maximal lipid accumulation was seen in week-3 with lipid droplets being observed floating in the medium prior to staining. The lipid vacuoles were visualised with Oil-red O staining (figure 5.7B). Adrenal cells harvested in MGPM managed to form cell pellets but no staining was detected with safranin O suggesting lack of glycosaminoglycans (GAG) formation (figure 5.8).
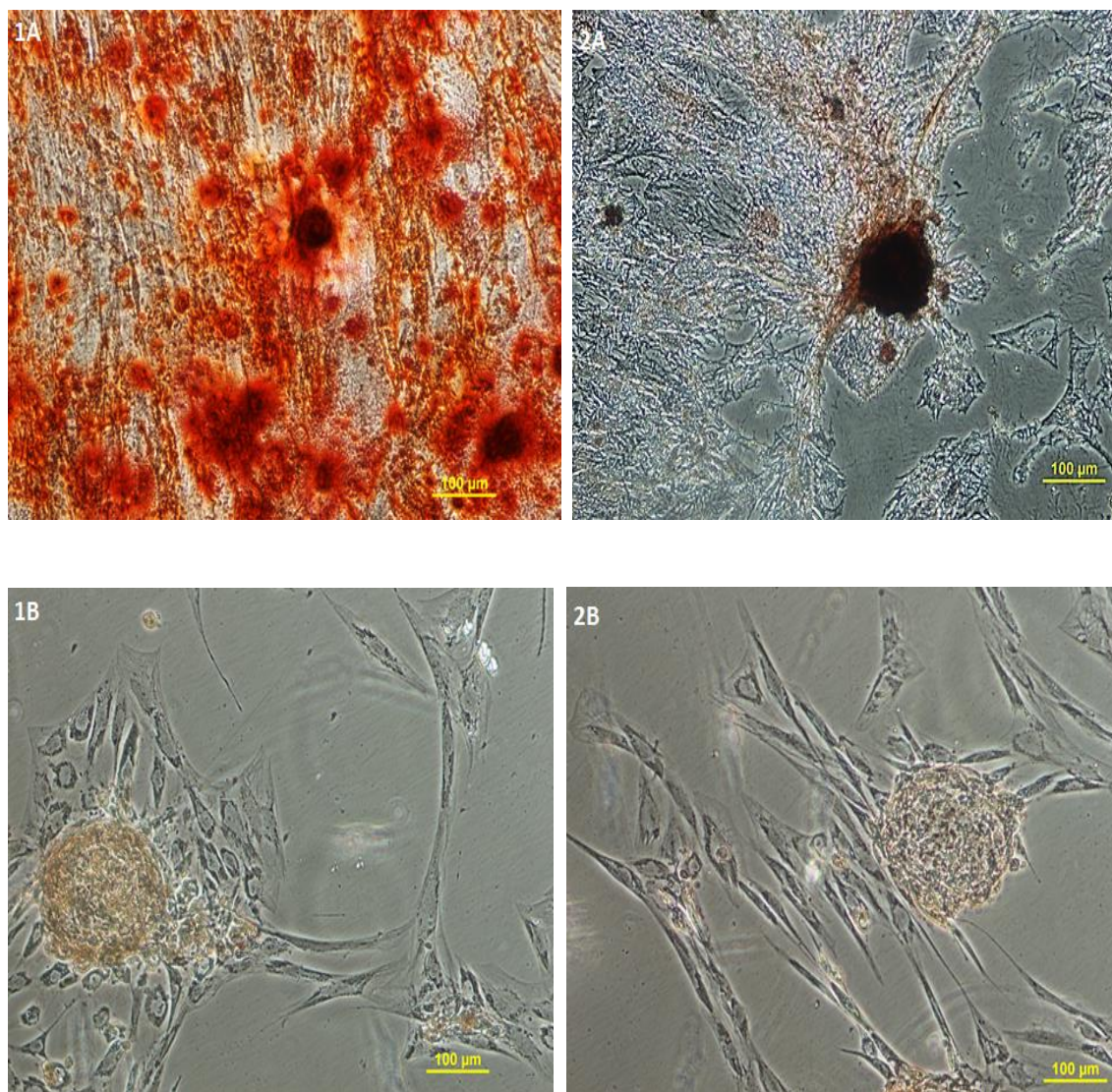
Figure 5.6. Osteogenic differentiation capacity of adrenal cortex-derived cells

Adrenal cells cultured in MGPM (1) and CM (2) were exposed to osteogenic differentiation medium. Cells from both media were shown to differentiate into osteogenic lineage at day-21 with enhancement of alkaline phosphatase activity as shown with the positive staining for Alizarin S (A). Figure B showed negative staining for non-induced controls. (Magnification x100)
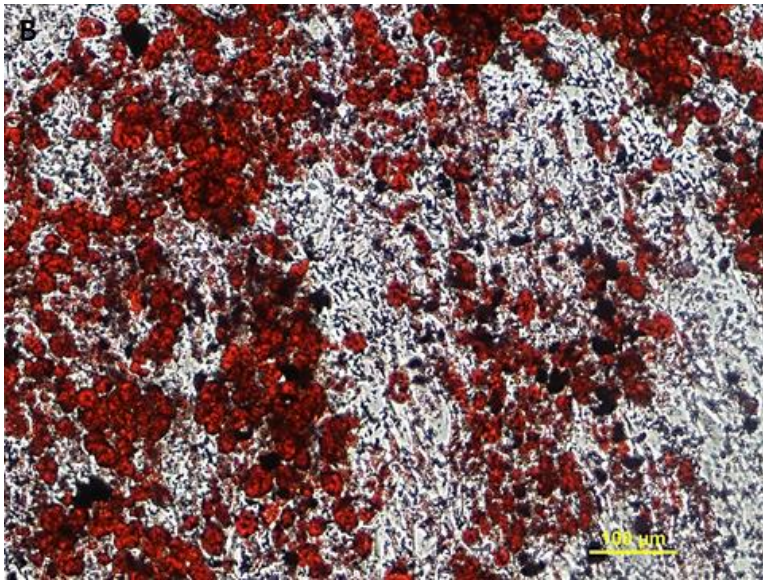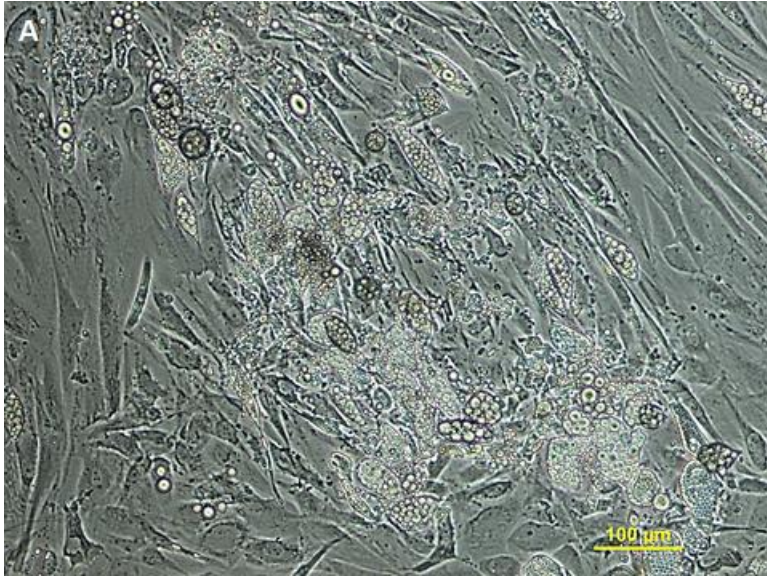
Figure 5.7. Adipogenic differentiation capacity of adrenal cortex-derived cells

Cells cultured in MGPM were exposed to adipogenic differentiation medium. Cells from MPGM were induced into adipogenic lineage, as shown by the cellular accumulation of lipid-rich vacuoles (A), which stained with Oil-red O (B). Figure C showed negative staining for non-induced controls (Magnification x100)
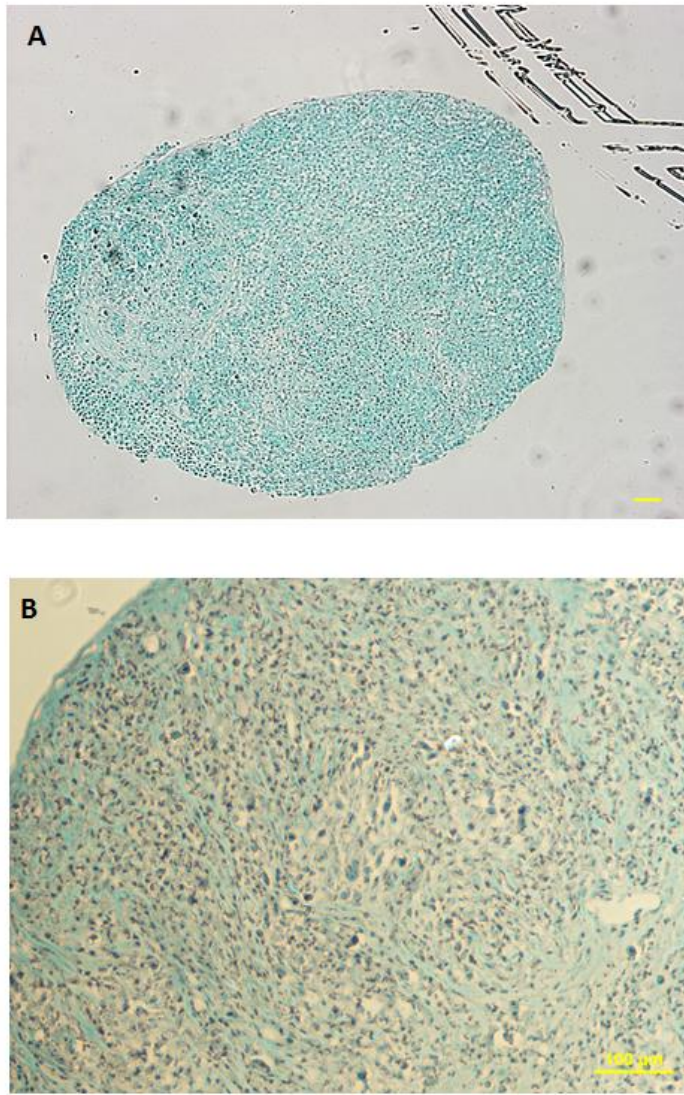
Figure 5.8. Chondrogenic differentiation capacity

Adrenal cells cultured in MGPM were processed into cell pellets and exposed to chondrogenic differentiation medium for 21 days. This figure showed 50x (A) and 100x (B) magnification of cell pellet following safranin O staining. The pellet had negative staining signifying a lack of GAG accumulation within the cell region. Adrenal cortex-derived cells cultured in either CM or MGPM were lacking in chondrogenic differentiation capacity under these conditions.

5.4.4 Flowcytometric analysis

I performed immunophenotyping using flow cytometer to characterise the antigens expressed by adrenal-cortex derived cells. The cell surface and intracellular marker evaluations were carried out at various stages of cultures. Adrenal cortex-derived cells are heterogeneous in their composition and hence no cell subset can be clearly discriminated on light scatter grounds. Nevertheless, adrenal cells harvested from MGPM have low side angle scatter (low cytoplasmic granularity) and forward angle scatter (smaller cell size) (figure 5.9). Neither the cells from CM or MGPM were positive for haematopoietic (CD45) or lymphocytic markers (CD19). Similar to the mesenchymal stromal cells (MSC) isolated from knee aspirate, which I used as a positive control, adrenal cortex-derived cells were strongly positive for MSC markers-CD44, CD90, CD105 and CD166. All these cells were also positive for MHC class I but negative for MHC class II markers (figure 5.9). The average percentage of cells positive for MSC markers was significantly less among those harvested from CM, in comparison to those from MGPM (CM vs MGPM (mean± S.D: 57.7±24.6% vs. 83.9± 14.5% ; p=0.006). The frequency or percentage of cells positive for each MSC marker cultured in CM and MGPM is summarised in table 5.8.

In addition, it was noted that the intensity of MSC marker expression was higher among cells from MGPM compared to those from CM, although this difference was not statistically significant (fluorescence intensity for CM vs. MGPM: 2639±611 vs.3194± 902; NS). When the cells harvested in MGPM were cultured in CM following the 5th and 7th passage, there was a reduction in the intensity of MSC surface marker expression (figure 5.10), but this was not statistically significant. The average frequency of cells positive for MSC markers was also decreased (90.5± 3.11% vs 78.0±9.62%, p=0.255,NS). There was no significant difference observed with respect to the intensity

of antigen expression or frequency of MSC marker-positive cells, between the various passages for both CM and MGPM-cultured cells.

Immunophenotyping using DAX1 and GLI1 intracellular markers was also carried out (figure 5.11). A significantly higher percentage of adrenal cortex-derived cells cultured in MGPM expressed GLI1 marker, compared to those harvested from CM (MGPM: 92.7±2.75% vs. CM:22.8±2.7; p=0.035). Cells from MGPM also showed stronger signals towards the GLI antibody, although this was not statistically significant (figure 5.12). GLI expression was significantly decreased following incubation with a synthetic adrenocorticotropic hormone ($ACTH_{1-24}$; synacthen) among MGPM-cultured cells (antigen expression: 31.6± 0.8%; P=0.03). A similar observation was noted when cells changed from MGPM to CM at passages 5 and 7 (antigen expression: 37.3± 9.8%; NS). Interestingly, DAX1 was highly expressed in cells cultured in MGPM but negligible in those harvested from CM, as the count was similar to those from controls (CM:3.95 ±1.05 vs MGPM: 94.9± 0.7% ; p=0.009). Double-immunnostaining of adrenal cells also revealed that MSC markers co-localized with GLI and DAX1staining (figure 5.13 & 5.14)

I attempted to study the expression of SHH and SF1 in adrenal cells using flow cytometry but failed. The anti-SHH and anti-SF1 antibodies purchased were probably not suitable for flow cytometry. Nevertheless, these antibodies showed good results in immunocytochemistry, as depicted in the next section.

Table 5.8. Immunophenotyping of MSC markers

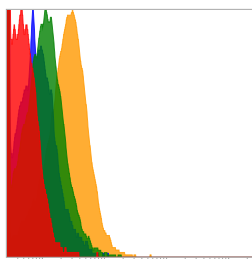| MSC markers	Percentage of positive cells (%+SEM)	
	MGPM (first passage)	CM (first passage)
CD44	89.7 ± 2.52	61.2 ±7.34
CD90	80.4 ± 14.4	44.6± 14.6
CD105	91.6 ± 0.9	83.7 ±.1.65
CD166	73.9 ± 8.12	41.4± 16.8

The differences between the frequency of positivity towards each MSC markers, cultured in CM and MGPM respectively, were non-significant (p>0.05).
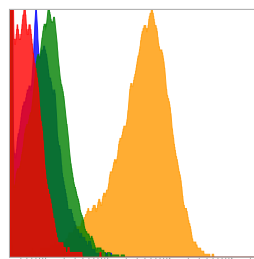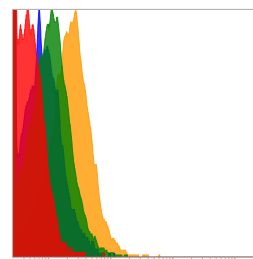
A

MPGM medium-Control

B

CM first passage-Control
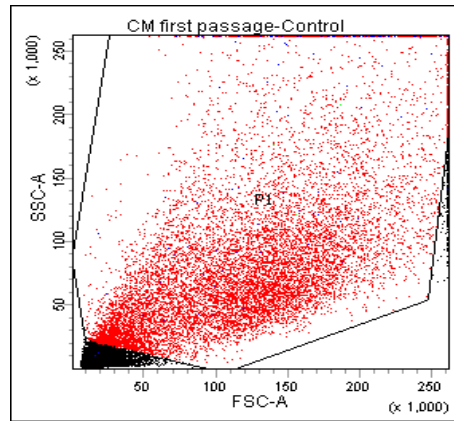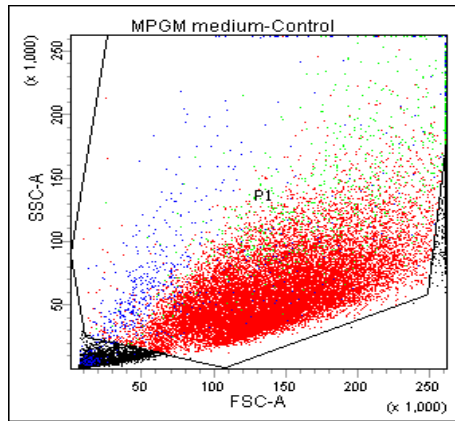
CD19

2-488/530/30-A

CD44

2-488/530/30-A

CD45

2-488/530/30-A

CD90
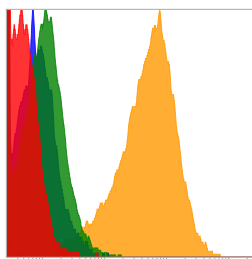
2-488/530/30-A

CD105
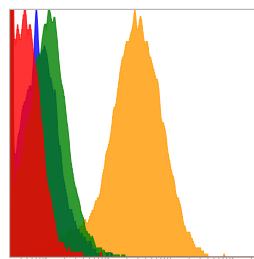
2-488/530/30-A

CD166
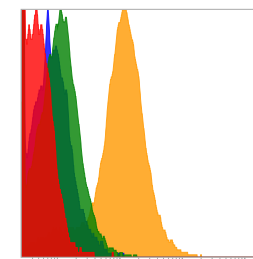
2-488/530/30-A

MHC Class I and II

HLA-DR

HLA-ABC
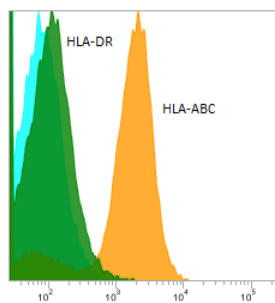
2-488/530/30-A

Figure 5.9. Representative MSC immunophenotype profile of adrenal cortex-derived cells cultured in CM and MGPM.

Graph A&B represent the scatter plot (side scatter vs. forward scatter FSC/SCC) for cells from MGPM and CM respectively. FSC/SCC represents the distribution of cells in the light scatter based on size and intracellular composition. More cells harvested from MGPM seem to have lower side angle scatter (low cytoplasmic granularity) and forward angle scatter (smaller cell size). The SSC/FCS gating of the total adrenal cells analysed was to exclude non-viable elements and debris.

The panel of histograms represent MSC immunophenotypes of adrenal cells. The adrenocortical cells displayed strong signals with CD44, CD90, CD105 and CD166 markers (as demonstrated by a shift to a higher fluorescent intensity from controls), but negative for haematopoitic and lymphocytic markers (CD19 and CD45). X-axes display signal (fluorescent) intensity and y-axes represent cell count.

(Red histogram: unstained cells; blue histogram: negative control with fluorochrome conjugated secondary antibodies only; green histogram: species-specific isotype contro; yellow histogram: The staining of cells with relevant antibody). The colour codes are applicable to all histograms except graph on MHC class I & II, where blue histogram indicates negative control, yellow and green histograms represent MHC class I (HLA-ABC) and MHC class II (HLA-DR), respectively.
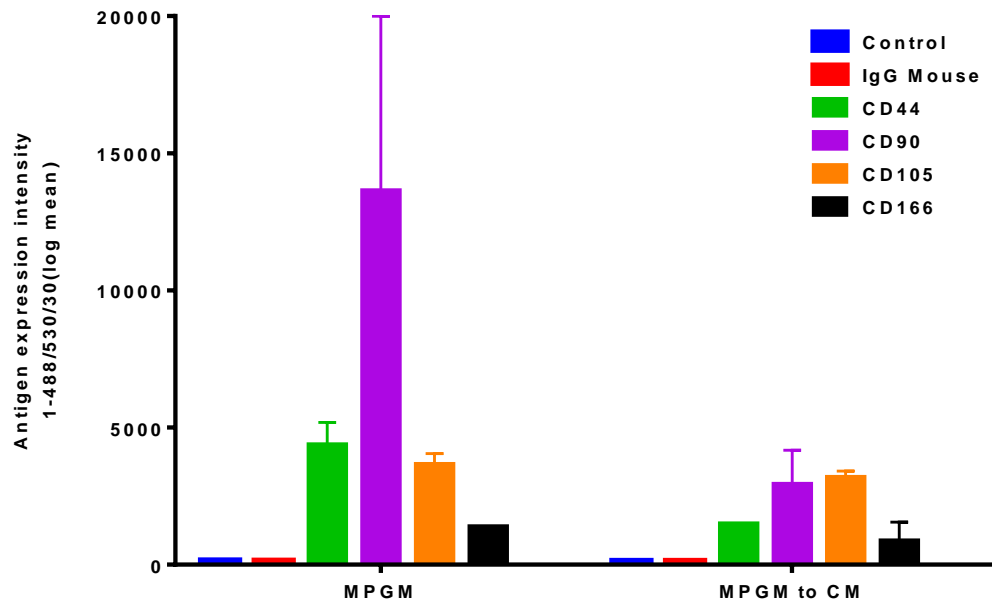
Figure 5.10. Effects of CM on MGPM-cultured cells with respect to MSC marker expression

The bar chart demonstrates reduced MSC marker expression among adrenal cells when they were switched from MGPM to CM (at passage 5 and 7). Y axes represent the signal intensity (mean with SEM) (All differences were statistically non-significant, $p > 0.05$).
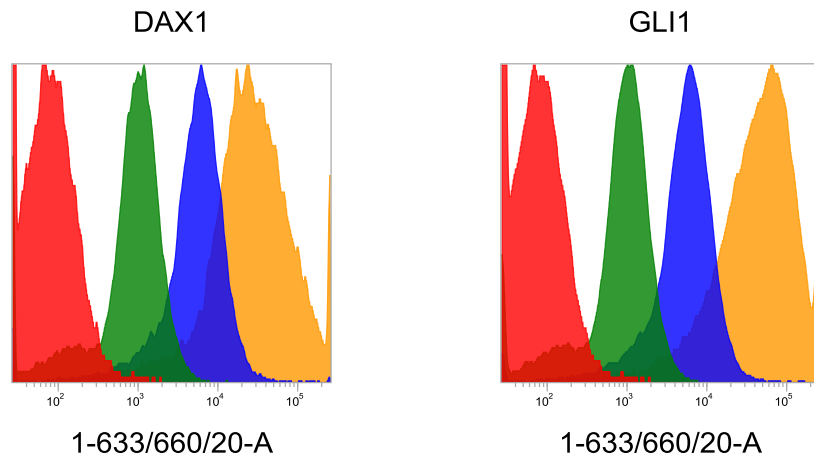
DAX1   **GLI1**

1-633/660/20-A   1-633/660/20-A

Figure 5.11. Representative FACS analyses of DAX1 and GLI1 immunophenotype of adrenal cortex-derived cells cultured in MGPM

The cells displayed positive signals with DAX1and GLI. The X-axes display signal intensity. (Red histogram: unstained cells; green histogram: negative control with fluorochrome conjugated secondary antibodies only; blue histogram: species-specific isotype control; yellow histogram: The staining of MGPM-cultured cells with relevant antibody)
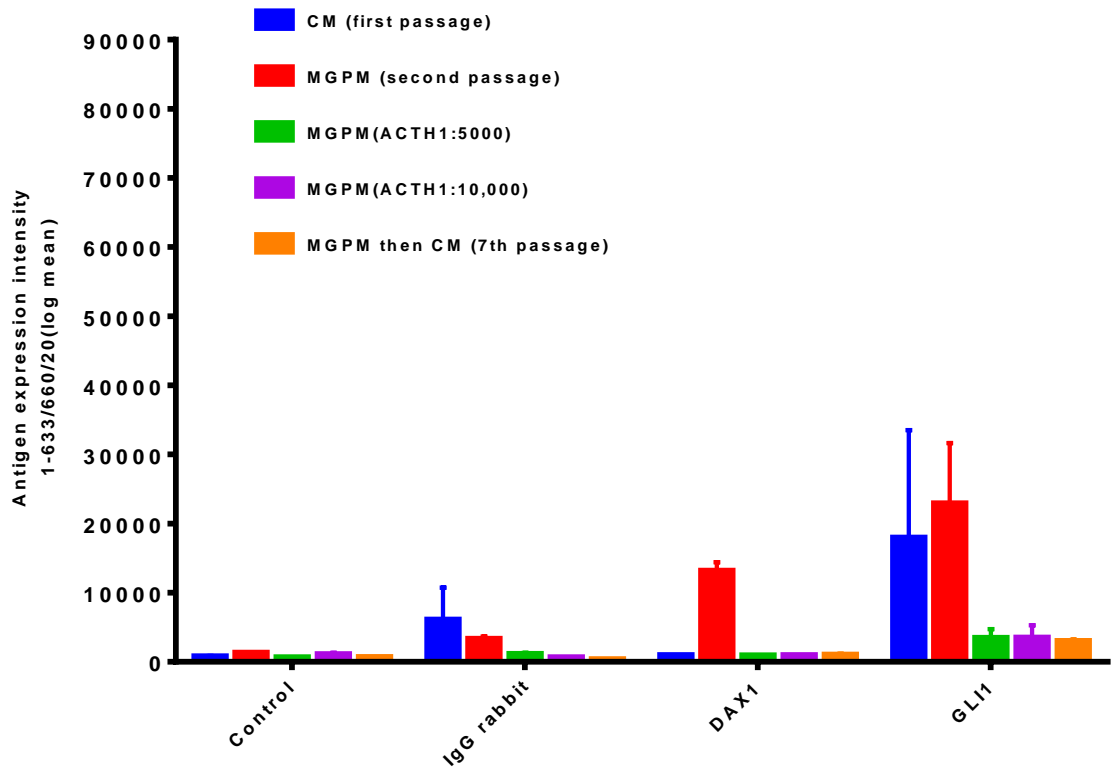
Figure 5.12. depicting the fluorescent intensity of GLI1 and DAX2 expression among adrenal cortex-derived cells.

GLI was richly expressed both in MGPM and CM, without significant difference (p=0.83). DAX1 signal was only expressed in cells from MGPM (P=0.08). GLI1 expression was reduced following incubation with ACTH at either 0.05µg/ml or 250ng/ml concentration (NS).
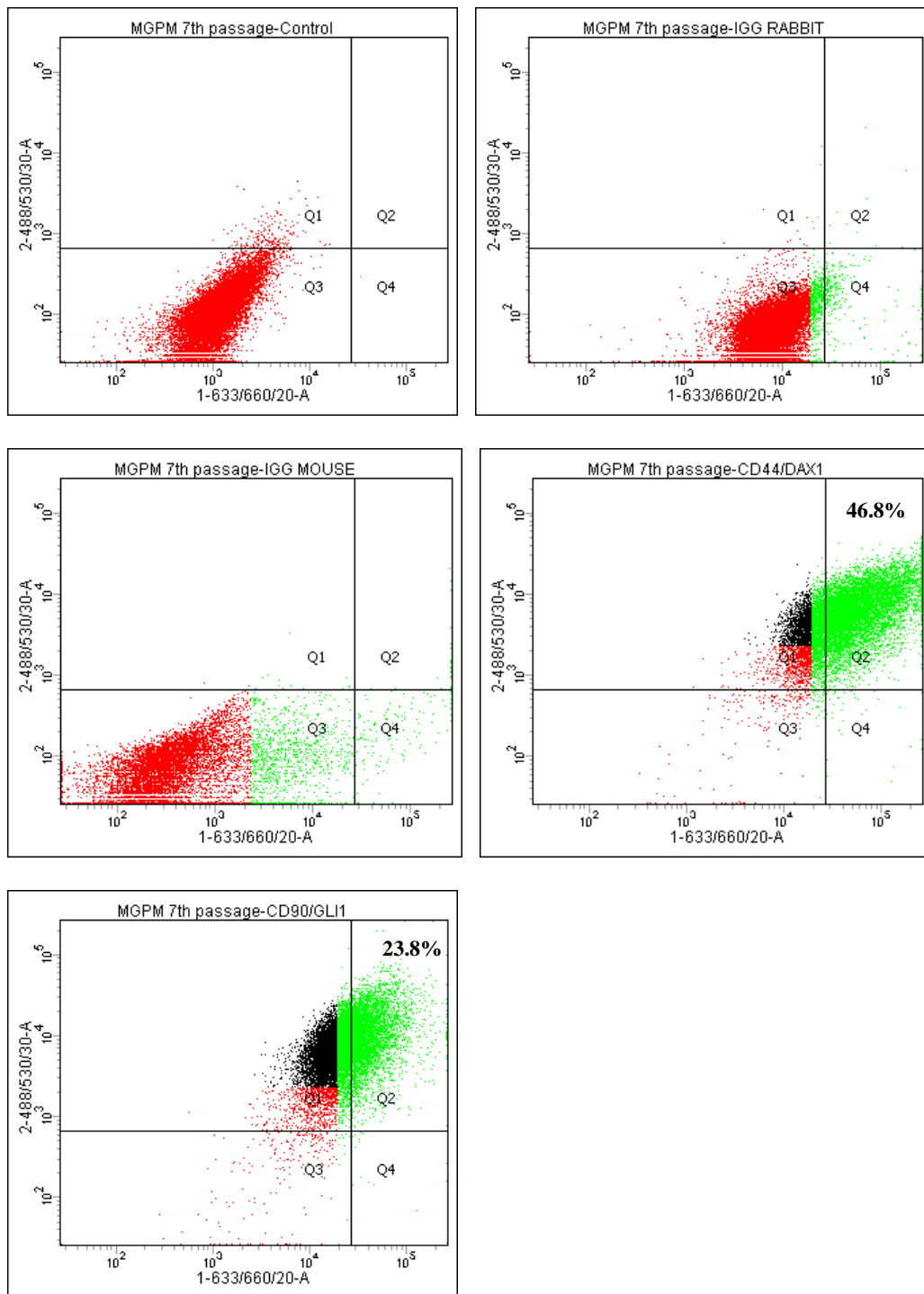
Figure 5.13. Dot plot for cells from 7th passage in MGPM, following dual staining with MSC (FITC labelled- 488/530/30) and DAX1/GLI markers (APC-633/660/20) as indicated.

Positivity with dual-staining was indicated by the Q2 region, in which >98% of the control and species-specific isotype controls fall outside this area. Approximately 46.8% of the cells co-expressed CD44 and DAX1, whereas 23.8% of cells co-expressed CD90 and GLI1.
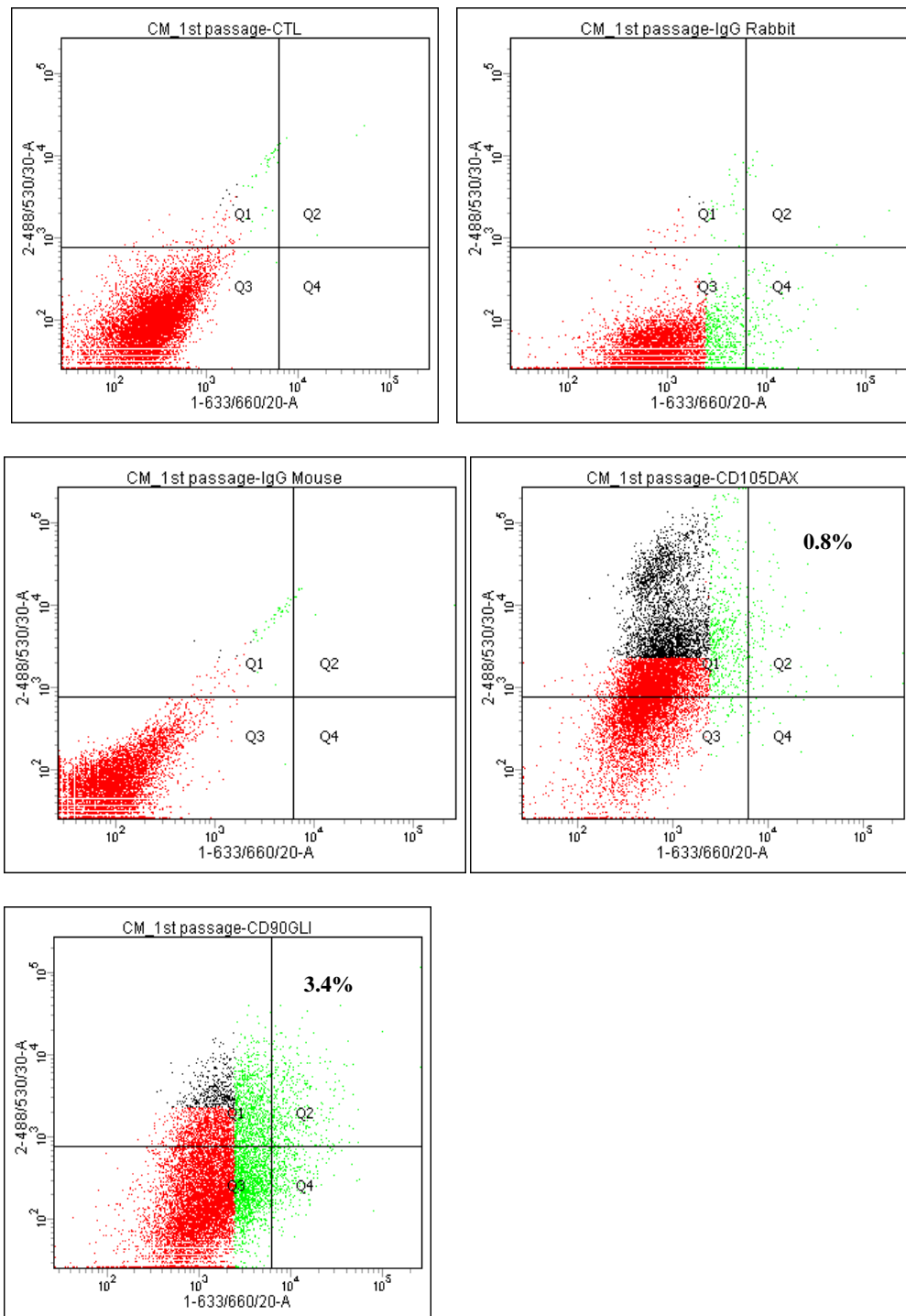
Figure 5.14. Dot plot for cells from first passage in CM, following dual staining with MSC (FITC labelled- 488/530/30) and DAX1/GLI1 markers (APC-633/660/20) as indicated.

Positivity with dual-staining was indicated by the Q2 region, in which >98% of the control and species-specific isotype controls fall outside this area. Approximately 1.3% of the cells co-expressed CD105 and DAX1, whereas 4.1% of cells co-expressed CD90 and GLI1.

5.4.5 *Immunocytochemistry study*

In order to verify the immunophenotyping results from FACS, I performed immunocytochemistry studies on cultured cells harvested from CM or MGPM. Adrenal cortex-derived cells cultured in either medium showed strong membranous staining towards CD44, CD90, CD105 and CD166 markers but negative for CD19 and CD45 antigens, as shown in figures 5.15 to 5.18. This was consistent with the results generated from FACS. Nevertheless, cells harvested from CM lost their positive staining towards CD 105/CD166 with only weak CD44/CD90 staining following second and third passages. In contrast, adrenal cells cultured in MGPM showed persistent positive staining towards all MSC markers used in this study despite repeated passaging.

I then examined the presence of adrenal/gonadal-specific markers (SF1, DAX1) and morphogenic signalling antigens (SHH and GLI1), among the cultured adrenal cells. Adrenal cells harvested from CM showed strong nucleus staining towards SF1 and GLI1 markers, with negative DAX1 and SHH staining (figure 5.20 to 5.22). In contrast, DAX1 and SHH were expressed among MGPM-cultured cells, as demonstrated by positive nucleus and cytoplasmic staining, respectively (figure 5.21 & 5.22). Strong GLI1 and SF1 staining was also observed among cells harvested from MGPM (figure 5.20 & 5.22). GLI1 was observed to be more widely expressed among the cell population from MGPM compared to those from CM.

Double-immunostaining revealed that MSC markers co-localised with SF1, SHH and GLI1 antigens (figure 5.23 and 5.24). However, I failed to demonstrate co-expression of DAX1 and MSC markers in either CM or MGPM- cultured cells.
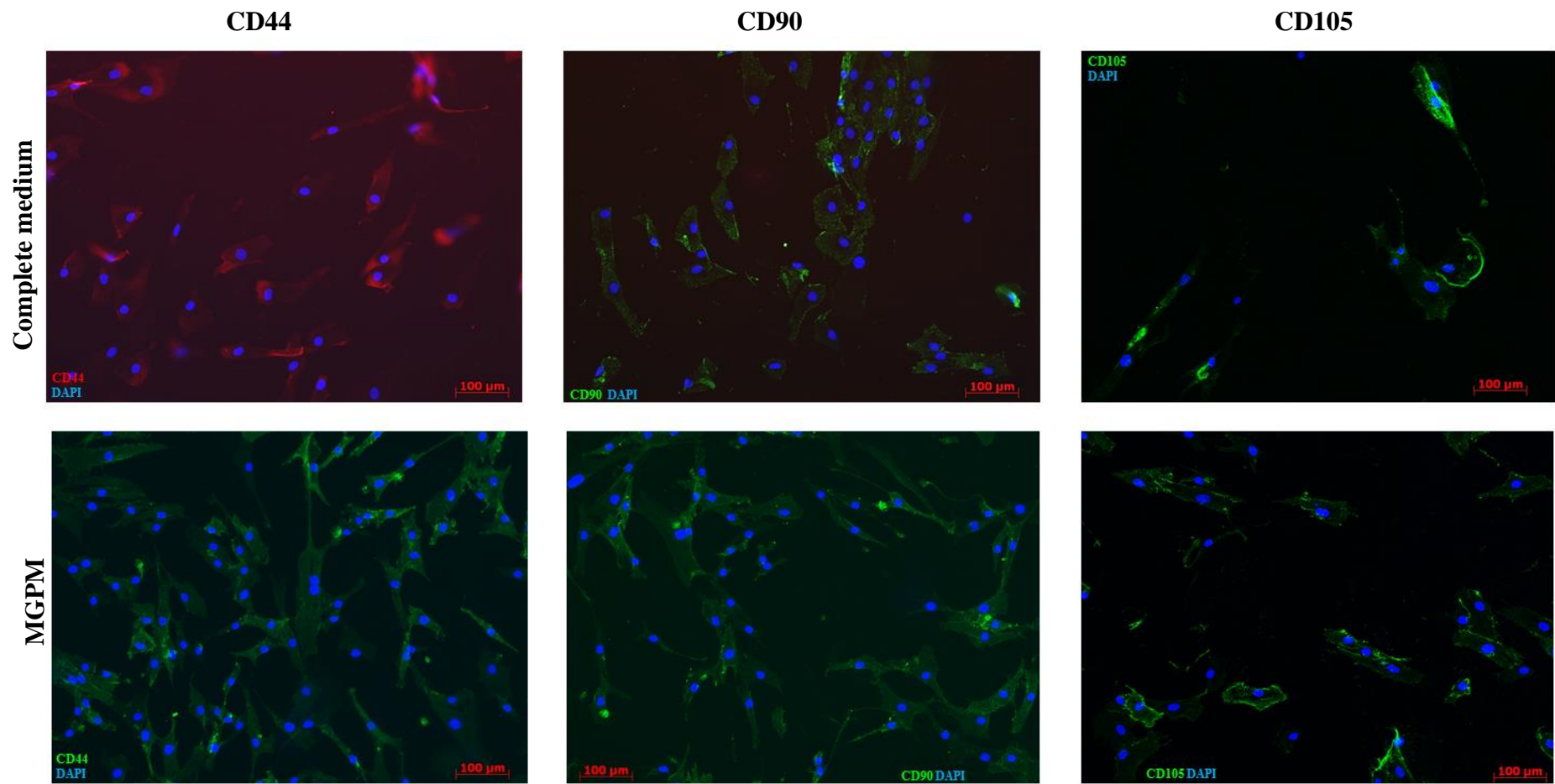
	CD44	CD90	CD105

Complete medium

MGPM

Figure 5.15. Immunolabelling of adrenal cells for MSC surface markers

Cells were cultured and maintained in CM and MGPM. Immunolabelling of MSC surface markers were carried out after passage 1 (day 10 in CM) and passage 3 (day 18 in MGPM) respectively. Negative controls were carried out in parallel to each immunolabelling reaction. The details of negative controls are depicted in figure 5.19. Cells from CM were larger and more sparsely distributed, compared to the smaller and more elongated cells from MGPM. Cells from both mediums stained positive for CD44, CD90, CD105 (Magnification x100). Pictures with higher magnification are depicted in figure 5.17 and figure 5.18.
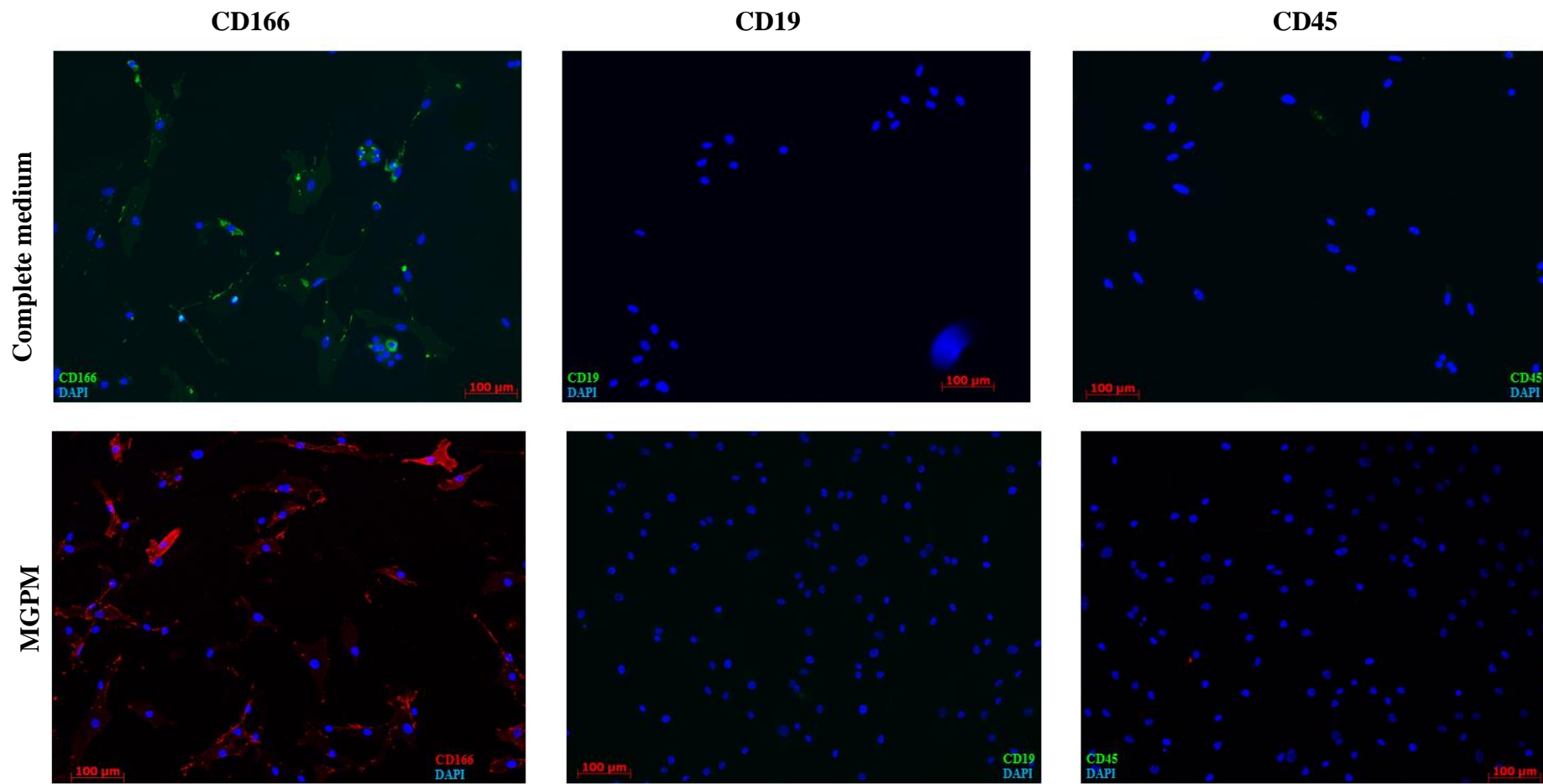
	CD166	CD19	CD45

Complete medium

MGPM

Figure 5.16. Immunolabelling of adrenal cells for MSC surface markers

Cells were cultured and maintained in CM and MGPM. Immunolabelling of MSC surface markers were carried out after passage 1 (day 10 in CM) and passage 3 (day 18 in MGPM) respectively. Cells from both mediums showed positive staining for CD166 but negative staining for CD19 and CD45 markers (Magnification x100). Pictures with higher magnification are depicted in figure 5.16.
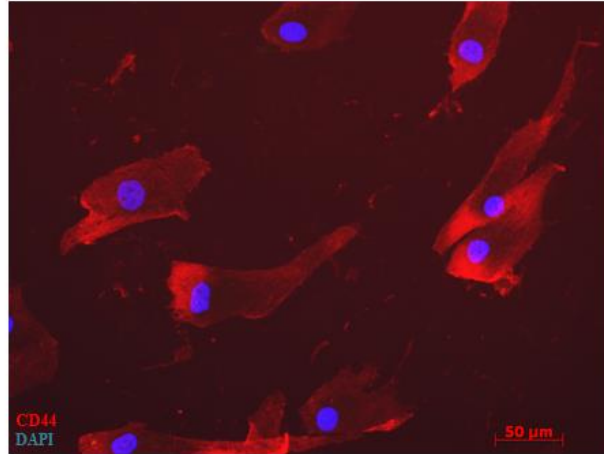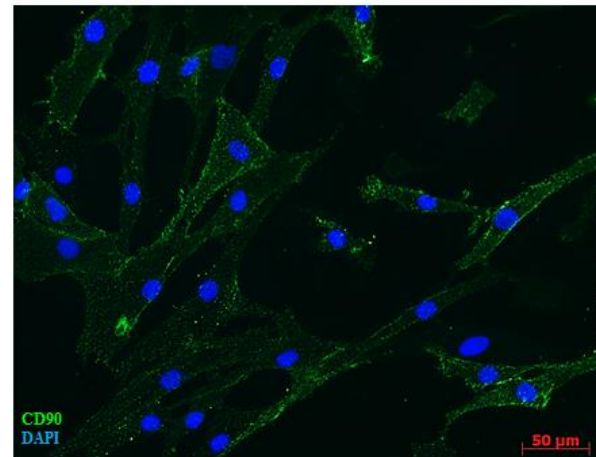
Complete medium (CM)　　　　　　　　　　　　　**MGPM**
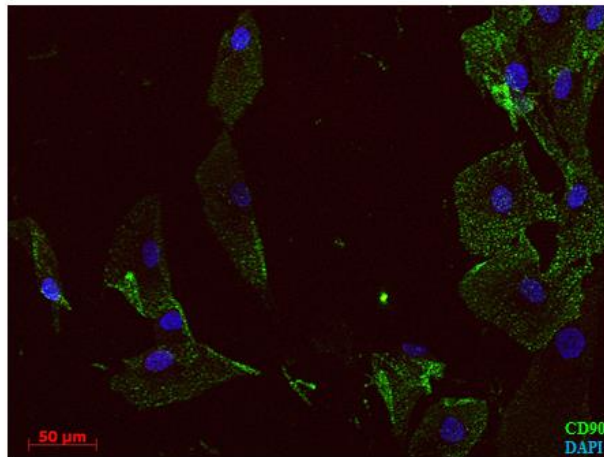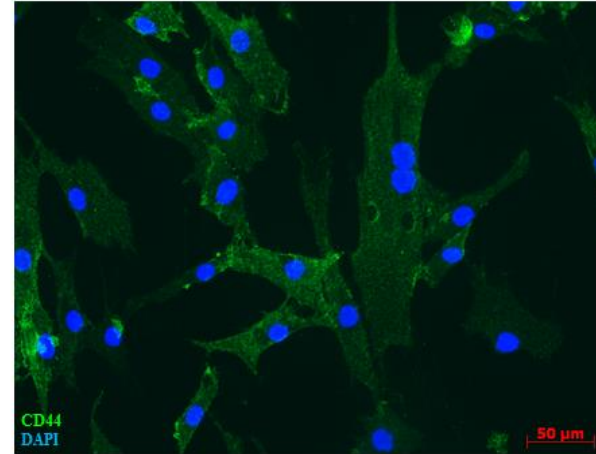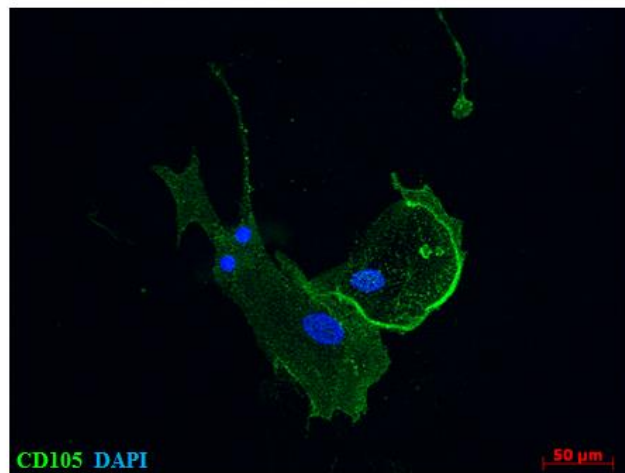
Figure 5.17. Immunolabelling of adrenal cells for MSC markers (CD44 & CD90), visualised at higher magnification, x200.
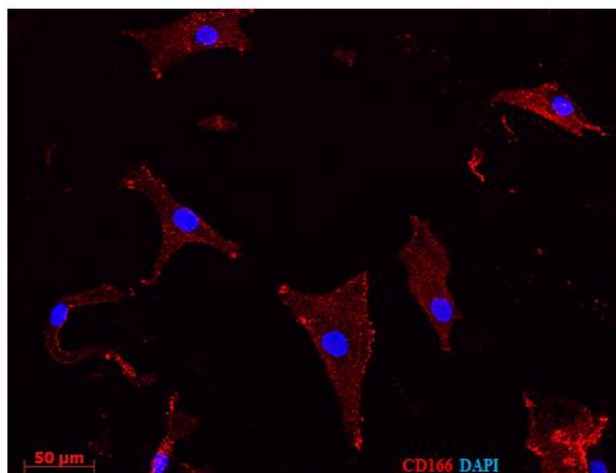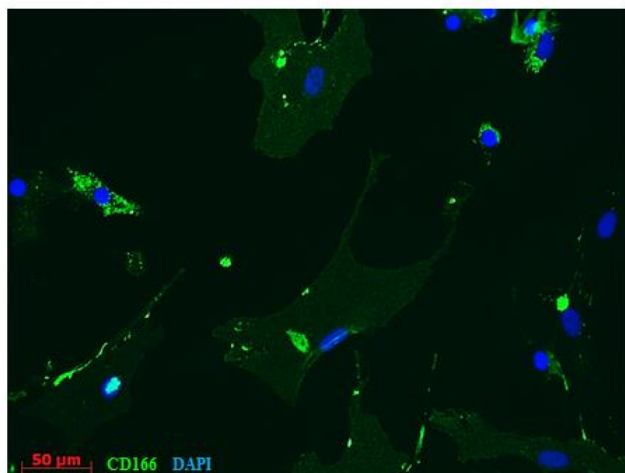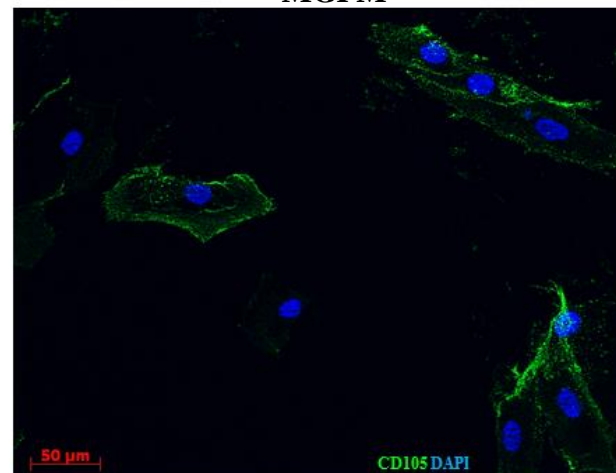
Figure 5.18. Immunophenotyping of adrenal cells for MSC markers (CD105,CD166), visualised at higher magnification, x200.
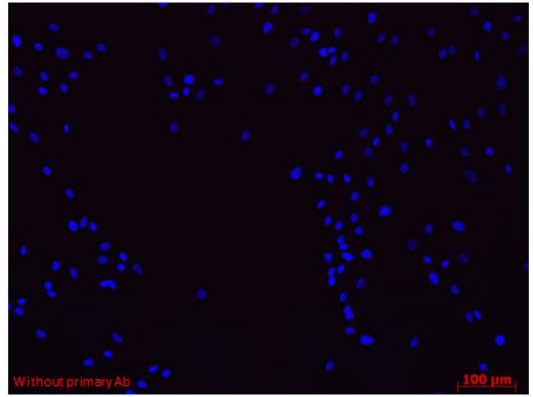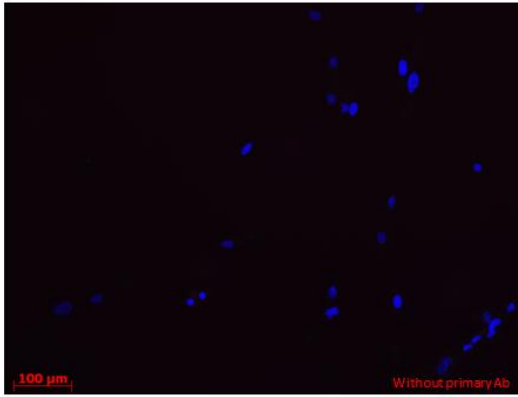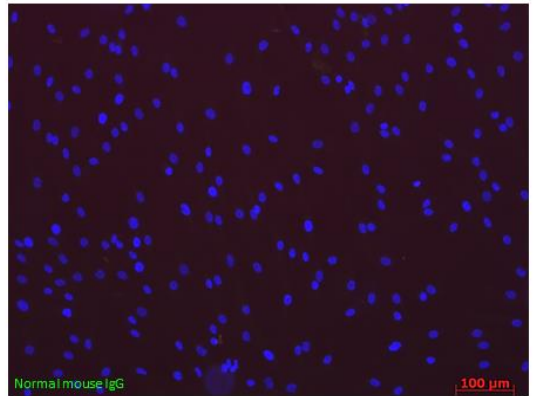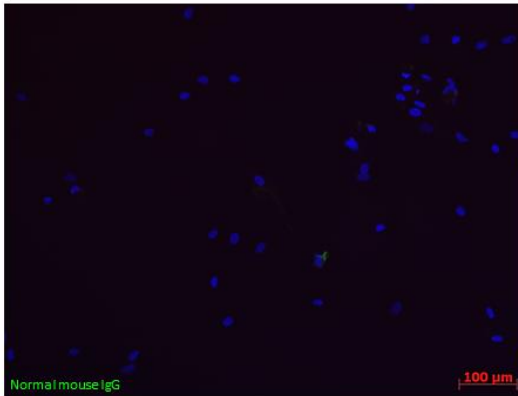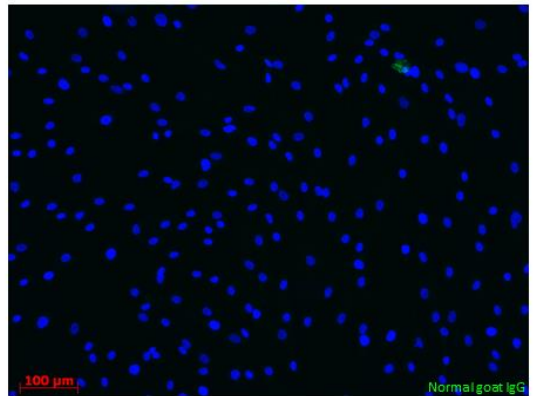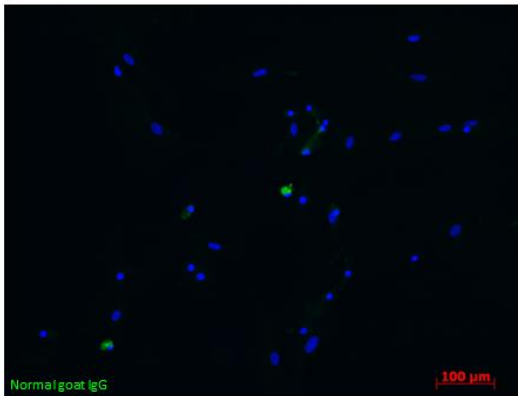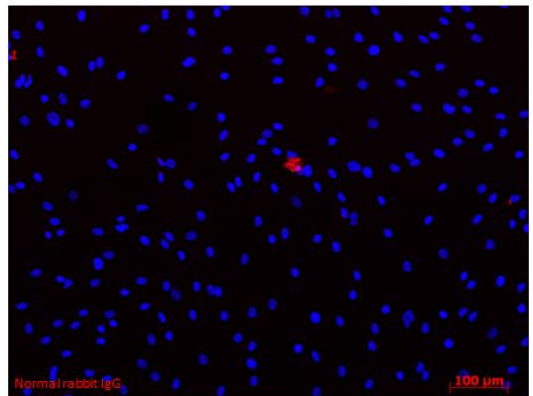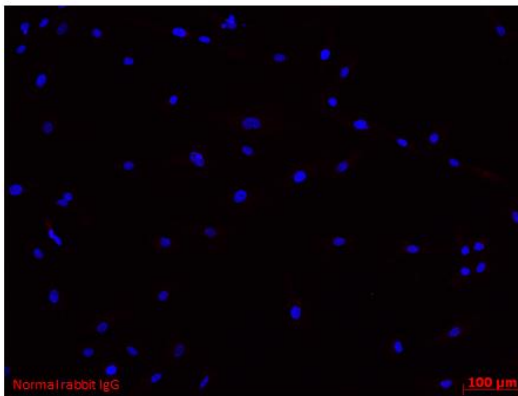
Figure 5.19. A representation of the negative controls used in this immunofluorescence study.

The top panel represents incubation with the second antibody without the primary antibody. To confirm the specificity of each antibody used in the immunochemical reactions, control immunolabelling reactions were carried out in parallel using species-specific pre-immune antibodies with the appropriate secondary antibodies, derived from mice, rabbits and goats (Santa Cruz Biotechnology). All isotype IgG controls were used in the same protein concentration as the relevant primary antibodies.

CM (SF1)   **MGPM (SF1)**

FITC

Merged
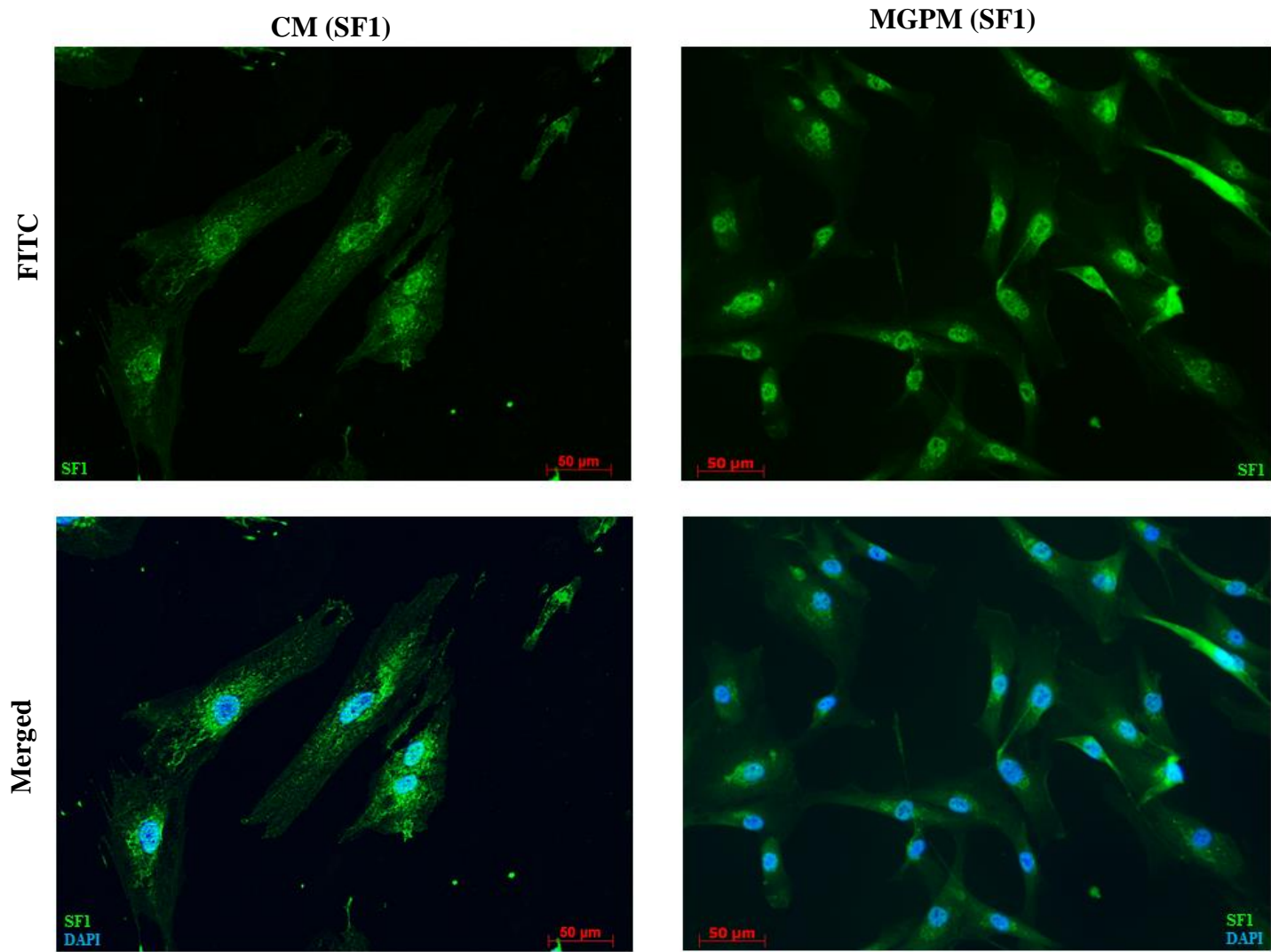
Figure 5.20. Immunolabelling of intracellular markers SF1

Immunolabelling of intracellular markers SF1 (Santa Cruz Biotechnology) were carried out after passage 1 (day 10) and passage 3 (day 18) for cells cultured in CM and MGPM respectively. Immunolabelling with SF1 revealed strong nuclear with weak cytoplasmic staining in >95% of the cells cultured in either CM or MGPM. (Magnification x200)

CM (DAX1)   MGPM (DAX1)

Texas Red

Merged
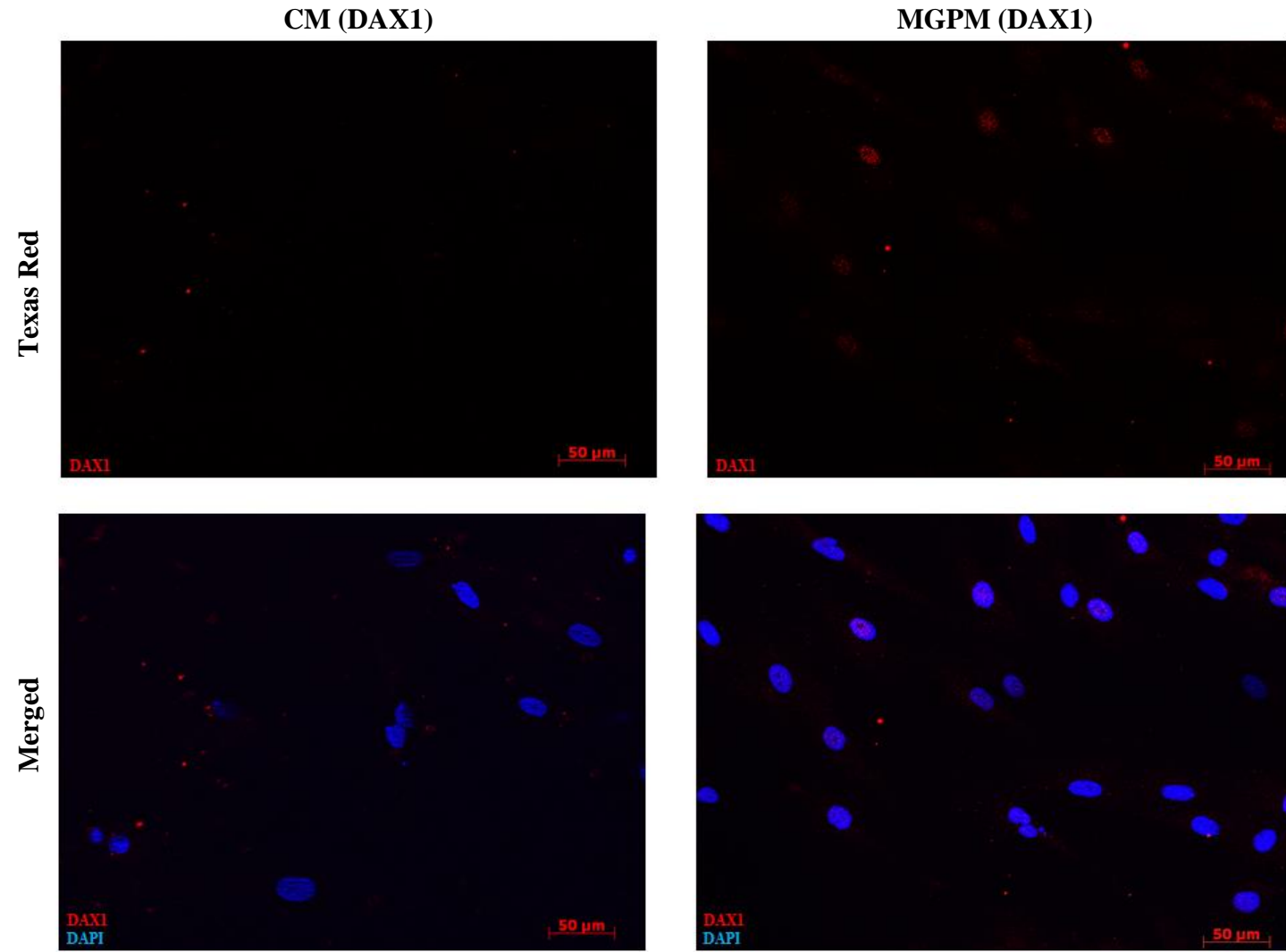
Figure 5.21. Immunolabelling of intracellular markers DAX1

Immunolabelling of intracellular markers DAX1 (Abcam) were carried out after passage 1 (day 10) and passage 3 (day 18) for cells cultured in CM and MGPM respectively. Positive DAX1 labelling was only found in cells harvested in MGPM, as demonstrated by the moderate degree of positive nuclear staining. (Magnification x200)

MGPM (SHH)   MGPM (GLI1)   CM(GLI1)
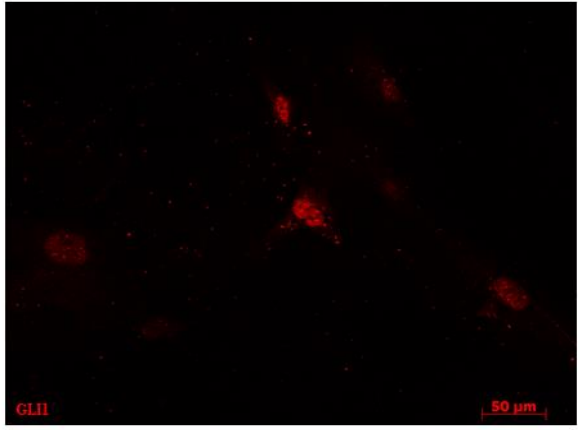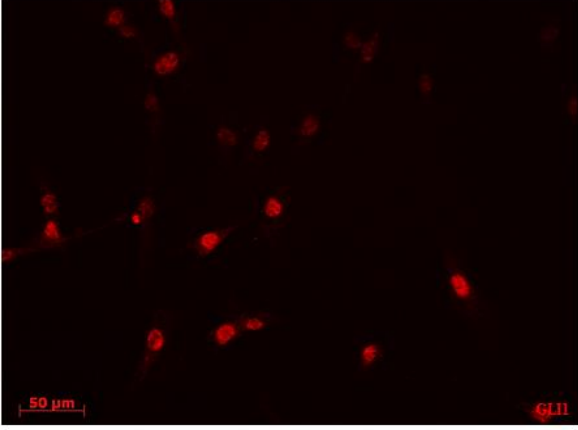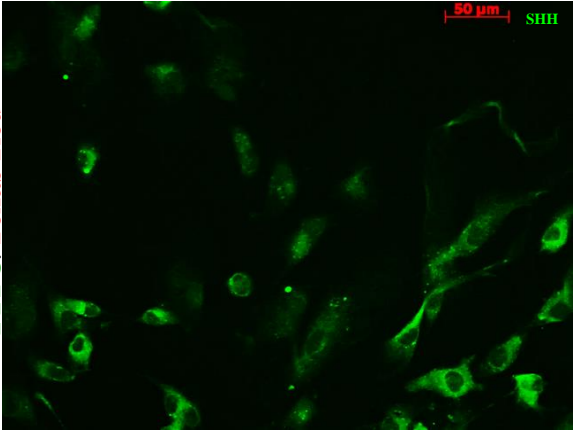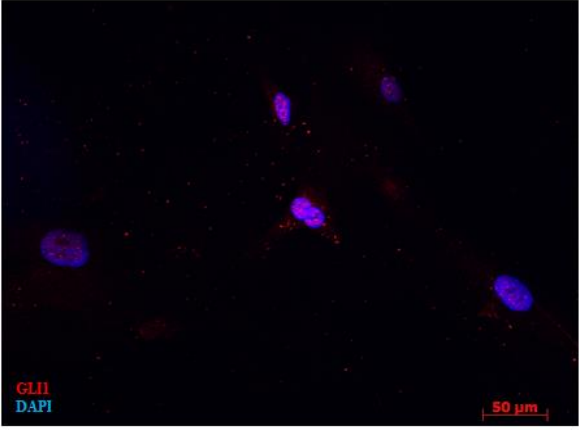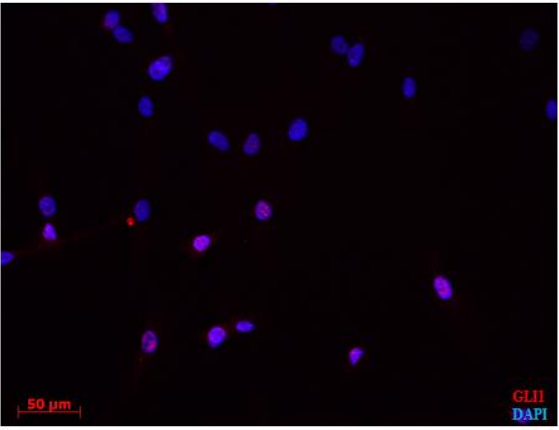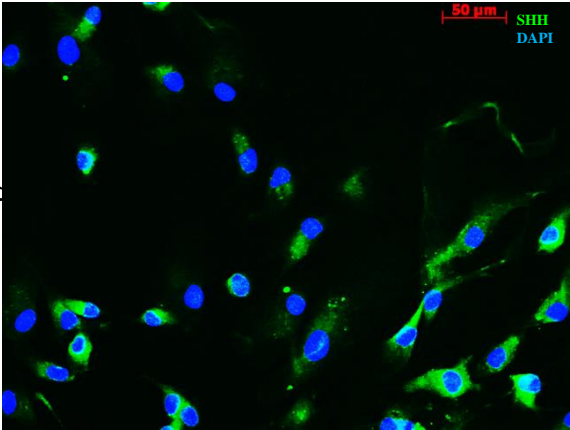
Figure 5.22. Immunolabelling of intracellular markers for SHH and GLI1

Cells were cultured and maintained in CM and MGPM. Immunolabelling of intracellular markers for SHH (Santa Cruz Biotechnology) and GLI1 (Santa Cruz Biotechnology) were performed after passage 1 (day 10) and passage 3 (day 18), in CM and MGPM respectively. Immunolabelling with SHH revealed strong cytoplasmic staining for cells cultured in MGPM only. GLI1 positivity was demonstrated among cells harvested from both cultures, as shown by strong nuclear staining.
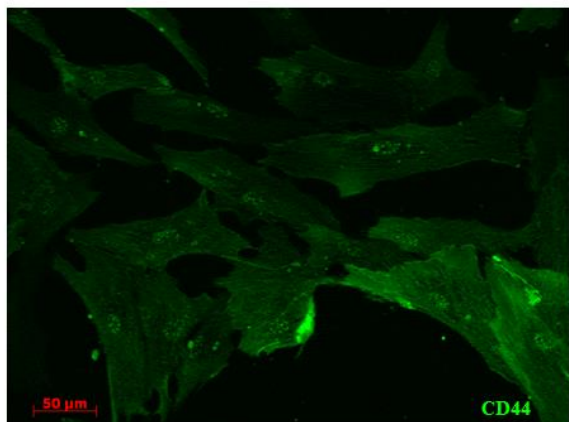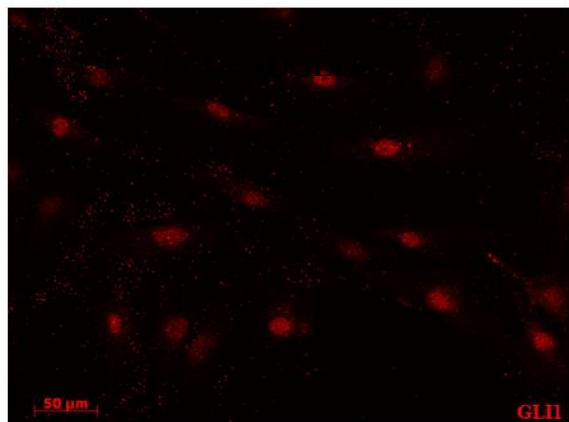
| MSC marker | GLI1/SHH | Merged image |
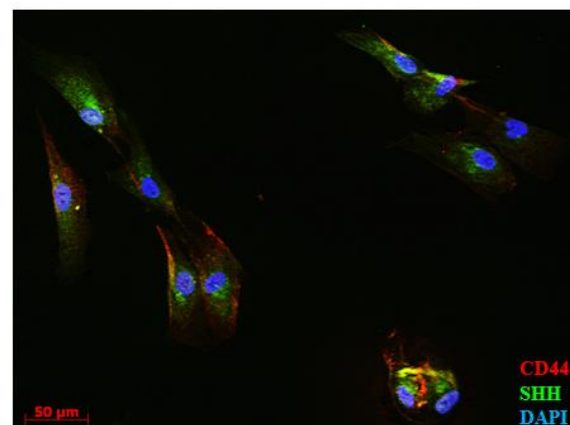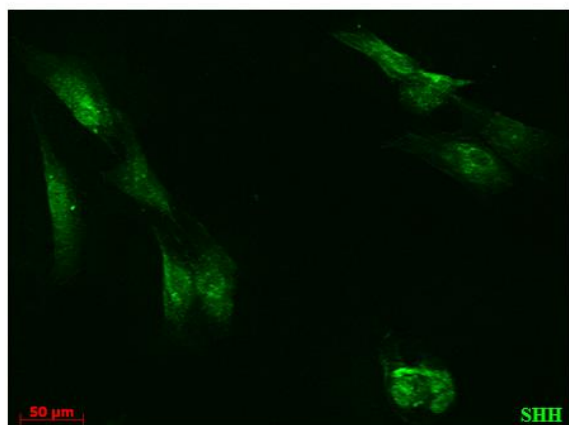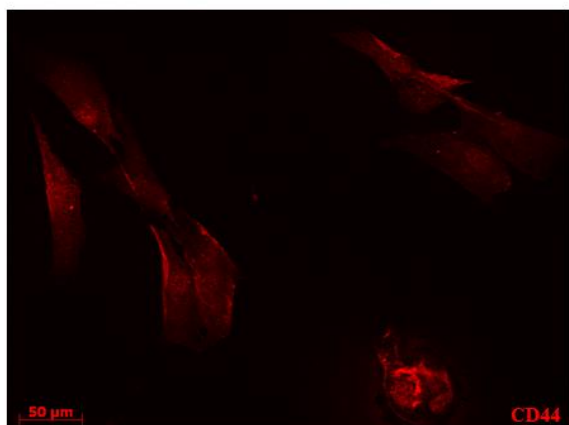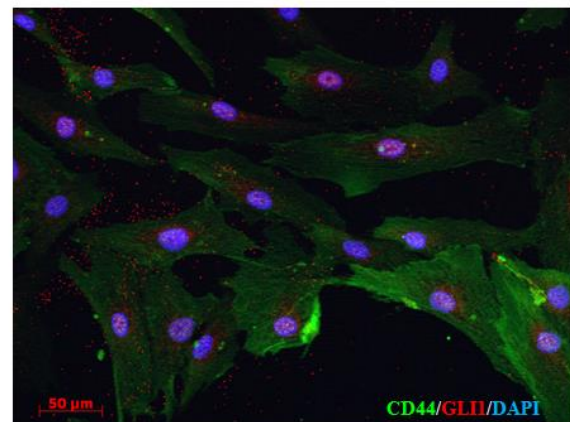
Figure 5.23. Double immunostaining of cells cultured in MGPM.

Cells cultured in MGPM co-expressed CD44 (membrane/cytoplasmic staining), GLI1 (nuclear staining) and SHH (cytoplasmic staining) markers.

(Magnification x200)

MSC markers	Steroidogenic factor-1	Merged image

MGPM

CM
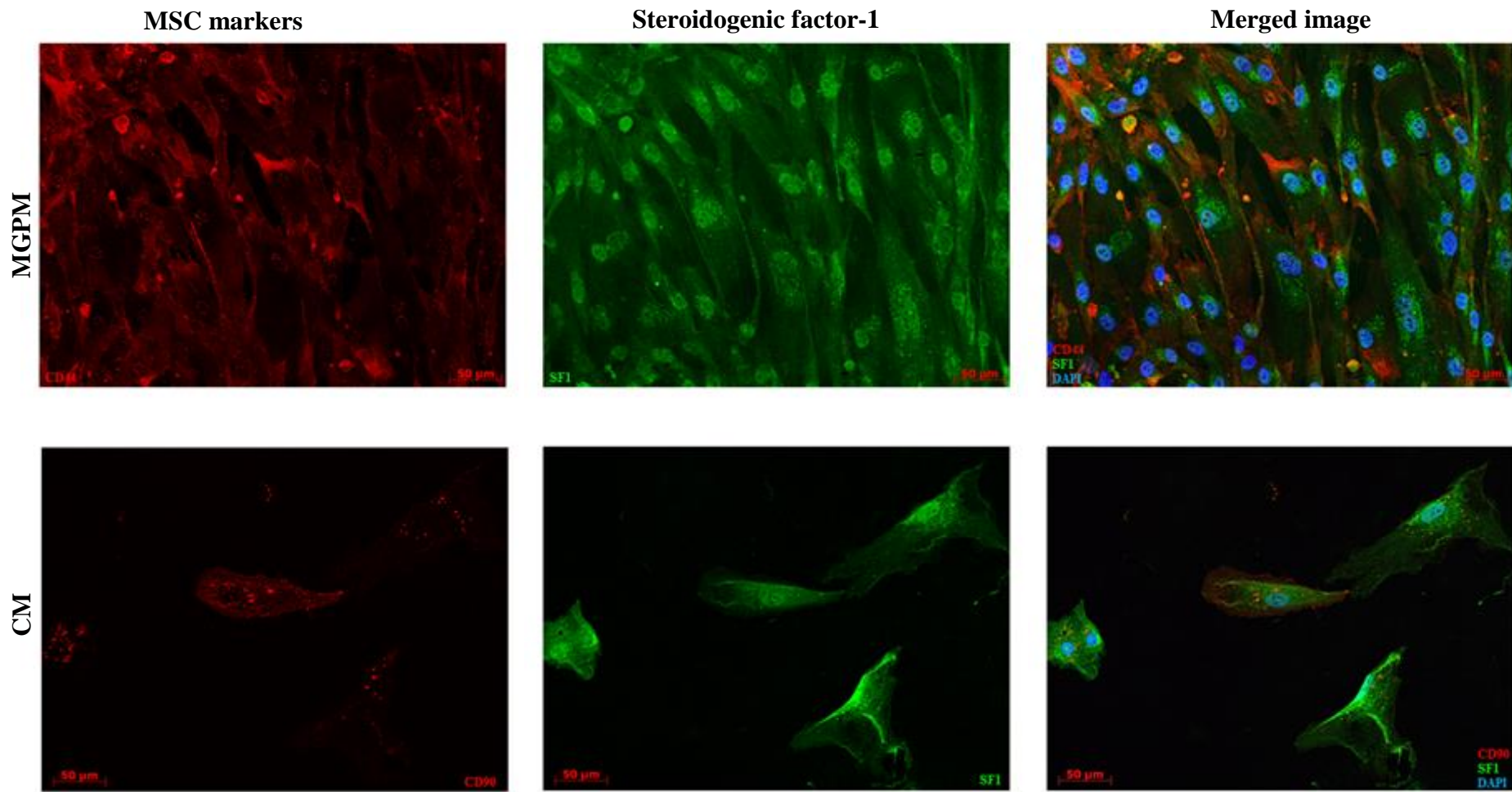
Figure 5.24. Double immunostaining of cells cultured in CM and MGPM as indicated.

MSC marker (CD44 or CD90) co-localised with steroidogenic factor 1 (SF1) in cells harvested from both CM and MGPM, as indicated by the uptake

of membranous staining (MSC) and nuclear/cytoplasmic staining (SF1). (Magnification x200)

5.5 Discussion:

I have identified a cell population that exhibits marker signatures for both mesenchymal stem cells (MSCs) and adrenocortical cells. This group of cells could potentially be the previously uncharacterized multipotent human adrenocortical progenitor cells. According to the International Society of Cellular Therapy (ISCT), the minimal criteria for defining MSCs includes adherence to plastic under standard culture conditions, expression of cell surface markers such as CD90, CD105 and the lack of expression of CD45, CD19 and HLA-DR (MHC class II). They also have the capacity to differentiate into osteocytes, adipocytes, and chondrocytes (Dominici et al. 2006). However, other markers have also been associated with MSCs in previous years, including CD44, CD166 and HLA ABC (MHC class I) (Schaffler & Buchler C 2007).

For the first time, it has been demonstrated that the human adrenal cortex harbours a cell population with properties consistent with MSCs that sustain long term culture. This MSC-like population is characterised by a low forward angle scatter (smaller cell size), and low side angle scatter (low cytoplasmic granularity). When plated onto a plastic vessel in the mesenchymal stem cell growth promotion medium (MGPM) under hypoxic condition, a homogenous cell population developed. They were morphologically fibroblast-like MSCs that were characterized by their ability to form colony-forming unit-fibroblast (CFU-F). They also had high proliferative capacity and exhibited multipotent potential towards osteogenic and adipogenic cell lineages. However, these cells failed to differentiate into the chondrogenic lineage. The exact reason for this is not clear but distinct features have been observed among MSCs isolated from different organs, with respect to their immunophenotype and secreted cytokines profile (Mitchell et al. 2006, Kilroy et al. 2007). Hence, the failure of induced-chondrogenesis among these adrenal-derived cells could be due to the lack of

innate chondrogenic differentiation potential or related to the *in vitro* cell expansion techniques.

Adrenal cells cultured in a complete medium were noted to have died following incubation with chondrogenic or adipogenic medium. This is likely due to the lack of MSC-like cells in the CM following incubation under normal oxygen tension (21%), resulting in failure to withstand high glucose and dexamethasone concentration in the differentiation medium. Indeed, a study has shown that MSCs usually present in stem cell niche under hypoxic condition and in vivo primary culture in normal atmospheric tension may exert oxidative stress on MSCs leading to apoptosis, as well as limit their proliferation and cell fate commitment (Mohyeldin et al. 2010).

As discussed in chapter 1, it has been postulated that adrenocortical progenitor cells reside at the subcapsular region of the adrenal cortex and co-express SF1 and SHH markers. This group of cells is essential for adrenal proliferation and to prevent cellular differentiation. DAX1 is the transcription factor for maintaining the 'stemness' of stem/progenitor cells, by turning off the cellular differentiation capacity. In this study, I found strong expression of SHH, GLI1 and SF1 among adrenal cells cultured in MGPM. SHH is the main initiator of the SHH-GLI pathway and GLI1 is the associated transcription factor. These markers were found to co-localize with SF1, the adrenal specific marker. This finding suggests that the MSC-like population expanded *in vitro* were predominantly adrenal progenitor cells, isolated from the subcapsular region of adrenal cortex and proliferated in MGPM. Furthermore, DAX1 expression was only observed among MGPM-cultured cell, indicating that 'stemness' was enhanced among an MSC-enriched medium. This accounts for the shorter doubling time, higher proliferative capacity and potential for long term culture.

This study has a few limitations. Firstly, it is difficult to isolate or sort the MSCs from the culture as the adrenal cortex-derived cells were comprised of a heterogeneous population of cells. This resulted in difficulties in quantifying and defining other molecular characteristics of this unique group of cells. Since this is the first study demonstrating isolation of MSC-like cells from the adrenal cortex, there is no previous published protocol with respect to the isolation and maintenance methods. Different isolation techniques, particularly the use of a digestive enzyme such as the recombinant fungal trypsin-like protease used in this study, might affect MSC function or differentiation capacity by non-specific degradation. Hence, future studies should attempt to explant the culture of MSC from adrenal cortex pieces.

MSCs have great potential in tissue engineering as they provide an autologous source of cells, without the issues of tissue construct rejection or immunosuppressive therapy. MSCs also lack HLA-DR antigens and the co-stimulatory B71 and B72 (Devine and Hoffman 2000, Majumdar et al. 2003), giving it great potential in allogenic applications. There is also increasing evidence that MSCs have the ability to migrate to the site of injury with anti-inflammatory and immunomodulatory properties (Griffin et al. 2013, Le Blanc et al. 2012,Prockop et al. 2012). For instance, T-cell lymphocyte proliferation was shown to be inhibited during co-culture with MSC, but the reversal of this inhibition was seen following removal of MSC (Di Nicola et al. 2002). They had also been shown to inhibit auto-reactive T and B cells (Deng et al. 2005) and promote production of CD4+CD25+ regulatory T cells in the peripheral blood of the patients with multiple sclerosis (Karussis et al. 2010). Hence, there has been increased interest in broadening the application of MSC to anti-inflammatory and immunomodulatory therapy, with some success already witnessed in conditions like diabetic foot ulcer

(Dash et al. 2009), Crohns' disease and graft vs. host disease (Le Blanc et al. 2008, Duijvestein et al. 2010).

MSC therapy certainly appears to have attractive therapeutic potential in autoimmune diseases. Experimental treatments of type 1 diabetes using MSC therapy in murine models have already shown some promising results. These studies demonstrated an increment in the number of pancreatic insulin-producing beta-cells with suppression of auto-reacitve T lymphocytes and inflammatory dendritic cells, leading to long term reversal of hyperglycaemia (Lee et al. 2006, Fiorina et al. 2009, Jurewicz et al. 2010). With this in mind, an understanding of the cell biology of adrenal-derived MSCs will be essential to regenerative medicine approach in AAD.

Further studies are required to confirm that this population of MSC-like cells consists of MSCs residing in the adrenal cortex. The location of adrenal-MSCs at the tissue level, as well as the genomic and proteomic properties of these cells, also warrants detailed investigations, potentially by qPCR or ELISA. A comprehensive high-throughput screening of the actions of various morphologic and transcription factors during differentiation of MSC to steroidogenic cells will also help identify the agents or factors essential for adrenal self-renewal or steroidogenesis. Finally, developing a method for differentiating MSCs into steroidogenic cells, in which the bone marrow or adipose tissues are a rich source for MSCs, could potentially lead to curative cell therapy for AAD.

Chapter 6.   Final discussion

Genetics studies on autoimmune Addison's disease (AAD) remain challenging due to the disease's complex genetic traits and rarity. Similar to most of the susceptibility alleles identified thus far, the common CD226 variant (*CD266 307*Ser*) identified in this thesis showed only modest disease effects in AAD. Most of the known susceptibility genes are downstream effectors of the immune system and are likely to have roles in the etiological pathway of AAD. However, these individual susceptibility variants are neither necessary nor sufficient to cause the disease. The other common problem encountered in genetic studies of AAD is the small collection of patient cohorts due to the disease's rarity, resulting in underpowered studies for detecting significant effects. Hence, future studies using either a candidate gene or linkage analysis approach will require more extensive international collaboration, perhaps cross-continent collaboration, to clarify the causal pathway involved in the development and progression of this condition.

Genetic studies in the last few decades have been focused on common genetic variants, detected either via a candidate gene study approach or genome wide association studies. However, HLA variants in chromosome 6 are thus far the only gene variants identified as having a strong impact on the pathogenesis of AAD. Although the SIAE rare variants that I found among the AAD cohorts are not statistically significant, the association analysis was calculated using the statistical method conventionally used for common gene variants, which might not be powerful enough for detecting significant effects. Hence, a more accurate and powerful statistical method should be developed for multiple rare variant models. A greater emphasis should be placed on the investigation of the association between rare gene variants and autoimmune susceptibility, even if this means labour-intensive deep genome sequencing and functional testing of each rare variant. On the other hand, AAD is caused by a complex interplay between genetics and

external factors. Future studies should also look into the role of epigenetics in the pathophysiology of AAD, as environment, lifestyle and cultural variances could have an impact on gene expression.

The work undertaken for this study has contributed to the advancement of knowledge that is relevant to the pathophysiology and treatment approach in AAD. The experimental medicine study using $ACTH_{1-24}$ (synacthen) therapy in AAD has made a critical conceptual advance in the treatment of this chronic disease. This study showed that established AAD could be ameliorated by treatment other than the conventional steroid replacement therapy. However, this therapeutic approach requires further exploration, especially with regards to treatment duration, patient selection, dosage regimen and long term side effect profile. Of particular interest is the benefit of synacthen therapy other than adrenal steroidogenesis. The majority of the participants reported significant improvement in quality of life following synacthen therapy, despite not having obvious increments in serum cortisol concentration. Although the ACTH receptor, melanocortin type -2 receptor (MC2R) is expressed predominantly in the adrenal cortex, it has also been found in human bone (Zhong et al. 2005) and skin (Slominski et al. 1996, Guo et al. 2005). In addition, the co-expression of MRAP1 (MC2R accessary protein 1) is essential in ACTH responsiveness (Metherell et al. 2005, Roy et al. 2007). Hence, it will be interesting to look for other tissues that express MC2R and MRAP, as well as the role of ACTH on these tissues. For instance, a study demonstrated that ACTH promotes osteoblast differentiation into mature osteoblast and counteracts dexamethasone-induced osteoblast apoptosis by stimulating the MC2R receptor on osteoblast with subsequent elevation of vascular endothelial growth factor (VEGF) (Isales et al. 2010). The observation of ACTH as the regulator of bone mass

showed the potential additive benefits of ACTH therapy in addition to re-establishing adrenal steroidogenesis in AAD.

On the other hand, the discovery of residual adrenal function in some patients years after diagnosis and treatment of AAD has opened up a new therapeutic window for this condition. New information from this thesis suggests that AAD is a heterogeneous disorder. Patients who have residual adrenal function were observed to be less likely to suffer from adrenal crisis and responded better to the synthetic ACTH treatment. This has made a paradigm shift in the understanding of the pathophysiology of AAD, from a chronic disease with lifelong dependency on steroid replacement to a potentially reversible and treatable condition for some patients. Future studies should be carried out to ascertain the prevalence of residual adrenal function in patients with AAD, as well as to establish the most sensitive biochemical markers for detecting this state, which could be well below the conventional lower limit of assay detection. The stratification of AAD patients based on their residual adrenal function will have an impact on both the treatment approach as well as in advancing the study of disease pathophysiology and genetics.

Although synacthen therapy has shown promising results, regular high-dose $ACTH_{1-24}$ therapy was not without adverse effects. The most significant of which were allergic cutaneous reactions and menstrual disturbance, which has been well described in the literature (Treadwell et al. 1964). Although anti-$ACTH_{1-24}$ antibodies developed during the course of the study might explain some of the side effects, they could not account for the menstrual-related symptoms. Melanocortin receptors (MC3R and MC5R) are expressed in the placenta and ovary (Wikberg et al. 2000) but little is known about the role of ACTH and melanocortin receptors in uterine function, which clearly warrants further investigation. Besides, the discovery of spontaneous anti-$ACTH_{1-24}$ antibodies

among patients with AAD and Graves' disease also raises the question of their role in endocrine autoimmunity, as well as other conditions typically attributed to steroid dysregulation.

The finding of mesenchymal stem cell (MSC)-like population in the adrenal cortex has contributed towards the understanding of adrenal plasticity. Although these adrenal cortex-derived MSC-like cells have yet to be proven as the adrenocortical stem cells, they have been found to exhibit features consistent with progenitor cells. Similar to non-bone marrow derived- MSC isolated from other tissues, the exact location of these MSC-like cells remains elusive. Many studies suggest that this group of cells resides on the luminal surface of endothelial cells in the perivascular niche, namely pericytes or adventitial cells (Crisan 2008, Corselli et al. 2012). They have also been shown to home in on injured or inflamed tissues, with anti-inflammatory and immmuno-modulatory properties. They promote tissue repair through the production of trophic factors which reduce inflammation and facilitate functional recovery of the damaged cells (Chopp & Li 2002, Lee et al. 2006, González et al. 2009).

Promising results have been demonstrated in experimental murine models of acute tissue injury and autoimmunity, such as autoimmune encephalomyelitis, glomerulonephritis and acute fulminant liver failure (Zappia et al. 2005, Kunter et al. 2006, Parekkadan et al. 2007). A few phase I clinical trials have also demonstrated clinical safety and the feasibility of MSC therapy in inflammatory bowel disease (Ciccocioppo et al. 2011), liver cirrhosis (Kharaziha et al. 2009) and myocardial infarction (Hare et al. 2009). In view of these encouraging preliminary results, the discovery of MSC-like cells in the adrenal cortex should prompt further investigation with respect to their true in-situ properties and biological function, to form a potentially efficacious tool that could serve as a long term cure for AAD.

The restoration of adrenal function in some but not all Addison's patients using synthetic ACTH suggests that ACTH may be necessary but not sufficient to induce adrenocortical stem/progenitor cell proliferation in all patients. Therefore, uncovering the interaction between ACTH and other endocrine and paracrine signals in the stem cell niche will be critical to the success of future regenerative medicine approaches in adrenal insufficiency. Key details, such as whether ACTH acts directly on ACSC, or whether an angiotensin-II signal is necessary for the transition from progenitor daughter cell to aldosterone-secreting zona glomerulosa cell, are unknown. Hence, a better understanding of the various molecular mediators and trophic factors involved in the regenerative and immunomodulatary process of adrenal-derived MSC-like cells is of paramount significance to bringing a therapeutic revolution in Addison's disease a step closer to reality.

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Appendix A

AddiQoL (Health-related quality of life in Addison's disease)

The following questions ask for **your views about your health over the last 4 weeks** and how you feel about life in general. Do not spend too much time answering, as your immediate response is likely to be the most accurate. Please answer every question.

	None of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time
I feel good about my health						
I can keep going during the day without feeling tired						
Normal daily activities make me tired						
I have to struggle to finish jobs						
I have to push myself to do things						
I lose track of what I want to say						
I sleep well						
I feel rested when I wake up in the morning						
I feel unwell first thing in the morning						
I am satisfied with my sex life						
I am relaxed						
I feel low or depressed						
I am irritable						
I find it difficult to think clearly						
I feel lightheaded						
I sweat for no particular reason						

	None of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time
I get headaches						
I get nauseous						
My joints and/or muscles ache						
I have back pain						
My legs feel weak						
I worry about my health						
My ability to work is limited						
I can concentrate well						
I am happy						
I feel full of energy						

	Strongly dis-agree	Dis-agree	Slightly disagree	Slightly agree	Agree	Strongly agree
I feel physically fit						
I get ill more easily than others						
I take a long time to recover from illnesses						
I cope well in emotional situations						

Appendix B

Protocols for making relevant solutions for SDS-PAGE

3x Gel Buffer:
- Dissolve 182g tris base in 300ml ddH2O.
- Adjust to pH8.45 with HCl.
- Add H2O to 500ml total volume.
- Add 1.5g SDS and store at 4°C.

AB-3 stock solution
- Dissolve 48g acrylamide and 1.5g bisacrylamide in 100ml water.
- Keep solutions at 7 – 10°C, crystallisation occurs at 4°C.

AB-6 stock solution
- Dissolve 46.5g acrylamide and 3g bisacrylamide in 100ml water.
- Keep solutions at 7 – 10°C, crystallisation occurs at 4°C.

10% APS
- 0.5g APS in 5ml SDW.

1x Cathode Buffer (load on top into wells)
- 12.11g Tris base
- 17.92g tricine
- 1g SDS
- Dilute to 1 litre with ddH2O
- Do not adjust pH
- Store at 4°C.

5x Anode Buffer (load bottom w/ 1x, gel apparatus tray)
- 121.1g Tris base
- 500ml H2O
- Adjust to pH 8.9 with conc HCl
- Dilute to 1 litre with ddH2O
- Store at 4°C.

10x Transfer Buffer
- 30.3g Tris base
- 144g glycine
- Add 800ml ddH2O, when dissolved make up to 1litre. Do not autoclave.

10x TBST
- 87.7g NaCl, dissolve in 500ml water.
- Add 250ml 2M Tris pH7.5
- Make up to 1 litre and add 5ml Tween 20.
- Do not autoclave.

Loading buffer

	1x buffer (10 ml)	Final concentration
Glycerol (100%)	4ml	40%
Tris/HCl (1mM; pH6.8)	2.4 ml	240mM
SDS	0.8g	8%
Bromophenol Blue	4mg	0.04%
Beta-mercaptoethanol	0.5ml	5%
DDH20	3.1ml	-

	1x buffer (10 ml)	Final concentration
Glycerol (100%)	4ml	40%

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