# *In vitro* and *in vivo* analysis of the effects of dehydroepiandrosterone on metabolic and vascular risk

**Samuel P. L. Rice**

**Submitted for the Degree of Doctor of Philosophy**

**Centre for Endocrine and Diabetes Sciences**
**Cardiff University**
**Wales**

**Submitted October 2008**





ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

# Summary

DHEA is an adrenal derived hormone with a unique secretory pattern with highest serum concentrations observed in middle age. Individuals with adrenal insufficiency exhibit gross DHEA deficiency though its replacement in this context is not commonplace. DHEA is known to behave as a pro-hormone with the ability to be converted into either androgenic or oestrogenic terminal hormones; whether this is its sole physiological role still remains unclear.

Various animal and *in vitro* studies have suggested that treatment with DHEA can precipitate improvements in body fat, adipocytokine profiles, insulin resistance and estimates of vascular disease. Human studies have demonstrated inconsistent results and have tended to focus on the physiological deficiency of DHEA associated with normal aging and not the pathological DHEA deficiency seen in adrenal insufficiency.

The aims of this thesis were: (1) To determine the effect of DHEA on preadipocyte (cell line and primary) proliferation and differentiation and to examine the mechanisms behind any observed effects. (2) To evaluate the effect of replacing DHEA on vascular function and body composition in subjects with primary and secondary adrenal insufficiency.

(1) DHEA inhibited proliferation in all preadipocytes examined secondary, at least in part, to cell cycle blockade. DHEA inhibited adipogenesis in omental but not subcutaneous derived preadiocytes. (2) Arterial stiffness and endothelial function was not affected the total population but stratification by study group showed that DHEA replacement reduced central blood pressure in patients with secondary adrenal insufficiency. Body composition was not affected in either subject group.

*I Nia ac Elgan*

# Acknowledgements

I would like to firstly thank my supervisors Dr Aled Rees and Dr Marian Ludgate for their help and support during these last few years.

I would also like to thank the head of our department Professor Maurice Scanlon and the other lead scientists (Dr Mark Lewis, Dr Jack Ham) and clinicians (Dr Steve Davies) based in the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales.

The help and encouragement received from my various friends and colleagues has been invaluable. I would like to mention in particular Dr Lei Zhang, Dr Neera Agarwal and Dr Hemanth Bolusani for their assistance with both the experimental and clinical components of this thesis.

I am grateful for the grant support provided for this work from the Wales Office of Research and Development, the Cardiff and Vale NHS Trust Small Grants Scheme and the 1 year fellowship the Royal College of Physicians (the Lewis Thomas Gibbon Jenkins of Briton Ferry Fellowship).

Finally a big thank you to my parents, without whom I would not be in the privileged position I find myself in today and of course my wife Nia.

# Abbreviations

| | |
|---|---|
| **ACTH** | Adrenocorticotrophic hormone |
| **ACE** | Angiotensin converting enzyme |
| **Ai** | Augmentation index |
| **AMV** | Avian myeloblastosis virus |
| **ARP** | Acid ribosomal phosphoprotein |
| **APRT** | Adenine phosphoribosyltransferase |
| **aPWV** | Aortic pulse wave velocity |
| | |
| **BAT** | Brown adipose tissue |
| **β-HSD** | B-hydroxysteroid dehydrogenase |
| **BMI** | Body mass index |
| **BP** | Blood pressure |
| **bPWV** | Brachial pulse wave velocity |
| **BR2** | Bradykinin receptor 2 |
| | |
| **CABG** | Coronary artery bypass graft |
| **cAMP** | Cyclic adenosine monophosphate |
| **CBG** | Cortisol binding globulin |
| **CD** | Compact disc |
| **cDNA** | Complementary DNA |
| **C/EBP** | CCAAT enhancing binding protein |
| **CLAP** | Chymostatin, Leucopeptin, Antipain and Pepstatin |
| **cm** | Centimeters |
| **CNS** | Central nervous system |
| **CRH** | Corticotrophin releasing hormone |
| **CT** | Computer tomography |
| | |
| **DEXA** | Dual-energy X-ray absorptiometry |
| **DHEA/S** | Dehydroepiandrosterone/sulphate |
| **DM** | Diabetes Mellitus |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribose nucleic acid |
| **dNTP** | Deoxynucleotide triphosphate |
| **dsDNA** | Double stranded DNA |

| | |
|---|---|
| **ELISA** | Enzyme-linked immunosorbent assay |
| **eNOS** | Endothelial nitric oxide |
| **ERK** | Extracellular related kinase |
| | |
| **FCS** | Foetal calf serum |
| **FMD** | Flow mediated dilatation |
| **FMH** | Family history |
| **FSH** | Follicle stimulating hormone |
| | |
| **GABA** | γ-aminobutyric acid |
| **GLUT4** | Glucose transporter 4 |
| **GH** | Growth Hormone |
| **GPDH** | Glycerol phosphate dehydrogenase |
| **GTN** | Glyceryl trinitrate |
| | |
| **HDL** | High density lipoprotein |
| **HIV** | Human immunodeficiency virus |
| **HOMA-IR** | Homeostatic model insulin resistance |
| **HPA** | Hypothalamic-pituitary-adrenal |
| **HSCRP** | High sensitivity C-reactive protein |
| | |
| **IDF** | International diabetes federation |
| **IL-6** | Interleukin 6 |
| **IGF** | Insulin like growth factor |
| **IMP** | Investigational medicinal product |
| **IRS** | Insulin receptor substrate |
| | |
| **JAK** | Janus kinase |
| **JNK** | c-jun N-terminal kinase |
| | |
| **Kg** | Kilogram |
| **KLF** | Krüpple like factor |
| | |
| **LDL** | Low density lipoprotein |
| **LH** | Leutenising hormone |
| **LPL** | Lipoprotein lipase |
| **LSCCE** | (P450) linked side chain cleaving enzyme |

| | |
|---|---|
| **MAPK** | Mitogen activated protein kinase |
| **MIF** | Macrophage inhibitory factor |
| **mg** | Milligrams |
| **MHRA** | Medicines and healthcare Products Regulatory authority |
| **mL** | Millilitre |
| **mM** | Millimolar |
| **MMLV** | Moloney murine leukaemia virus |
| **mRNA** | Messenger RNA |
| **MRI** | Magnetic resonance imaging |
| **$Na^+$** | Sodium |
| **ng** | Nanogram |
| **NHS** | National Health Service |
| **nM** | Nanomolar |
| **NO** | Nitric oxide |
| **NMDA** | N-methyl-D-aspartate |
| **OD** | Optical density |
| **PAGE** | Polyacrilamide gel electrophoresis |
| **PAI-1** | Plasminogen activator inhibitor-1 |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PET** | Positron emission tomography |
| **pg** | picogram |
| **PGC** | PPAR-γ cofactor |
| **$PGEI_2$** | Prostaglandin $EI_2$ |
| **PGK** | Phosphoglycerate kinase |
| **PI3K** | Phosphatidyl inositol kinase |
| **PKA** | Protein Kinase A |
| **PhosphoMAPK** | Phosphorylated MAPK |
| **PLC** | Phospholipase C |
| **PMSF** | Phenylmethanesulphonyl fluoride |
| **POMC** | Pro-opoimelanocortin |
| **PPAR** | Peroxisome proliferator activated receptor |
| **PVDF** | Polyvinylidene fluoride |
| **PWV** | Pulse wave velocity |
| **QPCR** | Quantitative PCR |
| **RNA** | Ribose nucleic acid |
| **RT** | Reverse transcription |
| **RT-QPCR** | Real time QPCR |
| **RXR** | Retinoic X receptor |

| | |
|---|---|
| **rpm** | Revolutions per minute |
| **SAE** | Serious adverse event |
| **SDS** | Sodium dodecyl sulphate |
| **SHBG** | Sex hormone binding glogulin |
| **SOCS** | Suppressor of cytokine signaling |
| **SREBP** | Sterol response element binding protein |
| **STAT** | Signal transducers and activators of transcription |
| **SUSAR** | Suspected unexpected serious adverse reaction |
| **TGF-β** | Transforming growth factor- β |
| **Tm** | Melting temperature |
| **TNF** | Tumour necrosis factor |
| **TRAF** | TNF receptor associated factor |
| **TUNEL** | Terminal UDT nick end labeling |
| **UCP-1** | Uncoupling protein-1 |
| **UDT** | Uridine deoxynucleotide transferase |
| **WAT** | White adipose tissue |

# Publications and presentation

**Abstracts:**

1. Sam Rice, Neera Agarwal, Lei Zhang, Eddie Wang, Mark Lewis, Martin Bullock, Aled Rees and Marian Ludgate. 2008. Dehydroepiandrosterone (DHEA) *in vitro* inhibits adipogenesis in omental but not subcutaneous adipose tissue. International Congress of Endocrinology.

2. Rice S, Agarwal N, Bolusani H, Ludgate M, Rees DA. 2008. Effects of DHEA replacement on arterial stiffness and metabolic function in primary and secondary adrenal insufficiency: a randomised, controlled trial. International Congress of Endocrinology.

3. Sam Rice, Neera Agarwal, Hemanth Bolusani & Aled Rees. 2008 Cardiometabolic disease in adrenal insufficiency: is the risk increased? *Endocrine Abstracts* **15** P121

4. Sam Rice, Neera Agarwal, Aled Rees & Marian Ludgate. 2007. Comparative analysis of DHEA action in preadipocyte cell lines and primary cultures. American Endocrine Society.

5. Sam Rice, Eddie Wang, Maurice Scanlon, Marian Ludgate, Aled Rees. 2006. Comparative analysis of the effects of dehyroepiandrosterone on white and brown pre-adipocyte proliferation/differentiation. *Endocrine Abstracts* **11** OC31

6. Sam Rice, Aled Rees & Marian Ludgate. 2006. Dehyroepiandrosterone differentially inhibits brown and white preadipocyte proliferation through phase specific blockade of the cell cycle. American Endocrine Society.

**Presentations (National/International):**

1. International Congress of Endocrinology 2008. Dehydroepiandrosterone (DHEA) *in vitro* inhibits human adipogenesis in omental but not subcutaneous adipose tissue. International Congress of Endocrinology (Oral)

2. International Congress of Endocrinology 2008. . Effects of DHEA replacement on arterial stiffness and metabolic function in primary and secondary adrenal insufficiency: a randomised, controlled trial (Poster)

3. Welsh Endocrine and Diabetes Society Meeting Spring 2008. *In vitro* and *in vivo* analysis of the effects of dehydroepiandrosterone on metabolic and vascular risk (Oral) (Borsey prize for best research presentation)

4. British Endocrine Society meeting 2008. Cardiometabolic disease in adrenal insufficiency: is the risk increased? (Poster)

5. American Endocrine Society 2007. Comparative analysis of DHEA action in preadipocyte cell lines and primary cultures (Poster)

6. American Endocrine Society 2006. Dehyroepiandrosterone differentially inhibits brown and white preadipocyte proliferation through phase specific blockade of the cell cycle (Poster)

7. European Congress of Endocrinology 2006. Comparative analysis of the effects of dehyroepiandrosterone on white and brown preadipocyte proliferation/differentiation (Oral)

**Presentations (University):**

1. Interdisciplinary Research Group (IRG) meeting 2008. Effect of DHEA on vascular function and body composition in primary and secondary adrenal insufficiency (Oral)

2. Postgraduate Research Day 2007. The effect of dehydroepiandrosterone on preadipocye cell lines and primary cultures (Oral)

3. Interdisciplinary Research Group (IRG) meeting 2007. Comparative analysis of DHEA action in preadipocyte cell lines and primary cultures (Oral)

4. Postgraduate Research Day 2006. Effects of dehydroepiandrosterone on white and brown preadipocyte proliferation/differentiation (Poster)

# Table of contents

# Index

## **Chapter 1**. General introduction

## **Chapter 2.** Effects of DHEA on proliferation and differentiation of preadipocyte cell lines

## **Chapter 3.** Effects of DHEA on proliferation and differentiation of human primary preadipocytes

**Chapter 4.** A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function and body composition in patients with primary and secondary adrenal insufficiency – Study design

**Chapter 5.** A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function and body composition in patients with primary and secondary adrenal insufficiency – results

## **Chapter 6**. General discussion

## List of tables

# List of figures

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Dehydroepiandrosterone

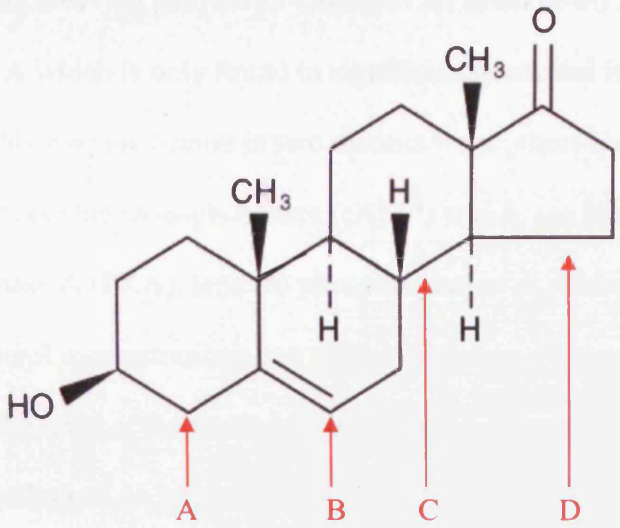
### 1.1.1 Overview

Dehydroepiandrosterone (DHEA) is a precursor sex steroid which, in common with all adrenal steroids, is derived from the parent molecule cholesterol. It was first identified in its sulphated form, dehydroepiandrosterone sulphate (DHEAS), in 1954 by Migeon and Plager [1] and was the first hormone with androgenic activity to be isolated from human plasma. Subsequent studies have shown that DHEA and DHEAS are present in the human and higher primate circulation in higher concentrations than any other steroid hormone [2], though levels decline dramatically after middle age [3, 4]. This age-related fall in concentration has led to speculation as to whether this physiological reduction in DHEA is causally related to disease processes normally associated with aging. This in turn has driven a market in sales of DHEA, particularly in the USA and on the internet where it is easily available as a food supplement. However, the precise physiological role of DHEA remains uncertain despite a variety of epidemiological studies demonstrating an inverse correlation with some features of the metabolic syndrome (obesity [5, 6], insulin resistance [7]) as well as cardiovascular morbidity and mortality [8, 9]. Furthermore, interventional studies in women (postmenopausal and those with hypoadrenalism) have demonstrated improvements in insulin sensitivity following DHEA therapy [10, 11]. Currently however, the somewhat limited evidence base for DHEA use in the clinical setting is largely confined to females with adrenal insufficiency in whom there is a failure to achieve adequate quality of life with or without reduced libido, despite optimal glucocorticoid and mineralocorticoid replacement [12, 13]. Nevertheless a plethora of

other uses have been suggested for DHEA in a diverse variety of conditions, ranging from infectious diseases to Alzheimer's disease [14-22].

### 1.1.2 DHEA Structure

All adrenal steroids which are derived from cholesterol share the same basic structure comprising a cylcopentenophenanthrene nucleus made up of three six-carbon rings (A, B, C) and one five-carbon ring (D). The rings are fused in the *trans* position to produce a planar structure with a hydrophobic side chain (Fig 1.1).




HO – hydroxyl group
$CH_3$ – methyl group

Fig 1.1 Structure of DHEA

Each DHEA molecule contains a double bond in ring B and a hydroxyl group in ring A. This hydroxyl group is substituted for a sulphate group to produce DHEAS and this small

change in the structure reduces membranous transport potential. Changes within the side chain located on ring D occur with the generation of different terminal hormones. Again, small changes here can provoke profound effects on activity and potency.

### 1.1.3 Synthesis of DHEA

Various enzymatically controlled pathways are involved in the generation of steroid hormones [23] .Cholesterol is the common precursor to all adrenal steroids. The primary and rate-limiting step in the conversion of cholesterol into functioning steroids involves the cleavage of a 6-carbon group. This reaction is governed by the mitochondrial enzyme P450-linked side chain cleaving enzyme (P450-LSCCE) also known as cholesterol desmolase or CYP11A which is only found in significant quantities in steroid-producing cells. Regulation of this enzyme occurs in two distinct ways: short-term regulation is governed by cyclic adenosine monophosphate (cAMP) which, via stimulation of cAMP-dependent protein kinase A (PKA), leads to phosphorylation of cholesterol-ester esterase and increases cholesterol concentrations, the primary reaction substrate. Long-term regulation occurs at the level of the P450-LSCCE gene whose promoter contains a cAMP response element. Binding of cAMP here increases transcription of P450-LSCCE. The system also possesses a negative feedback loop, whereby cholesterol inhibits the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA reductase). This enzyme catalyses the first step in the cholesterol production pathway and is also the target element for the Statin group of pharmacological agents. Activity of the P450-LSCCE produces pregnenolone which is the common precursor to glucocorticoids, mineralocorticoids and adrenal androgens. Once generated, pregnenolone passes into the

cytosol of cells within the zona reticularis of the adrenal cortex (Fig 1.2). The steroid-producing cells located in this zone possess CYP17 (17, 20 Lyase/17α-hydroxylase/P450c17) located at the endoplasmic reticulum. Firstly this enzyme catalyses the 17α-hydroxylation of pregnenolone producing pregnenolone sulphate then cleaves 2 carbon atoms to produced the 19 carbon ring structure of DHEA.

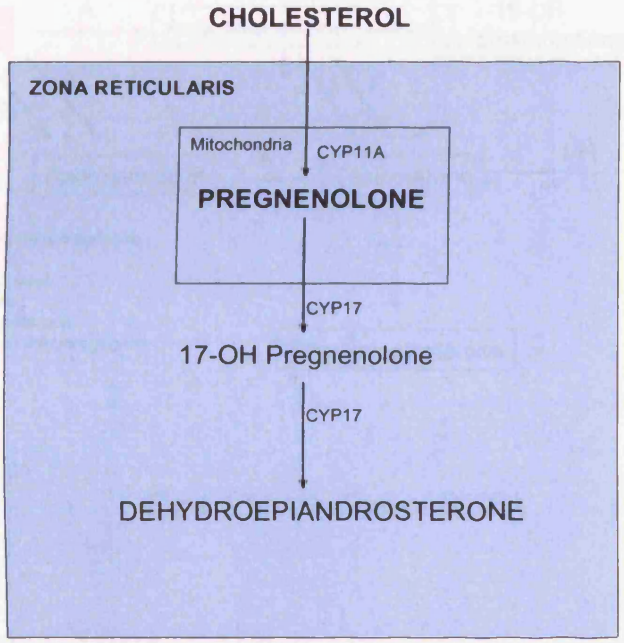



Fig 1.2 Synthesis pathway of DHEA

Once synthesised, DHEA is exposed to the enzyme 3β-hydroxysteroid dehydrogenase to generate androstenedione (Fig 1.3). Cells located within the other zones of the adrenal

cortex contain enzyme cascades that generate the terminal hormones aldosterone and cortisol.

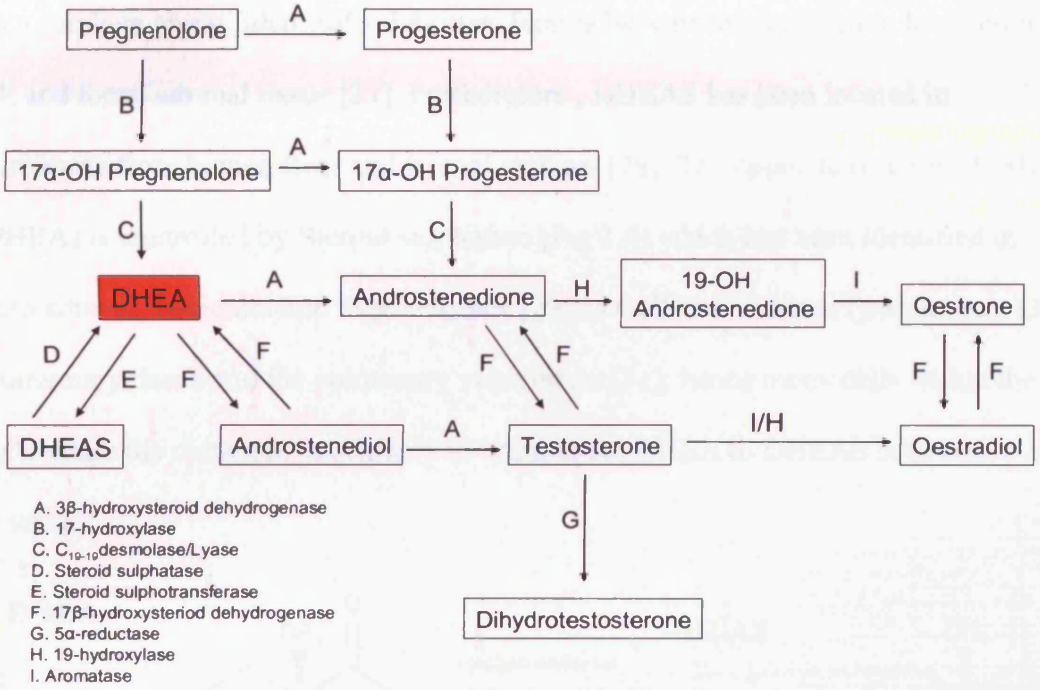



Fig 1.3 Metabolism of DHEA

### 1.1.4 Interconversion of DHEA and DHEAS

DHEAS, the sulphated conjugate of DHEA is quantitatively the most important androgen secreted by the adrenal cortex and is almost exclusively produced in the adrenal gland. DHEAS can be considered the hydrophilic circulating form whereas DHEA is lipophylic and can undergo membranous transport. DHEAS may be produced either from sulphation of DHEA or directly from pregnenolone sulphate [24, 25]. DHEA and DHEAS have been demonstrated to have androgenic activity relative to testosterone; DHEA exhibiting

relatively more androgenic activity than DHEAS [26]. There is continual flux between DHEA and DHEAS under the influence of converting enzymes. DHEA is converted to DHEAS by the activity of Sulphotransferase which replaces the terminal hydroxyl group with a sulphate group. Identical sulphotransferases have been identified in both human adult and foetal adrenal tissue [27]. Furthermore , DHEAS has been located in supernatants from human liver and jejunal cultures [28]. The opposite reaction (DHEAS to DHEA) is controlled by Steroid sulphatase (Fig 1.4) which has been identified in human adrenal, testicular and ovarian tissue [29] as well as on human lymphocytes [30], in pulmonary tissue and the pulmonary vasculature [31]; hence many cells within the body contain the metabolic machinery to bring about DHEA to DHEAS conversion and *vice versa.*

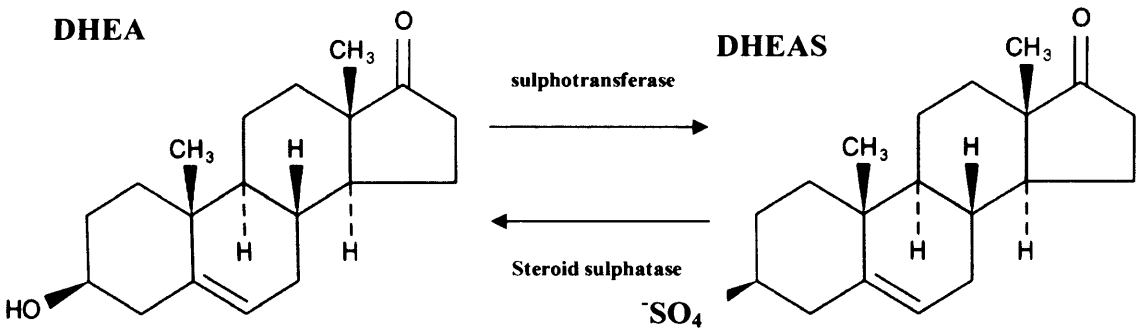



Fig 1.4 Interconversion of DHEA to DHEAS

However, recent evidence suggests that DHEAS is less readily converted back into active DHEA, hence DHEA/DHEAS ratios will vary predominantly according to Sulphotransferase activity [32].

DHEAS is routinely assayed during endocrinological assessment of androgenic status in females; approximately 20% of Caucasian and up to 30% of black females with a diagnosis of polycystic ovarian syndrome have elevated serum concentrations [33].

### 1.1.5 Protein binding

DHEA exists in the circulation mostly bound to albumin [34] with a smaller portion bound to sex hormone binding globulin (SHBG) (Fig 1.5), in contrast to testosterone and oestradiol which are bound principally to SHBG. More DHEA is found bound to SHBG in females particulary during pregnancy when SHBG levels rise. Furthermore, a larger percentage of DHEA exists in the circulation in the unbound state compared to testosterone and oestrogen. All three hormones are similar in that very little binding occurs with cortisol binding globulin [35].

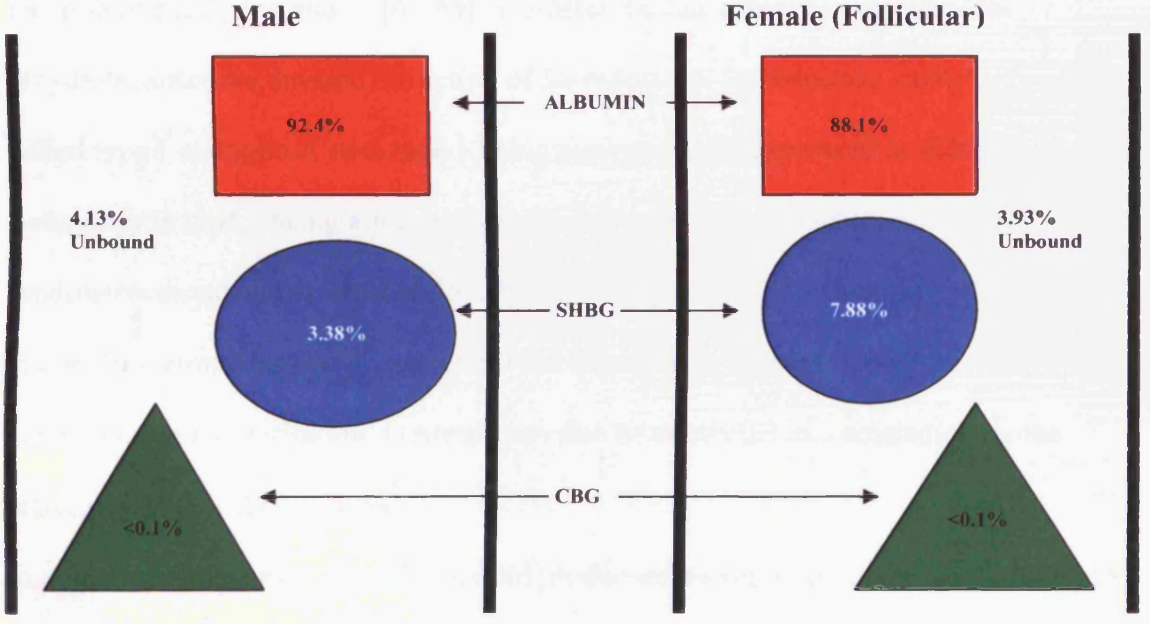



Fig 1.5 Transport of DHEA as a percentage concentration in each fraction (SHBG – sex hormone binding globulin, CBG - cortisol binding globulin) adapted from Dunn *et al* [35]

### 1.1.6 Metabolism

Once in the intracellular compartment DHEA can be converted enzymatically into either androgenic or oestrogenic terminal hormones. DHEA is converted to androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD). The structure of this membrane bound enzyme was only determined in 1989 [36] and its expression has since been confirmed in skin, adipose tissue, breast, lung, endometrium, prostate, liver, kidney, epididymis and brain tissue as well as in placental, adrenal, ovarian and testicular sites [37-40]. This once more indicates that there is widespread peripheral utilisation of DHEA. Because of its position upstream of oestradiol formation, inhibition of 3β-HSD has been proposed as a potential treatment for oestrogen sensitive malignancies [41, 42]. Androstenedione can be converted to testosterone by 17β-hydroxysteroid dehydrogenase (17-βHSD). A number of different types of 17β-HSD have been identified, some catalysing the forward or reduction reaction (types 1, 3, 5, 12 and 13) and others the reverse or oxidative reaction (types 2, 4, 6 and 8) [43-46]. Testosterone can in turn be converted to dihydrotestosterone through the action of 5α-reductase. 5α-reductase exists in 2 isoforms, called type 1 and type 2, with type 1 being predominantly expressed in skin [47] and a deficiency in type 2 being a recognised cause of male undermasculinisation [48]. Androstenedione is converted to 19-OH androstenedione by 19-hydroxylase before undergoing aromatisation to oestrone. Testosterone is converted directly to oestradiol, again through aromatisation. Oestrone can also be converted into oestradiol via the activity of 17β-HSD (Fig 1.3).

In both men and women most sex steroid production therefore proceeds through DHEA [49] and in post menopausal women all oestrogens and most androgens are generated in peripheral tissue from DHEA. The relative amounts of androgen or oestrogen produced

depends on the tissue concerned and the relative expression and activity of converting enzymes present. These enzymes are distributed extensively within the body indicating widespread terminal hormone production [39, 50].

Utilisation of DHEA may be sex-dependent. In DHEA deficient individuals, DHEA replacement results in a sexually dimorphic pattern of hormone production: in DHEA deficient men there is preferential bioconversion of DHEA to oestrogens and in women to androgens [51-53]. DHEA replacement may therefore exert different metabolic effects in men and women mediated through oestrogen and androgen respectively.

### 1.1.7 Secretion

DHEA is synthesised at rates of 16.34 ± 2.60 mg/24 hours in men and 16.19 ± 1.78 mg/24 hours in women [54] and has a normal adult plasma concentration range of 2.4-9.5 ng/mL [54-56]. DHEA has a short plasma half-life (approximately 25 minutes) which is attributed to rapid conversion into the sulphated ester DHEAS [57]. Concentrations of DHEAS are higher (1839 ± 320 ng/mL) than DHEA [56] and DHEAS is thought to represent a circulating pool ready to be converted into DHEA and then into further downstream metabolites.

Serum concentration of DHEAS is slightly higher in males compared to females. Furthermore, serum levels of DHEAS are significantly greater than those of DHEA (μM compared to nM), and vary less over a 24 hour period [55]. DHEAS also has a lower metabolic clearance rate than DHEA [57], these factors therefore dictate that a large plasma pool of DHEAS exists and the majority of DHEA is excreted as the sulphated conjugate [34].

Secretion of adrenal androgens and glucocorticoids is predominantly under the control of adrenocorticotrophic hormone (ACTH) which is generated by the pituitary gland through the action of hypothalamic-derived corticotrophin releasing hormone (CRH) (Fig 1.6). In young adults concentrations of DHEAS in peripheral blood are far greater than any other steroid hormone, with a dramatic fall in levels after the third decade [3, 4], in contrast to glucorticoids and mineralocorticoids whose serum concentration varies little with age [58, 59]. Adult concentrations of DHEA and DHEAS are reached in males at a mean age 13 years and 14 years respectively, while in females adult concentrations are reached at a mean age of 12 years and 15 years respectively [60]. It is likely that genetic influences play a significant role in determining serum DHEAS concentrations with some analyses suggesting a heritability of up to 65% [61].

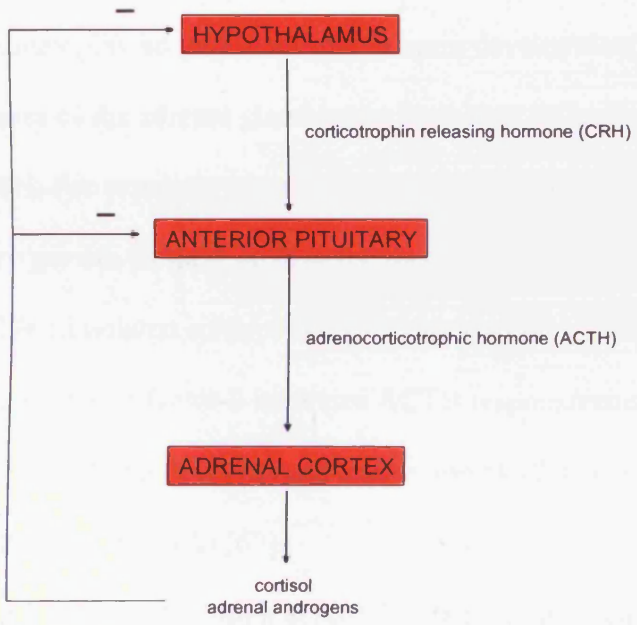



Fig 1.6 Hypothalamic-pituitary-adrenal axis

DHEA and DHEAS, like cortisol, are predominantly regulated by pituitary ACTH, resulting in pulsatile release, although the pulse magnitude for each is less than cortisol and more apparent for DHEA than DHEAS [55]. Similarly, circadian and also seasonal rhythms are in operation with higher DHEA values present in the mornings [62] and in autumn/winter [63].

For the most part, the observed changes in DHEA concentration are governed by changes in ACTH release from the pituitary gland. However, patterns of ACTH release can not explain fully the unique pattern of DHEA production through life (Fig 1.7).

DHEA is produced in high concentrations in the foetal state and is the major product of the foetal adrenal gland [64]. For reasons that are not entirely clear DHEAS levels are approximately 100 times higher than cortisol in umbilical cord blood with no gender difference [65]. DHEA may play an important role in brain development *in utero* [66]. It is likely that the responses of the adrenal gland in the foetus are influenced by other hormones beyond ACTH. For example, activation of the insulin-like growth factor-1 receptor (by Insulin like growth factor-1 or 2) in the foetal zone of the gland has been shown to increase ACTH stimulated cortisol and DHEAS secretion. Furthermore, local production of insulin-like growth factor-2 increases ACTH responsiveness as measured by steroid production (cortisol and DHEAS) and expression of 17 α-hydroxylase (CYP17) and P450- LSCCE (CYP11A) [67].

Levels of DHEA/DHEAS are therefore high at birth but fall rapidly thereafter [68], remaining low until approximately 10 years when the zona reticularis of the adrenal gland

matures and begins to increase production (adrenarche) [69]. The trigger and controlling mechanisms for this process are not fully understood. Levels continue to rise through puberty and peak in early adult life with a steady fall thereafter [3], despite no alteration in the production of ACTH during adrenarche, puberty or normal aging. This suggests reduced ACTH responsiveness and is illustrated by Synacthen (synthetic ACTH) testing in elderly subjects, whereby peak cortisol responses are similar to young adults but responses for DHEA are reduced [70]. Divergent cortisol and DHEA responses are also seen in acute illness [71] and conditions of low calorific intake [72] where cortisol, but not DHEA levels rise. The precise cause for the aberrant cortisol to DHEA ratios in these circumstances is unclear but is likely to be multifactorial involving the influence of extra-adrenal factors including pro-opiomelanocortin (POMC)-derived peptides [73], relative and tissue-specific concentrations of converting enzymes and perhaps the proposed adrenal androgen-stimulating hormone, the existence of which has not been confirmed [74].

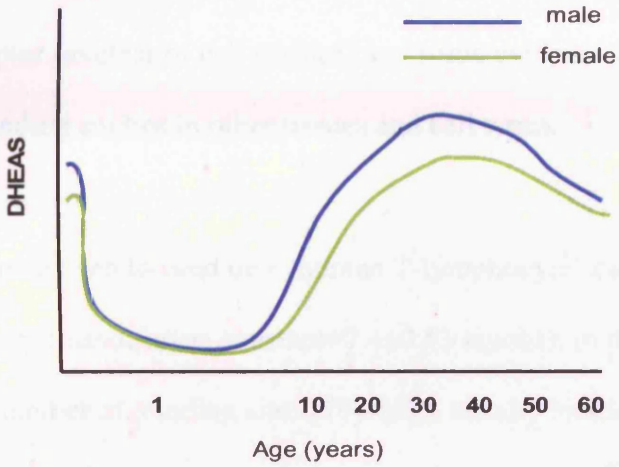



Fig 1.7 Variations in concentration of DHEAS levels with time

### 1.1.8 Mechanism of action

Labrie's review of DHEA action [75] suggested that it was not actually a hormone at all but exerted all its actions as a prohormone, though much recent evidence challenges this assumption. Studies have shown direct DHEAS action as a neurosteroid: at concentrations of 2nM it functions as an allosteric antagonist of the γ-aminobuteric acid A ($GABA_A$) receptor in rat brain samples. This effect was reduced by barbiturates (Phenobarbital) suggesting that DHEAS and barbiturates act at the same receptor sites [76, 77]. Low concentrations of DHEA ($10^{-9}$ molar) have also been shown to act on the N-methyl-D-aspartate receptor (NMDA) in embryonic neuronal primary cultures where it stimulates calcium influx, the rapidity of this effect suggesting action via a cell surface receptor. Further studies have shown that DHEA can promote neuronal growth [66].

Much of the DHEAS present in central nervous system (CNS) may be generated locally as evidenced by the preserved DHEAS levels in the CNS of rats that have undergone adrenalectomy and orchidectomy [78]. These CNS studies suggest that DHEA may possess a receptor (nuclear or cell surface) and some evidence for the existence of this comes from binding studies in other tissues and cell types.

Binding sites have been located on a human T-lymphocyte cell line that binds DHEA with high affinity (dissociation constant=7.4±0.53 nmol/l); in these cells DHEA actually increased the number of binding sites [79]. High affinity binding sites have also been located on $G\alpha_{i\,2,3}$-protein coupled receptors on membranes of bovine aortic endothelial cells ($K_d$ 48.7pM) that in turn activate endothelial nitric oxide synthase (eNOS) [80, 81]. DHEA may also exert direct action through binding to the androgen or oestrogen receptors. A study by Chen *et al* [82] demonstrated that DHEA bound to the androgen receptor with a binding affinity of 1μM and in turn reduced gene transcription in breast and prostate tumor cell lines. This study also revealed that DHEA bound to oestrogen receptors α and β with a low binding affinity of 1.1μM and 0.5μM respectively, but with preferential agonistic activity seen at the oestrogen receptor-β.

However, the major metabolic effects of DHEA are most likely to be indirect, mediated by tissue specific bioconversion into downstream metabolites of the sex steroid pathway (Fig 1.3).

A further and interesting effect of DHEA is seen in individuals with secondary adrenal failure receiving growth hormone replacement, whereby DHEA supplementation leads to

an increase in Insulin like growth factor-1 (IGF-1) necessitating a reduction in growth hormone dose [83]. A similar finding has also been observed following the supplementation of DHEA in elderly men and women in association with a reduction in IGF-1 binding protein levels [84]. Again it is not known if the mechanism of this effect is direct or indirect but the results suggests that there is likely to be continuous interaction between DHEA/DHEAS, other hormone systems, and the cell signaling pathways that they trigger.

### 1.1.9 Species differences

The dramatic age-related alterations and high serum concentrations of DHEA and DHEAS as well as the large circulating pool of adrenal-derived androgens is only seen in humans and higher primates. Other animal models used in the laboratory setting generate androgen and oestrogen solely from ovarian or testicular tissue and much lower serum concentrations are thus observed [56]. This leads to difficulties in extrapolating results from studies that utilise these animals to the human system. Effects that may be seen in the rodent following what is physiological exposure for humans cannot necessarily be related with confidence to the human situation as these concentrations are grossly supra-physiological for that animal. Similarly, experimental work utilising cell lines generated from animals will only provide a suggestion as to what the situation may be within the human body such that work with human or higher primate cells and cultures remains critically important.

## 1.2 DHEA and adipose tissue

### 1.2.1 Overview

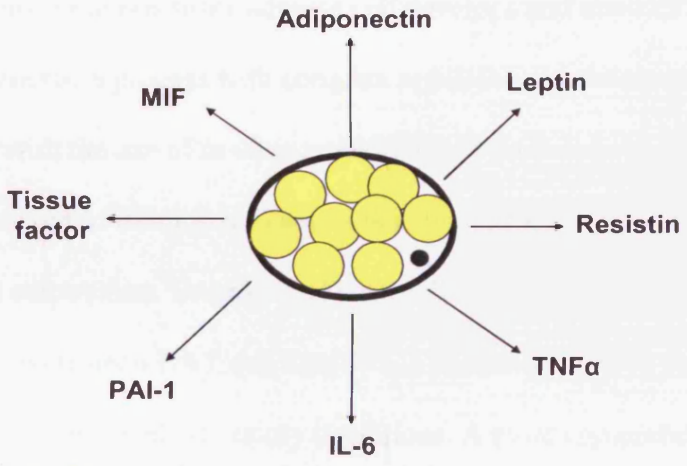
Adipose tissue contains both significant quantities of DHEA [85] and possesses the appropriate enzymatic machinery for its metabolism [86, 87]. DHEAS is also present in adipose tissue but at lower concentrations relative to DHEA due to increased polarity, and thus reduced lipid solubility, which in turn limits transport across the cellular membrane, although organic anion-transporting polypeptides B, D and E have been been postulated to facilitate internalisation of DHEAS in adipose tissue [87]. As previously discussed DHEA is present in high concentrations in plasma relative to other hormones; in adipose tissue this concentration is notable, where levels are 10 times greater than in the circulation [85]. Recent advances have led to changes in the perception of adipose tissue function such that it is no longer thought of solely as an energy reservoir. The effects of DHEA on adipose tissue have been studied using both *in vitro* and *in vivo* techniques.

### 1.2.2 Adipose tissue

Developmentally adipose tissue arises from mesenchymal stem cells along with bone and cartilagenous tissue and forms the body's largest energy reservoir. Traditionally it was believed that energy storage, along with insulating and cushioning properties were its only functions. Under the influence of various hormones and cytokines surplus energy is stored in the form of triacylglycerides (TAGs). This reserve can then be mobilised during times of low calorific intake. However, it has now become clear that the adipocyte has major metabolic influence affecting many tissues and systems including muscle, the hypothalamus, clotting cascades, the pancreas and the immune system.

Two types of adipose tissue exist: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT is not normally thought to be present in significant quantities in humans beyond the neonatal period and is more prevalent in smaller mammals. However small foci of BAT have been found in human adult WAT and mRNA markers of BAT have been identified in adult human WAT utilising sensitive techniques (quantitative real time polymerase chain reaction) [88]. Moreover, in cases of phaeochromocytoma, an adrenomedullary tumor which secretes catecholamoines, larger amounts of BAT can develop [89] illustrating not only the importance of adrenergic drive in the stimulation of BAT production but also the potential capacity of human adult WAT to generate BAT. Cold exposure is also an important BAT modulator and this or forced adrenergic stimuli *in vivo* has been shown to increase the amount of BAT-specific genes [90]. The primary function of BAT is thermogenesis, hence its preferred locations, for example in the thorax surrounding the great vessels or in the interscapular region. BAT possesses a unique protein termed uncoupling protein-1 (UCP-1) which mediates this response, converting energy storage to energy expenditure; this is in turn dissipated in the form of heat [91]. BAT differs morphologically to WAT in that the fat droplets of triacylglycerides are smaller and BAT adipocytes contain a far greater number of mitochondria. As will be discussed in more detail later, both WAT and BAT are under the control of a complex cascade of transcription factors that can in turn be modified by various external stimuli. Peroxisome proliferator activated receptor-γ (PPARγ) is probably the most important of these transcription factors in both adipose types. In BAT a specific co-activator for PPARγ has been identified termed PPARγ cofactor-1 (PGC-1); this is involved in BAT activation following cold exposure [92].

WAT is distributed widely though preferentially accumulates in particular locations (e.g. subcutaneous, visceral (omental), gluteo-femoral). It is now known that WAT distribution is important in determining metabolic risk, notably centrally distributed visceral or omental WAT is associated with the development of the metabolic syndrome [93]. This term refers to a collection of risk factors which cluster in individuals with an increased central adiposity or waist circumference (raised triglyceride levels, reduced HDL cholesterol, increased insulin resistance). WAT distributed subcutaneously or around the hips is not linked to such abnormalities to the same extent. The metabolic influence that WAT exhibits is in part a result of the production of an array of adipocytokines. The specific cytokines and their effects are summarised in Fig 1.8 and table 1.1 and will be discussed in more detail later in this chapter.




TNFα -Tumor necrosis factor-α
IL-6 - Interleukin 6
PAI-1 - Plasminogen activator inhibitor-1
MIF – Macrophage inhibitory factor

Fig1.8 Apidocytokines produced form adipocytes

| Adipocytokine | Metabolic effects |
|---|---|
| **Adiponectin** | Gluconeogenesis and glucose uptake<br>Lipid breakdown and removal (TAG clearance and β-oxidation)<br>Central appetite suppression and weight loss<br>Endothelial function |
| **Leptin** | Central (hypothalamic) suppression of appetite |
| **Resistin** | Activating inflammatory cytokines<br>Possibly insulin resistance |
| **TNFα** | Insulin resistance |
| **IL-6** | Inflammatory state |
| **PAI-1** | Antifibrinolytic<br>Insulin Resistance |
| **Tissue factor** | Coagulation |
| **MIF** | Inflammation<br>Insulin resistance |

Table 1.1 Adipocytokines and summary of metabolic effects

### 1.2.3 Adipogenesis and *in vitro* models

The process by which the precursor adipose cell develops and matures (or differentiates) is termed adipogenesis; a process with complex regulatory mechanisms that have often been determined with the use of *in vitro* models. These models have tended to use cells that are predetermined to develop into adipoctyes that have lost their multipotent potential, termed adipoblasts. Examples of such cells include NIH 3T3-L1 (murine WAT cell line) and PAZ6 (Human BAT cell line) which can be stimulated to undergo adipogenesis under controlled laboratory conditions. A more comprehensive review of these cells and adipogenesis models is provided in chapter 2.

### 1.2.4 Control of adipogenesis

Adipogenesis is controlled by a cascade of transcription factors that in turn can be influenced by extracellular factors. Rosen and Macdougald [94] termed the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) the 'master regulator' of adipogenesis. PPARγ is the most conserved member of the PPAR family and controls gene expression through heterodimerisation with the retinoic X receptor (RXR). Evidence for the critical importance of PPARγ in adipogenesis comes from several sources. For example PPARγ overexpression in isolation is sufficient to induce adipogenesis *in vitro* [95] whereas PPARγ-2 knockout mice exhibit reduced adipose tissue mass [96]. Furthermore PPARγ expression may be necessary to maintain mature adipocytes in the differentiated state [97]. Recent descriptions of dominant-negative mutations in the human PPARγ gene in subjects with partial lipodystrophy add more evidence to the key role of this gene in adipocyte differentiation [98]. An array of other transcription factors influence adipogenesis, often through either stimulation or inhibition of PPARγ (Fig 1.9) [99].

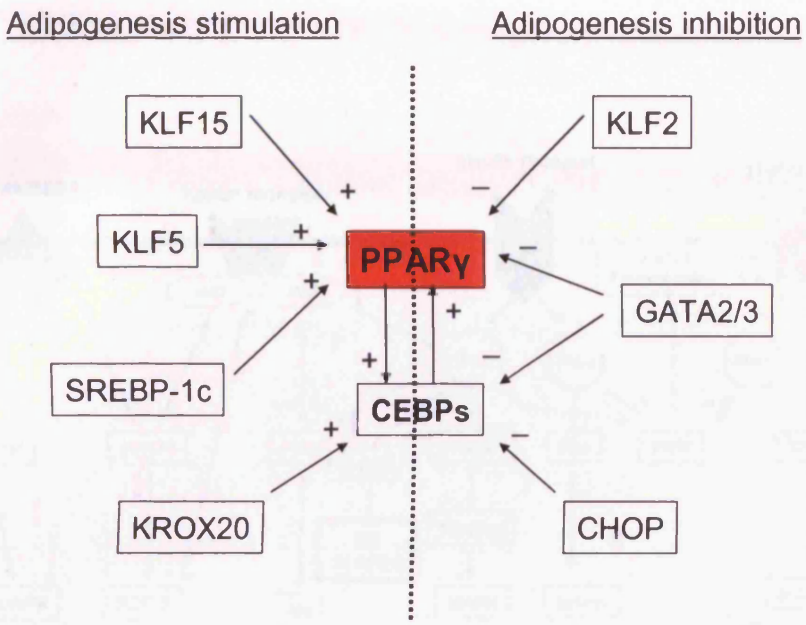
There are 3 PPARγ (PPARγ-1 and PPARγ-3 mRNA code for the same protein) isoforms derived from the same gene through the use of alternate promoters [100, 101]. PPARγ-1 is found in many cell types whereas PPARγ-2 is adipose tissue specific. PPARγ-3 has been found in adipose tissue, macrophages and colonic epithelium [100, 101]. The precise roles of these differing isoforms of PPARγ are yet to be determined.

Another family of transcription factors that play an integral role in adipogenesis are the CCAAT-enhancer binding protein (C/EBP) family which interact directly with PPARγ (C/EBP binding sites are located within the PPARγ-2 promoter) in a coordinated manner

to stimulate adipogenesis. A number of members of this family are involved in adipogenesis including C/EBPα/β/γ/δ and transcription factor homologous to CCAAT-enhancer binding protein (CHOP). C/EBPβ and δ are involved early in adipogenesis whereas C/EBPα influences the later stages. Glucocorticoids, known to promote adipogenesis, cause a transient rise in expression of C/EBPα and δ during differentiation [102]. C/EBPα acts to sustain PPARγ expression during differentiation but may have differential roles in WAT and BAT as C/EBPα knock-out mice have almost no WAT at all; however, BAT remains relatively unchanged [103].

Many other factors have a role in adipogenesis such as the Krüpple-like factors (KLF); these are zinc finger proteins some of which promote adipogenesis (KLF5/15/6) while others inhibit it (KLF2). KLF 15, as well as stimulating differentiation also enhances glucose transport through increased expression of glucose transporter 4 (GLUT4) [104, 105] while KLF2 inhibits PPARγ [106]. KROX20 is another pro-adipogenic factor that promotes C/EBPβ expression in 3T3-L1 cell differentiation [107]. Sterol response element binding protein-1c (SREBP1c) triggers PPARγ expression possibly by elaboration of a PPARγ ligand [108].

Anti-adipogenic factors include KLF2 and the GATA family of proteins which are reduced in adipogenesis and can inhibit the PPARγ-2 promoter [99].



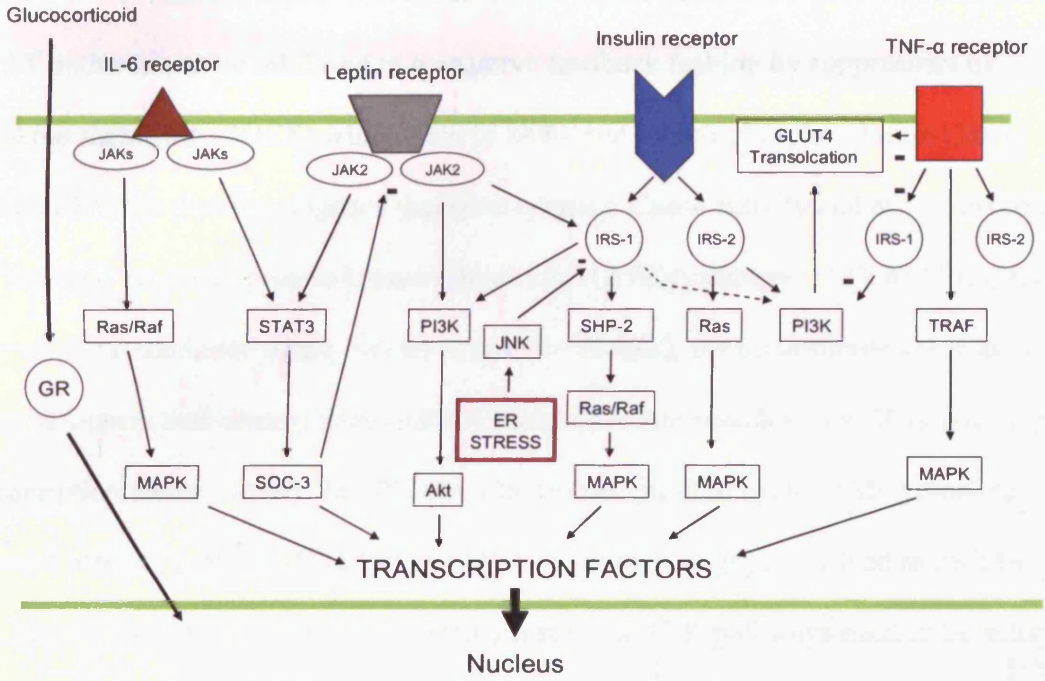

KLF 15/5/2 – Krüpple-like factors 15/5/2
SREBP1c – Sterol response element-binding protein-1c
CHOP – Transcription factor homologous to CCAAT-enhancer binding protein
PPARγ – Peroxisome proliferator activated receptor γ
CEBPs – CCAAT-enhancer binding protein family

Fig 1.9 Stimulatory and inhibitory transcription factors in adipogenesis

### 1.2.5 Adipocyte signal transduction

Adipogenesis is under the constant influence of extracellular factors that can alter expression of adipogenesis transcription proteins through binding to specific transmembrane receptors that in turn switch on (or off) a variety of cell signalling pathways. These cell signalling pathways demonstrate considerable cross-talk such that

the balance of extracellular constituents at any one time will define adipose cell transcript expression rather than any one single factor (Fig 1.10).




GR – glucocorticoid receptor
JAK- Janus Kinase
MAPK- Mitogen activated protein kinase
STAT-signal transducers and activators of transcription
PI3K- Phosphatidyl inositol 3 kinase
IRS- Insulin receptor substrate
GLUT-glucose transporter

SOCS-3- Suppressor of cytokine signalling-3
Akt- protein kinase B
TRAF-TNF receptor associated factor
ER – Endoplasmic reticulum
JNK- c-jun N-terminal kinase

Fig 1.10 External factors and adipocyte cell signalling adapted form Frühbeck *et al* [109]
Adipoctye cell signalling involves a number of signal transduction pathways activated by cell surface receptor-ligand binding that stimulates phosphorylation/dephosphorylation and thus pathway activation.

Janus kinases (JAK) are intracellular tyrosine kinases involved in the JAK-STAT (signal transducers and activators of transcription) cell signalling transduction pathway. When

the cell surface receptor is activated by ligand binding (e.g. leptin binding to its receptor) 2 JAKs dimerise by autophosphorylation. This then enables phosphorylation and activation of STATs. STAT proteins as their name suggests can enter the nucleus and directly affect transcription or can act as further signal transducers [110]. This JAK-STAT pathway can be inhibited in a negative feedback fashion by suppressors of cytokine signalling (SOCS) which bind to JAKs and inhibit phosphorylation [111].

Ras and Raf are proto-oncogenes that have tyrosine kinase activity and are involved in the mitogen activated protein kinase signalling (MAPK) pathway [111]. MAPKs, also known as extracellular signal regulated proteins (ERKs), are microtubule associated protein kinases that can modulate mRNA translation into proteins as well as affecting transcription factor activity. MAPK can alter transcription of cyclic AMP response element binding proteins (CREBs) and MYC, a proto-oncogene involved in cellular proliferation and differentiation. It appears that the MAPK pathways need to be activated to initiate preadipocyte differentiation and then deactivated to allow differentiation progression. This is illustrated by the observation that MAPK phosphorylation is required for 3T3-L1 cell proliferation. In contrast during later stage differentiation MAPK phosphorlyates and so deactivates PPARγ [112]. The importance of MAPK signalling in adipogenesis is also demonstrated by studies in MAPK knockout mice which display reduced amounts of body fat [113].

Akt, also known as protein kinase B is a serine/threonine specific protein kinase involved in protein synthesis and inhibits apoptosis. It has a protein domain that binds phosphoinositides e.g. Phosphatidyl inositol 3 kinase (PI3K). PI3K can be activated by the insulin receptor via IRS-1. Akt is involved in the translocation of the glucose

transporter 4 (GLUT4) to the cell membrane following insulin stimulation; Akt knockout mice thus develop diabetes [114]. Activation of the insulin receptor phosphorylates the Insulin receptor substrate (IRS). Activity of these increases the amount of cell surface GLUT4, as well as triggering the signalling cascade which culminate in MAPK activation and altered transcription. Tumour necrosis factor associated factors (TRAFs) are major signal transducers for the TNF system and MAPK is also a terminal component of this protein cascade [115].

A number of other signalling pathways other than those described are involved in adipogenesis such as the Wnt pathway [116], transforming-growth factor β and bone morphogenic protein pathways, Notch and DLK1/PREF-1 pathways as well as others. Hormones that exert effects via nuclear receptors such as cortisol, testosterone or oestradiol can also influence transcription directly with the binding of the hormone receptor complex to the appropriate DNA response elements. Glucocorticoids for example are known to promote preadipocyte differentiation into mature adipoctyes [117]. Other intra-adipose tissue factors can also influence cell signalling. In mice with an impaired endoplasmic reticulum stress response, insulin resistance develops through over-activation of c-jun N-terminal kinase (JNK) leading to phosphorylation of IRS-1 [118].

### 1.2.6 Adipocytokines

The adipocyte has important endocrine and paracrine capabilities mediated through the production and action of numerous substances known as adipocytokines (Fig 1.8 and table 1.1) which exert effects over neighbouring as well as distant cells and tissues.

Adiponectin is a 22 amino acid polypeptide whose serum concentrations correlate inversely with body fat mass. It exists in 2 form: a low-molecular weight dimer and a high molecular weight complex [119] which may be more metabolically active. Adiponectin concentrations are lower in subjects with type II diabetes [120] but are higher in women compared to men [121]. Concentrations can be increased by exercising, perhaps explaining the benefits of exercise in improved insulin sensitivity, probably through reduced hepatic glucose production and increased fatty acid β-oxidation in skeletal muscle [122]. Adiponectin may thus protect against the development of insulin resistance, type II diabetes and coronary artery disease.

Tumour necrosis factor-α (TNF-α) is a 212 amino-acid transmembrane protein which is secreted by a number of cell types including adipocytes and macrophages. Serum concentrations of TNF-α rise with increasing obesity [123]. It functions predominantly as a regulator of the immune response but its metabolic effects include induction of insulin resistance through serine phosphorylation of IRS-1 [124].

Leptin is a 167 amino acid protein secreted by WAT and to a lesser extent BAT. It binds to receptors in the appetite centers located within the ventral medial nucleus of the hypothalamus and may act as a satiety signal [125]. Leptin levels rise in parallel with increasing adipose mass and acts as a signal centrally indicating the relative amount of adipose tissue stores. Leptin secretion has also been implicated in the development of insulin resistance partially through serine phosphroylation of IRS-1 [126] though other lines of evidence suggest a beneficial effect of leptin on glucose tolerance [127, 128].

PAI-1 is an inhibitor of the fibrinolyic system that is mostly produced by the vascular endothelium but is also produced by adipocytes. PAI-1 levels correlate with levels of

insulin resistance and it has been suggested as a possible link between obesity and insulin resistance [129].

Resistin is a 108 amino acid protein whose levels correlate with obesity. It has been suggested that it plays a role in the development of insulin resistance [130]; however other studies have refuted this claim [131]. What is not in doubt is that it can function as a proinflammatory cytokine and can activate other inflammation associated cytokines such as IL-6.

Interleukin-6 (IL-6) concentrations also correlate with body mass index. Although it can be produced from many cell types it is thought that up to a third is derived from adipose tissue. IL-6 concentrations have been positively associated with cardiovascular and all cause mortality [132] and is likely to be a component of the chronic inflammatory state observed in obese individuals [133].

Tissue factor is a potent initiator of the coagulation cascade and is related to the cytokine class II receptor family. Expression of tissue factor has been shown to be upregulated in adipocytes of genetically obese mice which could, in part, explain the hypercoagulable state that is linked to obesity [134].

Macrophage inhibitory factor (MIF) has also been implicated in the pathophysiology of insulin resistance [135] and weight loss has been shown to inhibit its production [136].

This list of adipocytokines is not exhaustive; new proteins are continually being characterised (e.g. Retinol binding protein-4 [137]).

In summary, a wide variety of hormones and cytokines will influence adipose tissue growth and development via the action of numerous cell surface or nuclear receptors and through the action of a cascade of interlinked intracellular signalling pathways. A number

of investigators have sought to examine the impact that DHEA has on adipose tissue and the adipocyte, and also to determine the underlying mechanisms in any observed affect.

### 1.2.7 Effect of DHEA on adipose tissue: *in vitro* and animal studies

Gordon *et al* showed that DHEA inhibited the differentiation of the murine 3T3 fibroblast cell line into mature adipoctyes [138], in keeping with other observations of reduced proliferation with DHEA in this cell line [139]. Accepting the limitations of rodent models, DHEAS supplementation in rats fed a high fat diet reduced carcass lipid, fat depot mass and retroperitoneal and epidydimal adipocyte number [140]. A further study by Hansen *et al* showed that DHEA protected against the increase in visceral fat and the decrease in insulin-stimulated glucose uptake in male Wistar rats fed a high fat diet [141]. This effect on glucose uptake and insulin sensitivity was corroborated by Ishizawa *et al* who showed that glucose uptake was related to alterations in the activation of phospholipase D [142]. They later demonstrated the involvement of protein kinase C and phosphatidylinositol 3-kinase in the glucose uptake process [143] and also a down regulation of PPAR-γ expression through DHEA treatment [144]. DHEA has also been shown to up-regulate resistin [145]. An *in vitro* study by Hernandez-Morante *et al* that utilised human adipose tissue samples cultured in DHEAS for 24 hours demonstrated upregulation of adiponectin gene expression in omental adipocytes [146].

These preliminary studies suggest that DHEA supplementation may have a beneficial effect on adipocyte number and function though the physiological relevance to humans requires further clarification.

### 1.2.8 Effect of DHEA on adipose tissue: human studies

In contrast to most *in vitro* and animal model studies, which have demonstrated an inhibitory action of DHEA on adipocyte mass, observational and interventional studies in human subjects have failed to demonstrate such convincing results. The various studies are reviewed in a paper by Tchernof and Labrie [147]. In both men and women, investigators have demonstrated a negative correlation between non-sulphated DHEA and measures of obesity (Body mass index, percentage fat) [5, 6, 148-151]. In addition, an increase in central adiposity, as measured by waist hip ratio or computer tomography (CT) assessment of visceral fat area, has been observed in men with lower DHEA levels [5, 6, 148]. However the findings in relation to DHEAS are inconsistent [7, 148-166].

A number of interventional studies have examined the impact that treatment with DHEA has on body composition and plasma lipids. The majority of these clinical trials used a randomised controlled design, either in parallel groups or cross-over. Some have used populations with adrenal failure [12, 167, 168], others have used healthy [169-175], menopausal [176, 177] or obese [178, 179] populations and one examined a group of middle aged men with hypercholesterolaemia [180]. Doses used (25-1600mg daily) and treatment phase duration has also varied (4-52 weeks). As in the observational studies results have been inconsistent. A number of studies demonstrated improvements in measures of body fat (fat mass, percent body fat and skin fold thickness) [169, 174, 176] whereas others showed no effect, including all studies in adrenal failure. The most consistent potentially beneficial finding was a reduction in total cholesterol [169, 171, 176, 181]. However a number of the studies also demonstrated a reduction in HDL-cholesterol, a potentially negative effect on the lipid profile [171, 176, 177, 181, 182] .

The discordant results seen in the human studies could be as a result of many factors. In the interventional studies DHEA dose and treatment duration has varied widely as have study populations and age ranges involved. Furthermore, observational studies may show association but do not necessarily imply causation: low DHEA levels in obesity may represent an epiphenomenon as chronic disease induces a shift of intra-adrenal biosynthesis that favours cortisol over DHEA. The physiological importance of DHEA in relation to stage of life may also be a factor and is discussed in a paper by Ebeling and Koivisto [183]. They suggest that the effect of DHEA may depend on the hormonal status at the time of treatment and discuss the different effects that DHEA replacement could have in the pre and postmenopausal states (oestrogen abundant versus oestrogen deplete). Thus treatment with DHEA may well be of benefit in a particular group or subgroup of individuals at a particular point in life. Further, the possibility of a sexually dimorphic response should also be considered and has been discussed earlier in this chapter.

## 1.3 DHEA and cardiovascular disease

### 1.3.1 Overview

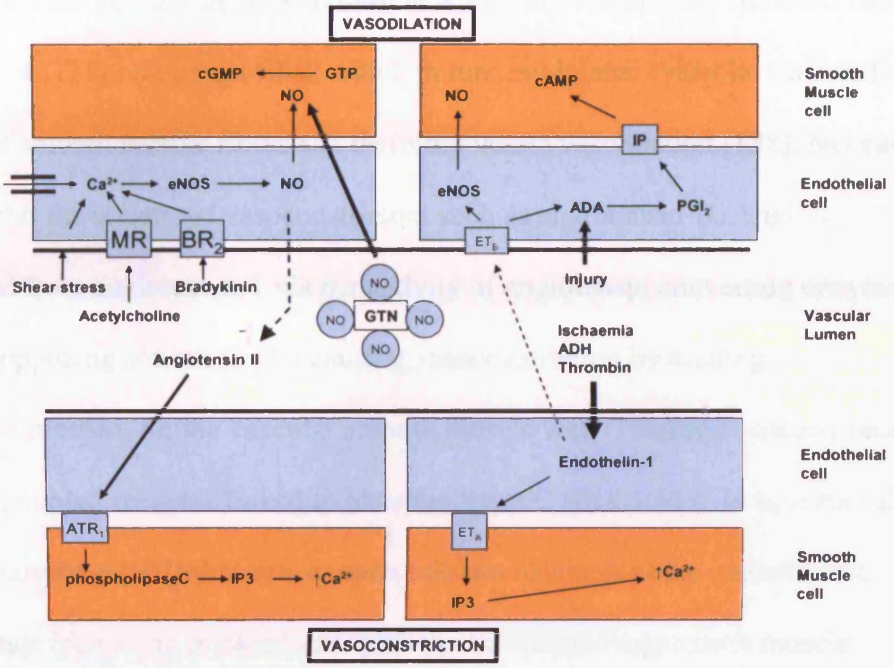
Cardiovascular morbidity and mortality occur as a result of atherosclerosis, a disease process which begins subclinically in childhood before manifesting itself through ischaemic events and myocardial infarction in later life. The earliest events in this process involve significant dynamic alterations in vascular biology [184] with the emergence of

the endothelium and arterial compliance as key regulators of vascular well-being [185, 186].

Epidemiological studies in aging populations have demonstrated a gender-specific inverse relationship between DHEAS levels and cardiovascular mortality in men [8] [9] though few interventional studies have examined the benefits (or risks) of DHEA supplementation on vascular outcome. Recent studies in middle-aged men [180] and elderly populations [187] have suggested a beneficial action of DHEA supplementation on endothelial function in the short term though no studies have yet examined these outcomes in pathological DHEA-deficient states. Interventional trials of physiological DHEA replacement in subjects with DHEA deficiencies may therefore be important in clarifying the vascular benefits of this compound.

### 1.3.2 The vascular endothelium

The vascular endothelium is a dynamic structure with the ability to modify blood vessel diameter in response to a variety of stimuli (posture, stress, exercise) through the release of, and in response to a number of vasoactive substances (Fig 1.11).




NO-nitric oxide
eNOS- endothelial nitric oxide synthase
MR-Muscarinic receptor
BR- Bradykinin receptor
GTP-Guanosine triphosphate
cGMP- cyclic guanosine monophosphate
ET-Endothelin receptor
cAMP- cyclic adenyl monophosphate
ADA-Arachidonic acid

$PGI_2$-Prostaglandin $I_2$
IP-Prostaglandin receptor
ADH- Antidiuretic hormone
ATR-Angiotesin II receptor
IP3-Inositol triphosphate
GTN- glyceryl trinitrate

Fig 1.11 Modulators of vascular tone

In health, vascular tone is maintained via the action of vasodilators and vasoconstrictors. Nearly all substances that induce vasodilation do so through nitric oxide (NO). NO is produced within the endothelium from the amino acid L-arginine via the activity of endothelial nitric oxide synthase (eNOS) [188]. Shear stress is the most important stimulating factor for NO production [189]; however, acetylcholine acting on muscarinic receptors (M1 and M3) and bradykinin acting on bradykinin receptors (BR2) [190] can also activate calcium channels in endothelial cells and generate NO via eNOS. NO is a lipophilic gas that can cross cell membranes. In the vascular endothelium NO crosses the

intima to reach the bundles of smooth muscle within the vessel wall. Here NO causes the degradation of GTP, releasing cGMP which in turn modulates cytosolic $Ca^{2+}$ and causes relaxation of smooth muscle fibres and therefore vessel vasodilation [188]. NO can also directly inhibit the actions of vasoconstrictors such as angiotensin-II. Angiotensin-II (AT-II), produced from angiotensin-1 via the activity of angiotensin converting enzyme (ACE), has opposing actions to NO causing vasoconstriction by binding to the angiotensin-1 receptor on the vascular smooth muscle wall. The angiotensin-1 receptor is a G protein-coupled receptor linked to phospholipase C (PLC). PLC in turn stimulates inositol triphosphate (IP3) that acts to open calcium channels at the endoplasmic reticulum, thus increasing intracellular calcium and stimulating smooth muscle contraction [191]. Another potent vasoconstrictor is endothelin-1. Endothelin-1 is generated within the endothelium from precursor molecules (preproendothelin and big endothelin). Endothelin-1 acts on the endothelin A receptor on the vascular smooth muscle wall in response to ischaemia to increase IP3 and intracellular calcium, in turn stimulating contraction [192]. Interestingly, endothelin-1 can also cause vasodilation through action on the endothelin B receptor. This triggers smooth muscle relaxation and vasodilation through prostaglandin $I_2$ ($PGI_2$) production from arachidonic acid and by stimulating eNOS. $PGI_2$, also known as prostacyclin, acts on the prostaglandin receptor (IP) to increase cAMP within the vascular smooth muscle cells inducing smooth muscle relaxation. The nitrate group of pharmacological agents classically used to treat angina (e.g. glyceryl trinitrate) are NO donors and effectively deliver NO directly to the vascular smooth muscle cells, bypassing the endothelial NO generating system. The type of

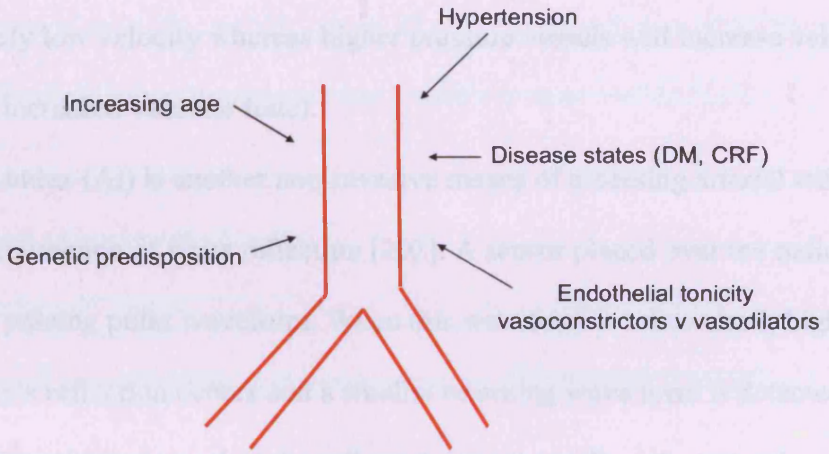
vasodilation stimulated by NO donors, in contrast to acetylcholine and other agents that act directly on endothelial cells, is therefore endothelial independent.

A fine balance exists between the various vasodilating and vasoconstricting substances, the combination of these substances along with the intraluminal microenvironment will govern vascular tonicity. An inability to respond to these stimuli or dysregulation of these mechanisms is known as endothelial dysfunction, a condition which precedes the development of atherosclerotic plaques, contributing to lesion development and subsequently clinical complications [193].

### 1.3.3 Arterial stiffness

Increased large artery stiffness is a function of aging and relates to a reduced ability of blood vessels to expand and recoil in response to pulsed cardiac output. This dynamic ability facilitates transduction of the intermittent cardiac pulse into steady blood flow and moves away from the traditional concept of blood vessels acting solely as conduits. The proximal aorta will dilate in response to increased volume generated in systole with an associated higher pressure (systolic pressure) and will then recoil during diastole, maintaining a lower pressure (diastolic pressure) and thus flow. The pulse pressure (difference between systolic and diastolic pressures) therefore, is both a reproducible and easily measured surrogate marker of artery stiffness. Increasingly stiffened vessels will have a reduced ability to accommodate and distend in response to the systolic pulse and so higher systolic pressures will be generated along with lower diastolic pressures secondary to impaired elastic recoil.

A number of factors are now known to regulate arterial stiffness (Fig 1.12). Traditionally it was thought to be secondary solely to alterations to structural components within the vascular wall such as elastin and collagen, both of which are located at the intimal medial layer. At higher pressures collagen primarily maintains vessels stiffness (e.g. in systole) whereas elastin functions at lower pressures. The ratio of elastin to collagen can therefore affect vessel stiffness whereby low elastin-to-collagen ratios are consistent with increased stiffness. Smooth muscle hypertrophy will also contribute to increasing vessel stiffness. However it is now clear that structural changes are not the only modulator of arterial stiffness as it is inexplicably linked to the vascular endothelium and the balance between vasodilating and vasoconstricting substances [194] as well as the inflammatory response [195].




DM – diabetes mellitus
CRF – chronic renal failure

Fig 1.12 Factors influencing large artery stiffness

Arterial stiffness has been shown to be an independent predictor of future cardiovascular disease [186] and may play a role in the development of hypertension [196] and atherosclerosis [197] as well as coronary artery disease. Further, it predicts cardiovascular risk in a number of disease states including dialysis dependent end-stage renal failure, hypertension and diabetes [198-200].

Pulse wave velocity (PWV) estimation or imaging (mostly ultrasound based) techniques can be used to determine arterial stiffness. Pulse wave velocity estimates stiffness by employing sensors placed cutaneously over a proximal (e.g. carotid) and distal (e.g.radial) palpable pulse to detect passing pulse waveforms. Velocity is then calculated as the distance between the 2 sensors (in meters) divided by the time taken for the waveform to pass from proximal to distal sensor (in seconds); this will be influenced by wall thickness, vessel radius and blood pressure [201]. A highly tortuous vessel may generate a falsely low velocity whereas higher pressure vessels will increase velocity (hypertension, increased vascular tone).

Augmentation index (Ai) is another non-invasive means of assessing arterial stiffness that utilises the phenomenon of wave reflection [202]. A sensor placed over the radial artery will detect the passing pulse waveform. When this waveform reaches small, high-resistance vessels reflection occurs and a smaller returning wave form is detected at the same sensor. The speed of travel of the reflected wave (as well as the out-going wave) is influenced by vessel stiffness and will travel at a higher velocity with increasing vessel stiffness such that the time difference between the out-going and reflected wave detected by the sensor will shorten. When a threshold velocity is reached the reflected wave will begin to summate or augment the out-going wave and the degree with which this

augmentation occurs is termed the augmentation index (Ai). Ai is thus a surrogate marker of arterial stiffness (the greater the extent of augmentation the stiffer the vessel). Ai will be influenced by vessel length which is often a function of height, gender differences are thus evident, Ai being lower in men than in women as well as blood pressure.

Both PWV and Ai can be measured non-invasively and with a high degree of reproducibility [203] and so are ideally suited for use in the context of clinical trials. The methodology involved in the measurements of PWV and Ai will be discussed in more detail in chapter 4.

Direct imaging techniques are another means of determining vessel stiffness and allow *in vivo* visualisation of wall thickness and vessel diameter. These techniques require considerable training and can be time consuming. Newer techniques still, such as MRI, are able to visualise entire vessels as well as flow and can therefore provide the most accurate measures of velocity, but the current expense and limited availability of these techniques render them, for now at least, inaccessible to most researchers.

### 1.3.4 DHEA and vascular dysfunction

A growing number of studies have attempted to address whether the age-related reduction in DHEA/DHEAS concentration is linked to diseases of the elderly including cardiovascular disease.

Cross-sectional and prospective epidemiological studies have shown an inverse correlation between DHEA/DHEAS levels and cardiovascular mortality, initially in older men [8, 204] and subsequently in young men [205] and pre and postmenopausal women

[206, 207]. A similar inverse relationship has also been found for DHEA/DHEAS concentration and cardiac graft stenosis as well as atherosclerosis extent [208, 209].

In lower order animals, supplementation of DHEA has been shown to reduce atherosclerosis [210] and potential mechanisms have been identified through *in vitro* studies. Liu and Dillon were able to demonstrate that DHEAS stimulates NO release from vascular endothelial cells through the activity of a plasma membrane G-protein ($G\alpha_{i2,3}$) coupled receptor [81, 211]. Further studies have shown inhibition of human vascular smooth muscle cell proliferation by DHEA involving the MAPK signalling pathway [212]. The same group also demonstrated an inhibitory action of DHEA on proliferation of bovine aortic endothelial and human umbilical vein endothelial cells [187, 212].

Interventional studies have shown beneficial effects on both established (cholesterol and body fat) [169, 171] and emerging (platelet aggregation and plasminogen activator type 1) [213, 214] cardiovascular risk factors as well as improvements in endothelial function, measured by flow-mediated dilatation, in middle aged men with hypercholesterolaemia [180] and in post-menopausal women [187].

The evidence would thus seem to support a beneficial effect of DHEA on cardiovascular risk, though the mechanism of this action, its gender dependence and reproducibility in pathological DHEA deficiency states have not been fully determined.

### 1.3.5 Testosterone and oestrogen: effects on the metabolic syndrome and vascular status

Clearly any effect that is proposed to be a consequence of DHEA deficiency or replacement may be a result not of DHEA itself, but secondary to the generation of active

terminal metabolites such as testosterone and oestradiol. Indeed Labrie's review on the mechanisms of DHEA action suggests that it is not a hormone at all and acts solely as a prohormone up-stream of terminal sex steroids [75]. As discussed earlier in this chapter, there are numerous studies that suggest this may not be the case but what is evident is that both testosterone and oestradiol themselves can exert actions on the various components of the metabolic syndrome (discussed further in chapter 3) as well as vascular health.
In post-menopausal females oestrogen ameliorates menopausal symptoms and protects against bone mineral density loss. Oestrogen may also have important vascular and biochemical effects in males. For example, men with abnormalities in oestrogen synthesis due to mutations in the aromatase gene develop early and advanced cardiovascular disease as well as increased levels of insulin, TAGs and LDL cholesterol [215-217] (all features of the metabolic syndrome). These inborn metabolic errors are discussed in more detail in chapter 4 and illustrate the importance of oestrogen on vascular function in males. Further, randomised trials of aromatase inhibition in young men showed impaired endothelial function, confirming that endogenous oestrogen production has an important role in maintaining vascular health in men [218]. Oestrogen can also stimulate the generation of the potent vasodilator nitric oxide through both genomic and non-genomic mechanisms again alluding to important vascular effects. Some authors have thus suggested that DHEA may exert its potential anti-atherosclerotic benefits through generation of oestrogen [219] though others have failed to confirm these findings [220].

Testosterone is also linked with vascular and metabolic health. Numerous observational studies show a clear association of subnormal testosterone concentrations in adult males

with the metabolic syndrome, obesity and type II diabetes [221] Furthermore, testosterone replacement can improve anthropometric measures of excess adiposity [222-224] and, in men with type II diabetes, improves insulin sensitivity and glycosylated haemoglobin levels [222] These benefits may be accompanied by changes to the adipocytokine profile with a reduction in leptin, indicating a reduction in fat mass, but also adiponectin [223] The mechanisms by which testosterone improves these parameters are not fully understood although one potential pathway may involve the androgen receptor whereby polymorphic variation in the CAG repeat sequence may influence body composition and testosterone levels [224]. Interventional studies have also shown that testosterone replacement improves functional capacity in men with heart failure [225] and augments ischemic thresholds in men with angina [226]. Further, testosterone has been shown to attenuate fatty streak formation in a mouse model of androgen insensitivity [227] suggesting mediation through mechanisms independent of the nuclear androgen receptor. Studies in men with prostate cancer undergoing induced hypogonadism also support an important role for testosterone in maintenance of vascular and metabolic health, with increases in measures of body fat and deterioration in insulin sensitivity [221] as well as impaired endothelial functioning [228] being observed.

Testosterone also influences lipid parameters, with the most consistent finding being a reduction in HDL cholesterol. However, this may not necessarily represent a detrimental change as the HDL affected demonstrates enhanced reverse cholesterol transport capabilities[229]. Further testosterone affects HDL subclasses differently with HDL3 reduced but HDL2 remaining unchanged [230]. It is not clear if this effect has long-term adverse cardiometabolic consequences. In contrast, testosterone has been shown to

increase HDL cholesterol concentrations in mice, although the mechanisms accounting for these inter-species differences are presently unclear [227]. Further beneficial lipid effects *in vivo* have also been shown with testosterone causing a reduction in total cholesterol [222]. It is clear from these discussions that both testosterone and oestrogen are intimately liked to lipid and vascular homeostasis such that any potential benefits of DHEA therapy on vascular integrity may well be mediated through downstream conversion.

### 1.4 DHEA and adrenal insufficiency

Adrenal insufficiency refers to a deficiency in hormones produced from the adrenal cortex and is classified as either primary, where the disease process occurs in the adrenal glands themselves, or secondary, where the deficiency is due to disturbance at the level of the hypothalamus and/or pituitary. Glucocorticoids (cortisol), mineralocorticoids (aldosterone) and the adrenal androgens exhibit varying degrees of deficiency in this group of conditions. These hormones are generated in different adrenocortical zones (Fig 1.13): cortisol and the adrenal androgens are, as previously discussed, synthesised in response to the pulsatile and rhythmic release of pituitary-derived ACTH controlled by negative feedback (Fig 1.6), while aldosterone production is largely under the control of the renin angiotensin system with comparatively little influence by ACTH. Aldosterone is intimately involved in the control of fluid balance, blood pressure and $Na^+$ homeostasis.

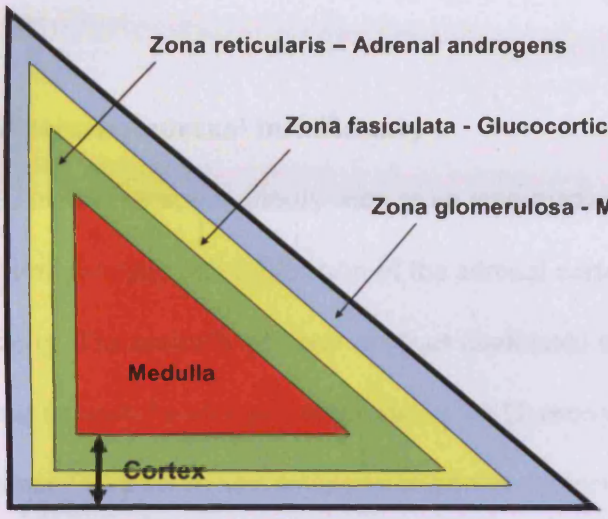



Fig 1.13 Functional zones of the adrenal cortex

### 1.4.1 Primary adrenal insufficiency

Primary adrenal insufficiency refers to conditions causing atrophy or destruction of adrenal cortical tissue resulting in reduced hormone production. This is now commonly known as Addison's disease after its first description in 1855 by Thomas Addison as a pairing of hyperpigmentation and wasting, a universally fatal condition at the time. In Addison's day the commonest cause for the condition was tuberculosis and this still remains problematic in less developed countries. The acid fast bacilli of tuberculosis have a particular predilection for adrenal tissue and up to 5% of individuals with active TB have adrenal involvement [231]. With the emergence of antimicrobial therapy, the incidence of Addison's disease has fallen, in the developed world at least, to current

disease estimates of 4.7-6.2 per million with a prevalence of 93-140 per million [232, 233].

#### 1.4.1.1 Causes of primary adrenal insufficiency

Addison's disease is now most commonly seen as an autoimmue process characterised histologically by lymphocytic cell infiltration of the adrenal cortex leading to macroscopic atrophy. The majority of cases are thus associated with high titres of antibodies directed against the adrenal cortex and/or 21-Hydroxylase enzyme [234]. Subjects with primary adrenal failure develop compensatory increased ACTH as a result of reduced negative feedback leading to hyperpigmentation through activation of melanocytes via the MC1-receptor.

In common with other autoimmune conditions Addison's disease occurs more commonly in females either in isolation or as part of a wider polyendocrine syndrome (APS 1 and 2). Other causes of primary adrenal failure are less common and are outlined in table 1.2.

| | |
|---|---|
| **Autoimmune** | Isolated<br>APS 1 + 2 |
| **Infective** | Tuberculosis<br>HIV<br>Fungal (histoplasmosis) |
| **Infiltrative** | Sarcoidosis<br>Amyloidosis<br>Haemochromatosis |
| **Haemorrhagic** | Anticoagulants<br>Trauma<br>Meningococcal septicaemia |
| **Bilateral infarction** | Antiphospholipid syndrome |
| **Metastatic** | Breast carcinoma<br>Malignant melanoma<br>Others |
| **Congenital** | Congenital adrenal hyperplasia<br>Congenital adrenal hypoplasia<br>Adrenoleucodystrophy |
| **Surgical** | Bilateral adrenalectomy |
| **Pharmacological** | Metyrapone<br>Ketoconazole<br>Aminoglutethimide<br>Etomidate |

Table 1.2 Causes of primary adrenal insufficiency

### 1.4.1.2 Investigations in primary adrenal insufficiency

Primary adrenal failure is characterised biochemically by a reduced or absent cortisol response to ACTH stimulation in conjunction with a raised serum ACTH level. Random cortisol levels, provided they are interpreted in the correct clinical context, can be useful

in that a value of >600 nmol/L almost always excludes the diagnosis and a morning value <100 nmol/L is strongly suggestive. A Synthetic ACTH test (synacthen 250μg) is used to confirm or refute the diagnosis (normal response > 550nmol/L) [235, 236].

Further biochemical investigation involves testing for antibodies directed against the adrenal cortex or 21-hydroxylase enzyme that are present in over 80% of newly diagnosed cases [234]. Serum concentrations of DHEA are invariably low, particularly in women [12]; aldosterone concentrations (which are not routinely performed) tend to be low or low/normal with increased plasma renin activity [236]. If the precise aetiology is not immediately clear further investigations may be required, such as abdominal CT scans or other depending on clinical suspicion.

#### 1.4.1.3 Pharmacotherapy in primary adrenal insufficiency

The mainstay of treatment for Addison's disease is hormone replacement in the form of glucocorticoid (most commonly hydrocortisone) and mineralocorticoid (fludrocortisone). Typical dosage regimens over 24 hours are 20mg of hydrocortisone divided in 2 or 3 doses and 0.05-0.15mg of fludrocortisone. Maintenance doses of hydrocortisone are typically doubled during intercurrent illness. Adrenal crisis, which is potentially life threatening, requires emergency treatment with large intravenous doses of hydrocortisone in conjunction with large volumes of 0.9% sodium chloride solution to correct volume and sodium depletion.

### 1.4.1.4 Morbidity and mortality in primary adrenal insufficiency

Primary adrenal failure has been shown to negatively impact on health related quality of life [237] particularly in fatigue, depression and anxiety domains [12]. However until recently mortality data in Addison's disease were lacking partly due to the scarcity of the condition and it was assumed that the mortality rates in optimally replaced patients was equal to that of the background population [238]. However, a recent Swedish population based study found a 2-fold higher risk ratio for death in patients with Addison's disease attributable in the main to cardiovascular causes [239]. Further studies in other populations are required to confirm these findings, though an as yet unpublished series from Denmark shows similar results.

### 1.4.1.5 DHEA replacement in primary adrenal failure

A number of authors have now demonstrated improvements in measures of quality of life in response to DHEA in primary adrenal failure, such as anxiety, depression and physical correlates in women [12], and mood and well-being in both sexes [240]. These studies have culminated in the current utilisation of DHEA in this patient group on an *ad hoc* basis, in females in particular, who complain of a reduced quality of life and/or reduced libido.

The effect of DHEA supplementation has on metabolic factors has been investigated in this population though the results have been, in general, disappointing and the majority of studies have been performed on subjects who do not have a pathological deficiency (e.g. healthy, obese, postmenopausal, perimenopausal, men with hypercholesterolaemia, physiological deficiency). No study has demonstrated improvements in adiposity in this

group, though one study performed in women with primary adrenal failure demonstrated significantly increased insulin sensitivity following a 12 week treatment phase of 50mg DHEA daily [10]. A rise in IGF-1 following DHEA therapy in women has been demonstrated in a further study [12], which could also influence glucose handling.

A recent study evaluating the effects of long term DHEA treatment in Addison's disease demonstrated a reversal in a trend for bone loss at the femoral neck measured by dual energy X-ray absorpitometry (DEXA) scanning [240].

To date there has been no definitive evidence that DHEA replacement in this group has any beneficial effects on cardiovascular outcomes and this is largely due to a paucity of studies particularly with regards to measures of cardiovascular health. However a recent study did attempt to address this issue in females with Addison's disease but failed to demonstrate any improvements in endothelial function or MRI assessment of cardiac status following 6 months of DHEA supplementation [241]. Therefore on the basis of studies performed to date and despite some intriguing *in vitro,* animal and human interventional studies (though not in pathological deficiency) DHEA remains a treatment that benefits quality but not quantity of life in primary adrenal failure.

### 1.4.2 Secondary adrenal insufficiency

Secondary adrenal insufficiency occurs where there is an absence or a reduction in the synthesis and secretion of ACTH, and is more common than primary adrenal insufficiency with an estimated prevalence of 150-280 per million [242, 243].

### 1.4.2.1 Causes of secondary adrenal insufficiency

In clinical practice, secondary adrenal insufficiency occurs most commonly following hypothalamic-pituitary suppression due to exogenous glucocorticoid therapy. Excluding iatrogenic disease, most other causes are secondary to, or occur as a result of treatment for, a pituitary adenoma. Secondary adrenal failure is found usually in conjunction with other hormone deficiencies of the anterior pituitary gland (i.e. hypopituitarism). These deficiencies may be partial or complete in which case the deficiency state is referred to as panhypopituitarism. The causes of secondary adrenal failure are outlined in table 1.3.

| | |
|---|---|
| **Tumours** | Adenoma (functioning/non-functioning)<br>Craniopharyngioma<br>Meningiomas, gliomas, hamartomas<br>Dysgerminomas<br>Pinealomas<br>Metastasis (lung, breast, prostate) |
| **Vascular** | Infarction (apoplexy, Sheehan's syndrome, post CABG)<br>antiphospholipid syndrome,snakebite coagulopathy)<br>Giant internal carotid artery aneurysm |
| **Infiltrative/inflammatory** | Lymphocytic hypophysitis<br>Sarcoidosis<br>Histiocytosis<br>Wegener's granulomatosis<br>Haemochromatosis<br>Vasculitis (e.g. systemic lupus erythematous) |
| **Infective** | Tuberculosis, Brucellosis, abscess<br>HIV, Syphilis |
| **Trauma/destruction** | Basal skull fracture with pituitary stalk transection<br>Pituitary surgery and/or radiotherapy<br>Cranial irradiation for central nervous system malignancy |
| **Functional** | Childhood deprivation<br>Chronic disease<br>Weight loss/low BMI<br>Anorexia nervosa<br>Athlete syndrome |
| **Congenital** | Single/multiple hormone deficiencies |

Table 1.3 Causes of secondary adrenal insufficiency/hypopituitarism

### 1.4.2.2 Investigations in secondary adrenal insufficiency

The biochemical diagnosis of secondary adrenal insufficiency, like primary adrenal insufficiency, is based on a reduced or absent cortisol response to a stimulus but unlike primary adrenal failure this is found in conjunction with undetectable or inappropriately low ACTH. There is often other associated hormone deficiencies depending on aetiology and the majority can be confirmed by basal blood sampling. Dynamic stimulatory tests

are however required to assess both integrity of the HPA and GH axis. The gold-standard investigation remains the insulin tolerance test which assesses cortisol and GH responses to induced hypoglycaemia (glucose <2.2 mmol/L). Further investigations are required to determine the cause including pituitary imaging (CT or MRI) and, if suspected investigations for hypersecretory pituitary adenomas.

#### 1.4.2.3 Pharmacotherapy in secondary adrenal insufficiency

The cornerstone of the management of secondary adrenal insufficiency is glucocorticoid replacement and doses identical to those used in primary adrenal failure are utilised. However, unlike primary adrenal failure mineralocorticoid replacement is not required, this being primarily under the influence of the renin-angiotensin-system. Frequently other anterior (thyroxine, testosterone/oestrogen, GH), and occasionally posterior (desmopressin), pituitary hormones are replaced simultaneously.

#### 1.4.2.4 Morbidity and mortality in secondary adrenal insufficiency

Quality of life is impaired in secondary adrenal failure although in this group this is not likely to be due solely to adrenal insufficiency. A number of studies have demonstrated an adverse impact on long term survival [244-247] and cardiovascular disease is again implicated here. A number of explanations have been put forward for this increased risk, including untreated GH deficiency, suboptimal gonadal hormone replacement, unphysiological glucocorticoid replacement (which may induce fat accumulation, hypertension and insulin resistance) and the use of radiotherapy. However no studies have examined the potential role of DHEA deficiency on cardiovascular risk to date.

### 1.4.2.5 DHEA replacement in secondary adrenal insufficiency

DHEA has been shown to be reduced in secondary adrenal failure [248] and, as in primary adrenal failure, replacement of DHEA in women with hypopituitarism has been shown to improve both libido and activity as judged by the patient's spouse after 6 months of treatment [249]. Clinical trials evaluating metabolic effects of DHEA replacement in hypopituitarism are scarce. However, it has been demonstrated that DHEA supplementation in hypopituitary patients on GH can stimulate an increase in IGF-1 levels necessitating a reduction in GH dosage [83]. No study to date has, however, specifically examined the effect that DHEA replacement has on markers of cardiovascular risk in this patient group.

The presence of pathologically reduced DHEA/DHEAS concentrations in primary and secondary adrenal insufficiency suggests that these are ideal conditions in which to examine the influence of DHEA replacement on cardiometabolic risk.

The evidence discussed in this chapter may point to beneficial alterations within the cardiovascular risk profile in response to treatment with DHEA. We hypothesised that DHEA would have a beneficial effect on endothelial function, arterial stiffness and cardiovascular risk markers in primary and secondary adrenal failure and postulated that these effects might occur, at least in part, through the modulation of body fat and the metabolically active substances that it generates.

## 1.5 AIMS

1. To assess the effect that DHEA has on adipose tissue by examining the influence of DHEA on preadipocyte cell lines and primary cultures and to investigate the mechanism behind any observed effects.
2. To determine whether treatment with DHEA in a group of patients with adrenal insufficiency has a beneficial effect on endothelial function and markers of cardiovascular risk.

# Chapter 2

# Effects of DHEA on proliferation and differentiation in preadipocyte cell lines

## 2.1 From body composition to the preadipocyte

The observation that DHEA supplementation can cause a reduction in body fat in both animal and human studies has been discussed in chapter 1. At the level of the adipocyte a number of possible mechanisms exist that may explain this. Any agent that has a negative effect on fat may do so directly or indirectly by reducing the number of fat cells *per se*, inhibiting the ability of the precursor cells to proliferate and/or differentiate or finally by reducing the amount of lipid present within the individual fat cells (induction of lipolysis or inhibition of lipogenesis). For the purpose of this study I have chosen to examine whether DHEA has a direct and negative impact on adipose tissue and have chosen to focus on the precursor fat cell the preadipocyte, which allows us to explore the possibility of inhibition on both proliferation and differentiation.

The preadipocyte represents an intermediate stage in the development of functioning cells of mature adipose tissue. Preadipoctyes are derived from the same embryological tissue as muscle and cartilage, termed mesoderm. During the third trimester of foetal development, pockets of what will form future adipose tissue organise around sites of large vascular structures (neck, shoulder, pelvis) and these sites retain their adipose tissue concentrations into adult life. The development of adipose tissue and vascular structures appears to be closely linked and, as discussed in the review by Ailhaud, Grimaldi and Négerl, adipose tissue secretes a number of substances that can modulate angiogenesis such as transforming growth factor-β (TGF-β); the release of these factors could explain the developmental link between these two systems [250]. The possibility of increasing the number of adipocytes that

an individual possesses after this developmental period is debatable. In cases of obesity, since mature adipocytes do not proliferate, immature precursor cells retained within mature adipose tissue may be stimulated to differentiate, and/or proliferate, in response to environmental and/or genetic factors. 'Fatness' therefore may be secondary to a combination of increasing lipid content of the adipocytes that then enlarge (adipocytes can vary in size from 20-200μm in diameter) coupled with the proliferation/differentiation of these latent precursors.

Adipocyte development can be organised into stages (Fig 2.1). Firstly the multipotent mesenchymal stem cell loses the ability to become bone and cartilage and is thus predetermined to become an adipocyte (unipotent). This cell can be termed an adipoblast but potentially still retains the ability to become either WAT or BAT, although the precise developmental interactions between these two types of adipose tissue are not fully understood. It is likely that unipotent adipoblasts can remain as such into adulthood or can become a preadipocyte. The preadipocyte stage is associated with growth arrest at the $G_1$/S phase, in culture often secondary to cell-cell contact (contact inhibition), although this not an absolute requirement [251], and at this point the cell is committed to becoming a mature adipoctye. The glucocorticoid dexamethasone has been shown to influence this point by inhibiting proliferation and then stimulating differentiation [252]. This stage is associated with the disappearance of the early differentiation markers such as PREF-1. In murine cell lines a limited number of growth cycles occur after this point, known as clonal expansion, although this may not be the case in human preadipocyte cultures [253].

Intermediate and late phases of preadipocyte differentiation are characterised by the expression of essential transcription factors such as PPARγ and CEBPβ. In the *in vitro* environment the addition of specific hormones is often necessary to trigger the terminal stages of differentiation (e.g. hydrocortisone/dexamethasone [254], insulin [255] and tri-iodothyronine [256]) although differentiation in cell lines (3T3-L1) can occur in the absence of these hormones. Sex hormones, for which DHEA is a precursor, are not a requirement for adipocyte differentiation. A number of experimental models of differentiation have been described each using different cocktails of hormones and these will be discussed in greater detail later in this chapter. The terminal stage of differentiation is characterised by an increase in expression of enzymes such as glycerol phosphate dehydrogenase (GPDH) and lipoprotein lipase (LPL) that perform routine adipocyte cell functions. With reference to DHEA, chronic (12 days) exposure of the preadipocyte cell line 3T3-L1 to DHEA has been shown to inhibit glucose 6 phosphate dehydrogenase activity and block differentiation when using a differentiation mixture containing both dexamethasone and insulin [257]. Furthermore DHEA has been shown to down regulate PPARγ in rat preadipocytes [144] and one study has demonstrated down regulation of C/EBPα in 3T3-L1 preadipocytes [258] whereas another study revealed no effect on this transcription factor in rat preadipocytes [144].

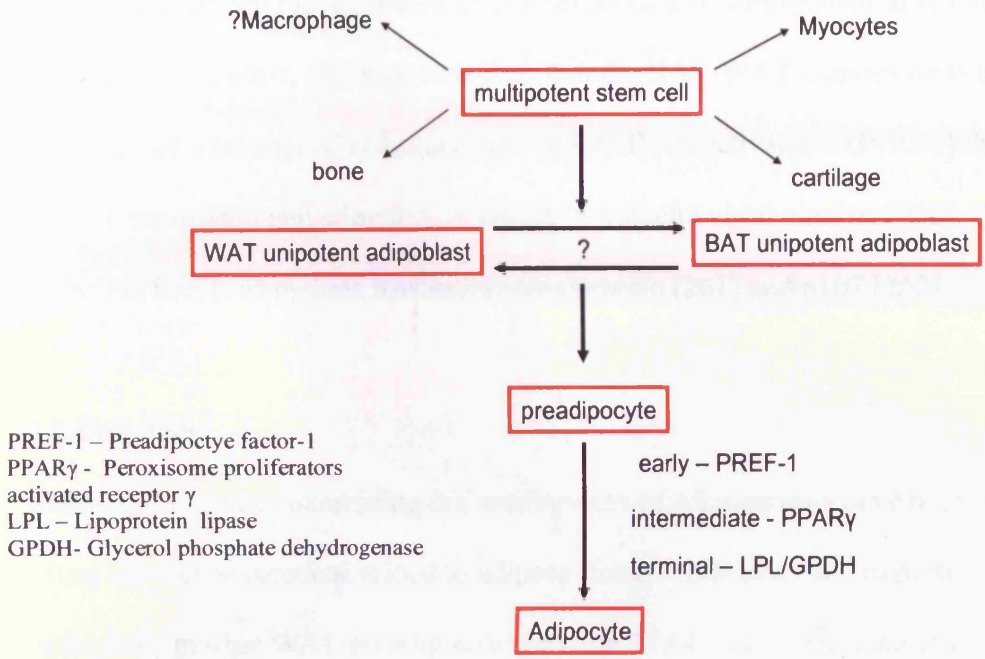



Fig 2.1 Developmental stages of the adipocyte

### 2.1.1 White and brown adipose tissue

The morphological, biochemical and functional differences between WAT and BAT have been discussed in chapter 1. The same transcription factor activation and hormonal stimuli are involved in the development of both adipose tissue types (PPARγ and C/EBPs) but in the case of BAT, cold and adrenergic drive are also of critical importance; the transcription factor FOXC2 is known to augment adrenergic signalling in BAT. WAT and BAT arise developmentally from distinct precursor cells; this is evidenced by the observation that precursor cells isolated from BAT deposits can be stimulated to produce BAT markers (uncoupling protein-1 (UCP-1) mRNA) but similar cells from WAT cannot [259]. What is not known is the exact point in development that WAT and

BAT precursor cell types diverge or at what point that commitment to either cell type becomes irreversible, although it is clear that the BAT-WAT conversion is under the influence of a number of cofactors such as PPARγ-coactivator-1 (PGC-1) that increases UCP-1 expression and stimulates increase in mitochondrial number [260]. Other cofactors involved include Retinoblastoma protein [261] and p107 [262].

## 2.2 Cell lines

Many of the studies examining the mechanisms of adipogenesis have been performed using adipoblast (predetermined to adipose lineage) cell lines and in particular the embryonic murine WAT preadipocyte cell line 3T3-L1 [263]. A number of other cell lines have been utilised (3T3-F442A, Ob17 and others), but as all of these are adipoblasts, it is the latter stages of differentiation that are better understood. Fewer studies have focused on BAT, potentially because questions remain over its relevance to human physiology beyond the neonatal period. However, owing to the presence of BAT in the foetal state and the high DHEA output from the foetal adrenal it was felt that it was important to determine if any relationship existed. In order to establish the effect that DHEA has on adipose tissue *in vitro* this study has utilised the mouse WAT preadipocyte cell line 3T3-L1 and the SV-40 (simian virus) transformed human BAT preadipocyte cell line PAZ6 [264].

### 2.2.1 3T3-L1

The 3T3-L1 preadipocyte cell line was obtained form the American Type Culture Collection. These cells are derived from isolates of the murine 3T3 fibroblast cell line.

The 3T3 cell line was established by George Todaro and Howard Green in 1963 from Swiss mouse (Mus musculus) embryonic tissue [265]. The 3T3-L1 line differs from the standard 3T3 line in that it possesses an exaggerated ability to accumulate lipid [266]. 3T3-L1, like 3T3 cells, grow as a monolayer on plates in culture until confluence is reached, at which point contact inhibition is demonstrated. It is following confluence that lipid droplets begin to accumulate. Lipid accumulation can be curtailed by keeping the cells in a continual state of growth and following trypsinisation the cells lose their lipid content and the resultant cells following passage appear more fibroblast-like once more (Fig 2.2). The process of lipid accumulation involves the differentiation of preadipocytes into adipocytes [267]. This process of differentiation can be augmented by the addition of a differentiation mix, containing a cocktail of hormones, into standard culture medium. This cell line has proved to be particularly hardy, maintaining functional capacity through a large numbers of passages.

3T3-L1 preadipocytes

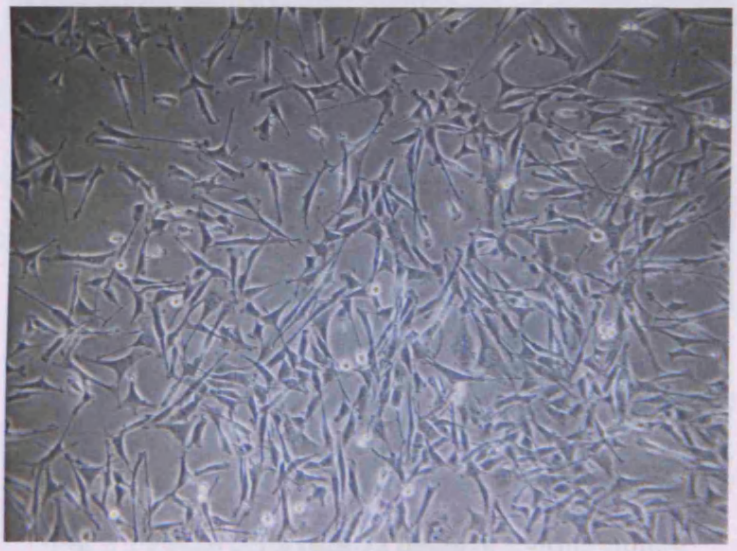


Fig. 2.2 3T3-L1 cells after 48 hours of routine culture

### 2.2.2 PAZ6

The PAZ6 cell line was kindly provided by Vladimir Zilberfarb (Institut Cochin, Université Paris Descartes). It is a human immortalised brown preadipocyte cell line, derived by transfecting human vascular stromal cells from infant brown adipose tissue with SV (simian virus) 40 antigen under the control of the vimentin promoter [264]. These cells can be cultured for prolonged periods (Fig 2.3) and withstand storage in liquid nitrogen. If the culture medium is altered to contain pro-differentiating factors the cells differentiate into mature brown adipocytes. This can be seen macroscopically with the development of lipid droplets and by polymerase chain reaction (PCR) measurement of markers of mature brown adipose tissue that are either specific to BAT such as uncoupling protein-1 (UCP-1) or are generic to all mature adipoctyes such as LPL [264].

PAZ6 preadipocytes

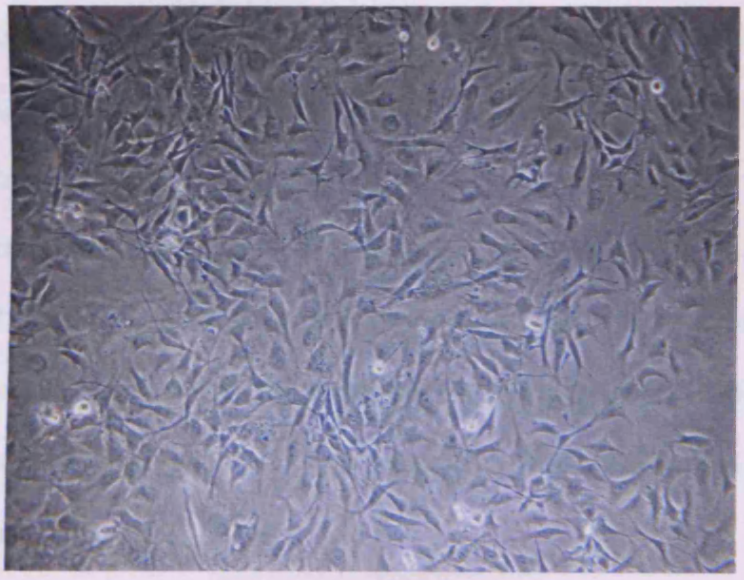


Fig 2.3 PAZ6 cells after 72 hours or routine culture

## 2.3 *In vitro* models of adipogenesis

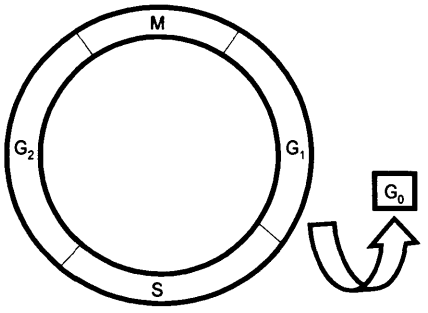
The term adipogenesis encompasses WAT and BAT adipocyte differentiation in all its stages. As previously discussed various substances can aid the process of differentiation in the laboratory setting and these are reviewed in a paper by Rosen and Spiegelman [263]. A number of *in vitro* models have been used to examine this process, which in turn has enabled investigators to assess the impact that various reagents have on differentiation.

Early studies suggested that cAMP was a requirement for differentiation and employed protocols containing methylisobutylxanthine (IBMX), a phosphodiesterase inhibitor that increases cAMP levels, in combination with insulin and the glucocorticoid dexamethasone. It is well established that insulin is a crucial component in differentiation regimens and increases the number of cells that differentiate per sample as well as augmenting lipid accumulation [256]. IGF-1, a component of foetal calf serum, may interact with insulin to augment differentiation. Dexamethasone reduces expression of PREF-1/DLK1, a requirement for terminal differentiation, as previously discussed, but the necessity for this class of hormone for differentiation is debatable as is the use of a number of other factors. Thyroid hormone, Growth hormone and Retinoic acid have all been employed in differentiation protocols to a varying degree. Previous endeavors in the Centre for Endocrine and Diabetes Sciences have optimised an *in vitro* differentiation protocol that utlises a number of the factors discussed here and this will be described in detail later in this chapter [268, 269].

## 2.4 Cellular proliferation and the cell cycle

The predipocyte by definition exists within the cell cycle and therefore maintains the potential to proliferate; only when stimulated to differentiate is this potential lost (excluding clonal expansion). Proliferation refers to normal cell division or mitosis and for differentiation to initiate, proliferation needs to cease, culminating in exit from the cell cycle.

Cell division can be divided into separate stages depending on the specific point of eukaryotic cell division, which is in turn determined by the amount of genetic material present within the cell and it is these stages that make up the cell cycle (Fig 2.4).




M – Mitosis
$G_1$ – GAP 1
$G_0$ – See text
S - Synthesis
$G_2$ – GAP 2

Fig 2.4 The cell cycle

The cell cycle can be divided into 2 stages, mitosis (M) and interphase, with interphase subdivided further into 3 stages, GAP1 ($G_1$), DNA synthesis (S) and GAP 2 ($G_2$) [270]. Cells often exit the cell cycle at $G_1$ and enter either permanently, or temporarily, a $G_0$ stage in which cells no longer undergo cell division; in the context of the preadipocyte it is at this point that differentiation occurs. Checkpoints exist at $G_1$/S (known as the

restriction point) and $G_2$/M so that if the external environment is not appropriate or if the cell is damaged and repair is required, mitosis can be blocked [271].

Cells within the $G_1$ stage carry out predetermined roles appropriate to the tissue in which that cell resides. During this stage the cell also generates various enzymes required for the next stage in the cycle. The S phase is characterised by DNA replication and once a cell has passed though the S phase it contains double the amount of DNA. The $G_2$ phase is concerned with preparation for mitosis in which microtubules are formed. During M phase the DNA and cytoplasm divides producing 2 daughter cells containing the same genetic material as the parent cell.

The cell cycle is controlled by sequences of cyclins and cyclin dependent kinases that can be activated by extracellular stimuli. Activation of these results in a phosphorylation cascade culminating in the stimulation of transcription factors that promote progression of the cell into the subsequent cell cycle stage [272].

### 2.4.1 Proliferation

Proliferation represents the balance between cell death and cell division which culminates in the rate at which a particular cell type increases (or not) in number. This will occur in all eukaryotic cells (within the cell cycle) assuming the environment is suitable. In standardised culture conditions a particular cell type will divide at a given rate and the addition of a non-toxic substrate can either, increase, decrease or have a neutral effect on the rate at which cell division occurs. Cell number determination at the end of an exposure period can be performed in a number of different ways. Direct visual counting using a haemocytometer allows for estimation of the number of cells present per mL of

cell suspension and this method, with the addition of an appropriate dye (e.g. trypan blue) can be used to determine cell viability and thus toxicity. Direct cell counting is open to observer bias and is time consuming and so electronic methods of direct cell or particle counting have been developed [273]. Cells under investigation are placed in a solution through which an electrical current is passed and this solution then flows through an aperture of known diameter. Each time a single particle or cell passes through this aperture it causes a drop in voltage by increasing aperture impedance. This drop in voltage is then amplified and can be recorded on an oscilloscope. The aperture can be configured to identify cells/particles of a particular size.

Other methods for assessing the proliferation rate include the incorporation of a radioactive isotope, such as $^{3}$H-thymidine, into the DNA of the dividing cells. This process relies on the principle that more radioactive isotope will accumulate in samples that are undergoing more cell division. A proportion of cells can be incubated with potential proliferation inhibitors/stimulants, while others remain in standard culture medium alone. Following this all cells are incubated with $^{3}$H-thymidine which is DNA incorporated. Reagents that inhibit/stimulate proliferation will generate samples that emit less/more radiation respectively compared to controls when assessed by scintillography.

### 2.4.2 Cell cycle analysis

The cell cycle has been discussed above and a number of experimental techniques exist to determine what impact an external stimulus will have on the distribution of cells within the different phases of the cell cycle.

As cells pass through the stages of the cell cycle the amount of genetic material varies as discussed. If cells treated with the reagent under investigation are fixed with ethanol and then exposed to a DNA stain, such as propidium iodide, a detailed cycle stage distribution map can be generated outlining the number of cells present in each stage at a given time point. If this resulting map is compared to untreated samples it is possible to ascertain if a particular reagent affects the progression through the cell cycle and hence proliferation. The technique of flow cytometry, when coupled with a DNA specific stain, can be used to determine cell cycle distribution of a particular sample. A suspension of cells that have incorporated the DNA stain is streamed such that each cell separates. At this point, cells are exposed to light (usually in the form of a laser) that excites the stain within the DNA, generating a specific scatter pattern of light. This is monitored by an array of detectors and varies depending on the amount of DNA present within the cell, and thus the stage within the cell cycle. As well as describing the relative amounts of cells within each cell cycle stage, flow cytometry can be used to determine if a reagent has triggered apoptosis. Apoptotic cells reside in a point just prior to the $G_1$ peak and a substantial amount of cells located here suggest exposure to an apoptotic stimulus. Other methods are available to determine whether a cell has been triggered to undergo apoptosis. The terminal uridine deoxynucleotide transferase (dUTP) nick end labeling (TUNEL assay) technique relies on attaching labeled dUTP onto damaged DNA of cells about to undergo apoptosis [274]. The attached dUTP can then be stained using avidin-conjugate peroxidase which can in turn be visualised, following agarose gel electrophoresis, by ultraviolet transillumination.

### 2.4.3 Analysis of cell signalling – phospho-specific antibody Western blotting

Intracytoplasmic cell signalling relies on numerous protein cascades triggered by external stimuli that interact both with each other and the cell nucleus. This has the effect of altering transcription and modifying cellular function in response to the external environment and thus control over both proliferation and differentiation. The proteins involved in cell signalling pathways often exist in two forms, phosphorylated and non-phosphorylated. In many, but not all cases the phosphorylated form is active (e.g. MAP kinase). This dichotomy of form can by quantified using Western blotting, a technique originally described by Burnette in 1981 [275], that can differentiate between the inactive and active forms of the specific proteins by identifying the phosphorylated protein state, thus allowing investigators to identify specific pathway involvement in response to an experimental stimulus. A schematic representation of the steps involved in Western blotting is shown in Fig 2.5. The use of phosphorylation state-specific antibodies in the investigation of cell signalling events is widespread and is discussed in the review paper by Mandell [276] . Following exposure to the reagent under investigation, cells are homogenised and the protein components separated by electrophoresis using gels loaded with negatively charged sodium dodecyl sulphate (SDS). This covers the proteins which then move towards the positive electrode. Smaller particles migrate faster through the gel and take up terminal positions corresponding to molecular weight which can be compared to a stained marker, placed in an adjacent lane that contains proteins of known molecular weight.

The gel is then electroblotted onto a membrane (nitrocellulose or polyvinylidene fluoride (PVDF)) to enable detection. After blocking to prevent non-specific antibody attachment,

the membrane is incubated under gentle agitation with a primary antibody (directed against the protein under investigation) and then subsequently with a secondary antibody (directed against a species specific component of the primary antibody). The secondary antibody typically contains a chemiluminescent conjugate that when activated emits luminescence, proportional to the amount of protein present, which is captured on photographic film. To confirm the identification of the protein under investigation a comparison is made between the pattern of bands on the developed film and the membrane blot.

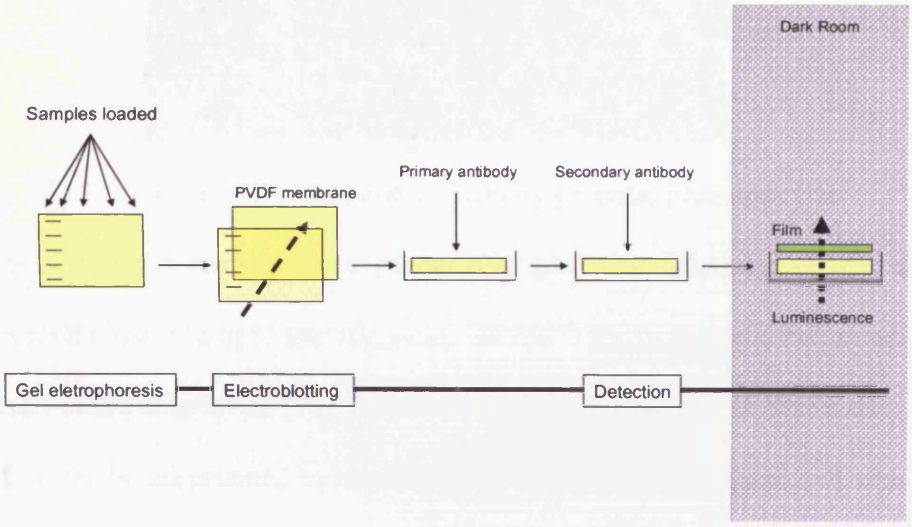



Fig 2.5 Western blotting schematic

### 2.4.4 *In vitro* assessment of differentiation

The extent to which a particular preadipocyte sample undergoes differentiation can be quantified by a number of methods. Most simply it may be appreciated visually through

light microscopy with the characteristic appearance of rounded mature adipocytes containing numerous lipid filled droplets (Fig 2.6). The number of differentiation foci per randomly chosen high power field can give an indication of the relative extent of differentiation occurring in different samples. This method may be strengthened by increasing the number of experimental samples examined but is however open to observer bias and the counts should be ideally performed by an independent observer.

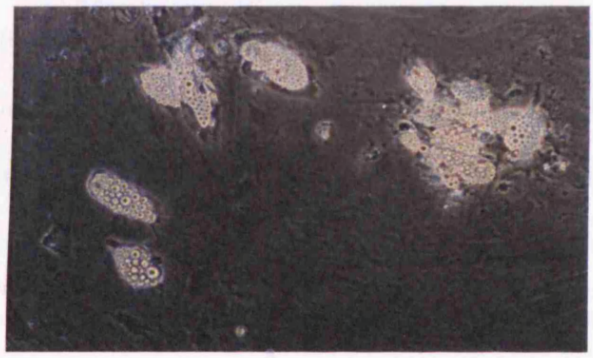


Fig 2.6 Foci of differentiation in primary omental preadipocytes

A further method of differentiation assessment is to determine the extent of lipid droplet formation with the use of a lipid specific stain. Oil red O stains the lipid component of ethanol fixed mature adipocytes (Fig. 2.7).The relative amount of stain and thus differentiation can be ascertained by absorbing the stain in 100% propan-2-ol which can then be quantified by colourimetry.

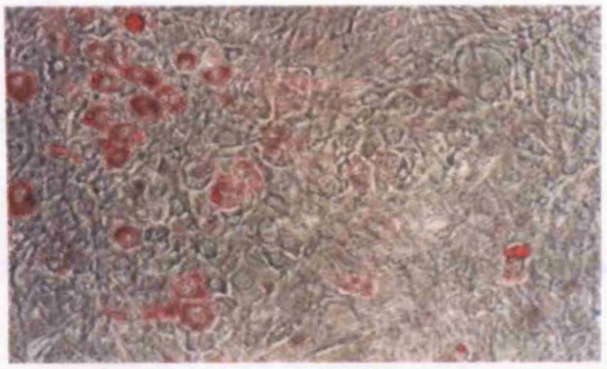


Fig 2.7 Oil red O stain in 3T3-L1 preadipocytes

A potentially more precise method to determine relative differentiation rates in control and reagent exposed samples is to measure transcripts for markers of various stages of adipogenesis. This involves RNA extraction followed by reverse transcription and real-time quantitative polymerase chain reaction (RT-QPCR) (Fig 2.8).

Extracted RNA is reverse transcribed to produce cDNA which is then amplified by PCR. With the use of primers directed to differentiation specific factors (PPARγ, GPDH, LPL) the extent of differentiation occurring in a reagent exposed sample can be accurately determined relative to a control.

The process of RT requires the use of one of two types of reverse transcriptase enzyme, avian myeloblastosis virus (AMV), or moloney murine leukeamia virus (MMLV). To generate cDNA these enzymes require the presence of a primer. For the purposes of this study the primer oligodT has been utilised and this binds to the poly (A) tail at the 3' mRNA terminus. Other primer types can be used such as oligonucleotides of random sequences using large numbers of diverse oligonucleotides or specific oligonucleotide sequences corresponding to a particular mRNA.

The cDNA generated by RT provides the template for PCR [277] which occurs according to a sequence of temperature sensitive events. Firstly a short high temperature is employed to denature the double stranded cDNA. Following this a cooling phase occurs during which predesigned oligonucleotide primers anneal to the cDNA sequences of interest. An elongation phase then follows in which a DNA polymerase triggers DNA synthesis initiating from the cDNA adhered primers, the reaction utlising pre-added deoxynucleotide triphosphates (dNTPs).

This tri-stage sequence is repeated numerous times increasing the amount of DNA exponentially. Eventually the rate of DNA production plateaus secondary to substrate depletion or product/primer competition for cDNA binding sites [278].

Each PCR reaction is, however, open to variability and the efficiency of each reaction can be affected by reaction conditions, target region and primer sequences and target region length. When quantifying results from PCR reactions it is necessary to attempt to cater for efficiency variability. Quantification of PCR (Q-PCR) is performed by comparing the results obtained per amount of input RNA to a series of consecutively diluted target sequence samples [279]. In order to accommodate potential efficiency discrepancies and loading errors each reaction is compared to a housekeeper gene such as human adenosine phosphoribosyltransferase (APRT) or acidic ribosomal phosphoprotein (ARP). Housekeepers are so termed, as they are involved in routine cellular functions, are found in all cells (APRT catalyses the conversion of adenine and 5-phosphoribosyl-pyrophosphate to AMP [280]) and are ubiquitously expressed despite alterations in experimental conditions. Q-PCR results from the gene under investigation are expressed relative to those from the housekeeper.

Following the RT step it is possible to perform a semi-quantitative PCR. The resulting PCR product can be separated by agarose gel electrophoresis and visualised using ethidium bromide. This conventional PCR provides a check point that confirms the RT reaction has generated the correctly sized product by comparison with an appropriate range base-pair ladder.

The amplification of mRNA and the detection of the PCR product can be combined such that results can be generated in real-time. PCR product detection is further aided by the use of DNA specific dyes. SYBR Green is one such dye that binds to double stranded DNA (dsDNA). The binding process increases dye fluorescence in line with product generation during PCR (Fig 2.8). The number of PCR cycles that are required for fluorescence to increase beyond an arbitrary baseline threshold is used as a quantification method and termed the crossing the threshold (Ct) value. Samples containing less input cDNA (more dilute) will require more cycles to cross this threshold and will therefore have a higher Ct value. As well as quantification the Ct values provides a quality check as the Ct value should occur on the exponential/linear portion of cDNA amplification; plotting the Ct values against Log concentration should generate a linear graph with a correlation coefficient close to 1.0. Another quality check is melting point analysis. At a specific melting temperature dsDNA will dissociate into single strands characterised by a dramatic reduction in fluorescence. Melting point analysis provides a quality control ensuring that the correct product has undergone polymerisation. The correct DNA product for a particular pair of primers will have the same melting temperature ($T_m$ – temperature where 50% of DNA becomes single stranded) dependent on base composition (GC rich DNA has a higher $T_m$ whereas AT rich DNA has the converse). At

the end of RT-PCR amplification the temperature is increased to the melting temperature for this purpose, the presence of other smaller peaks can indicate contaminating DNA or primer-dimer.

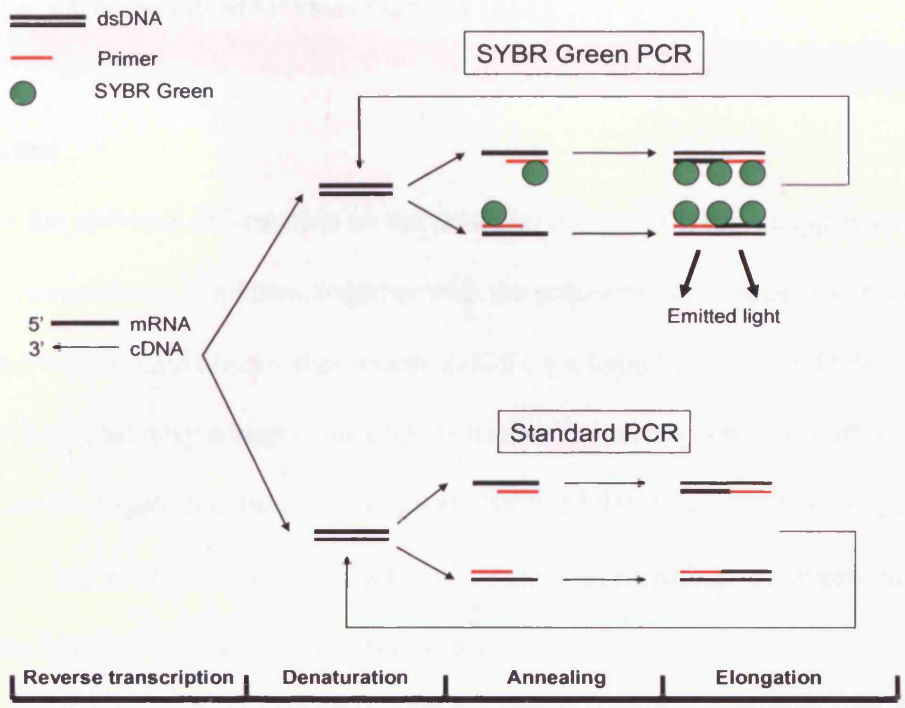



Fig 2.8 PCR schematic

## 2.5 Previous studies

A number of previous *in vitro* studies have attempted to examine the influence that DHEA has on adipose cell biology. These studies have focused on WAT with no BAT studies performed to date. DHEA has been shown to inhibit conversion of 3T3 fibroblasts into adipocytes and also the proliferation of 3T3-L1 cells [138, 139]. In terms of the mechanisms by which adipogenesis may be inhibited a study by Kajita *et al* demonstrated that DHEA can bring about a down regulation of PPARγ expression in adipocytes [144] and as already discussed this transcription factor is the major controller of adipogenesis.

Furthermore the differentiation of 3T3-L1 fibroblasts into adipoctyes was found to be blocked by DHEA [257]. In relation to cell signalling DHEA has previously been shown to reduce the proliferation of vascular smooth muscle cells and this was demonstrated to involve alterations in MAP kinase activity [212].

## 2.6 Aims

Given the previous information on the potential impact of DHEA supplementation on body composition in humans together with the reduction of fat mass and protection against weight gain observed in rodent models, we hypothesised that DHEA exerts a direct and inhibitory action upon adipose tissue. The work presented within this chapter aims to investigate this further through analysis of DHEA action on preadipocyte cell line (3T3-L1 and PAZ6) proliferation and differentiation, including an investigation of the mechanisms involved in any observed effects

## 2.7 Materials and Methods

All culture medium constituents were obtained from BioWhittaker (Belgium) unless otherwise stated. A number of experimental reagents have been used during this project, DHEA, DHEAS, β-oestradiol, testosterone [all Sigma-Aldrich Company Ltd, Dorset, UK] and trilostane [donated by Bioaccelerate Inc, London, UK]. All reagents were dissolved in dimethyl sulfoxide (DMSO) [Sigma-Aldrich Company Ltd, Dorset, UK] to produce stock solutions of $10^{-2}$ molar. As DMSO has been demonstrated itself to have an impact on 3T3-L1 adipogenesis [281] further dilutions were made using cell type appropriate culture media so a maximum concentration of DMSO of 0.1% was present in

any one experiment, furthermore 0.1% DMSO was included in the control in all experiments.

### 2.7.1 Culture Conditions

3T3-L1 and PAZ6 cells were grown in 75cm$^2$ Nunclon$^{TM}$ delta surface flasks [Nunc, Roskilde, Denmark]. 3T3-L1 medium consisted of Dulbecco's modified Eagle medium (DMEM) (with 4.5g/L glucose without sodium pyruvate) and Ham's F12 complete medium (ratio 1:1) supplemented with 10% foetal calf serum (FCS) (EU approved), 2mls sodium pyruvate, 3mls of 1.5% sodium bicarbonate, 100U/mL penicillin [Cambrex, Berkshire, UK] and 100µg/mL streptomycin [Cambrex, Berkshire,UK].

The PAZ6 culture medium contained DMEM and Ham's F12 complete medium (ratio 1:1), heat inactivated fetal calf serum (8%), 3ml Hepes buffer, 2ml pyruvate, 100u/mL Penicillin and 100µg/mL Streptomycin.

All cell culturing was performed in humidified 5% $CO_2$ atmosphere at 37°C, the culture medium being changed every 3$^{rd}$ day.

### 2.7.2 Routine maintenance of cell lines

On reaching confluence the cells underwent passage. To remove the cellular monolayer culture medium was aspirated and cells washed with 2mL 1% trypsin [Cambrex, Berksire, UK] then incubated with a further 2mLs of 1% trypsin until the cells were in suspension (5-10 minutes). To the resultant suspension 8mLs of culture medium warmed to 37°C was added for trypsin inactivation. The solution was then transferred to fresh flasks or further diluted with culture medium and plated for experiments.

To replenish the cell line bank, excess cells were trypsinised as above and centrifuged at 1000rpm for 5 minutes. The remaining cell pellet was then re-suspended in 500 μL Freezing mix (FCS containing 10% DMSO) and transferred to 2mL sterile cryo-tubes and placed into an insulating freezing box containing propan-2-ol [Fisher Scientific UK Ltd, Leicestershire, UK] for slow freezing (rate of 1°C/min) in a -80°C freezer [New Brunswick Scientific] for 24 hours until transfer to liquid nitrogen (-190°C).
To thaw samples from liquid nitrogen, cells in freezing mixture were warmed in a water bath and immediately placed in 9.5mLs of standard medium and centrifuged for 5 minutes at 1000rpm. Medium was aspirated and the resulting cell pellet was resuspended in 10mls of fresh medium and transferred to a flask and incubator as above.

### 2.7.3 Direct cell counting

Culture medium was removed and cells trypsinised as above. The approximate number of cells per ml of solution was then estimated using a haemocytometer (Camber counter, [Weber Scientific International Ltd, Middlesex, UK]) and then diluted, depending on suspension density, to produce intra-well concentrations of $10^3$ or $10^4$ cells. Following transfer to 24 well plates (1mL per well of appropriate culture medium) cells were incubated at 37°C for 24 hours allowing optimal cell-to-plate attachment. DHEA was then added to wells in decreasing concentrations ($10^{-6}$ molar to $10^{-9}$ molar) in quadruplicates. To the first column no reagent was added and to the second column 0.1% DMSO was added as a control. All samples were incubated for 24 and 48 hours then the medium was removed and each well was trypsinised (0.5mLs trypsin per well). The resulting suspension was transferred to a cell counting cuvette containing 10mLs of

isoton (Coulter Isoton II Diluent [Beckman Coulter, Gmbh, Germany]). Each well was also washed with 0.5mLs of isoton which was transferred to the same cuvette. Cells were counted using a particle counter [Particle counter size analyzer $Z_2$, Beckman Coulter Gmbh, Germany] with particle size set between 3.8nm and 9.0nm. Each sample was counted twice and an average value used in the statistical analysis.

### 2.7.4 Toxicity assay – Trypan blue

3T3-L1 and PAZ6 cells plated in 24 well plates (Cell concentrations $10^4$ or $10^3$ per mL per well) were incubated with DHEA $10^{-5}$ molar and $10^{-9}$ molar and DMSO 0.1% and untreated cells (standard culture medium only) for 48 hours. Cells were then trypsinised producing a suspension that was then centrifuged at 1000rpm for 5 minutes. The resulting cell pellet was resuspended in 50μL of culture medium to which was added an equal volume of Trypan blue (0.1%) [Sigma-Aldrich Company Ltd, Dorset, UK] (from stock solution 0.4% diluted with PBS). Cells were immediately examined under a light microscope using a haemocytometer. Cells exposed to a toxic substance have damaged membranes and take up the blue dye.

### 2.7.5 Flow cytometry

3T3-L1 and PAZ6 cells ($10^3$ per mL) were plated into 6 well plates [Nunclon$^{TM}$ delta surface, Nunc, Roskilde, Denmark] in standard medium (3mL per well), after 24 hours DHEA was added to produce an intra-well concentration of $10^{-6}$ molar. In separate wells DMSO 1% was added as a control. After 48 hours the medium was removed and the well contents trypsinised and centrifuged at 1000rpm for 5 minutes producing a cell pellet, to

which 1mL of cold (4°C) 70% ethanol was added drop-wise. The solution was transferred to 1.5mL tubes and stored at -20°C.

The ethanol was removed following centrifugation at 1000rpm for 10 minutes and the samples washed twice with PBS. Propidium iodide (50μg/mL in PBS) and RNAse A (50μg/mL) were added and incubated for 20 minutes at 37°C. The resulting samples were then detected using a FACSCalibur flow cytometer [Dakocytomation, Cambridge, UK]. For these experiments reagent provision and FACSCalibur flow cytometer operation was kindly performed by Dr E. Wang, Department of Immunology, School of Medicine, Cardiff University.

### 2.7.6 Western blotting

Sample collection: Cells grown in 6 well plates until confluence were washed ×3 with 1mL of PBS then incubated in serum free medium (DMEM only) for 2 hours followed by fresh serum free medium for a further 2 hours. DHEA dissolved in standard culture medium was spiked into wells ($10^{-7}$ molar) at time points of 5, 10 and 20 minutes. For controls 0.1% DMSO was spiked into separate wells at the same time points. Cells were then washed 3× with cold (4°C) PBS-V (1mL sodium vanadate [Sigma Aldrich Company Ltd, Dorset, UK] per 100mL PBS) then homogenised on ice with a 1mL syringe plunger in 200μL of SDS-PAGE Loading buffer (Appendix II) containing 100mM sodium vanadate (10μL/mL), 100mM phenylmethanesulphonyl fluoride (PMSF) (10μL/mL) and CLAP (chymostatin, leucopeptin, antipain and pepstatin) (1μL/mL) [both Sigma Aldrich Company Ltd, Dorset, UK] and stored at -80°C.

Gel Electrophoresis: 50µL of each homogenate was boiled for 5 minutes and then centrifuged for 5 minutes at 13,000rpm then loaded into stacking gel wells (Appendix II) and separated on a 10% acrylamide gel, (Appendix II) (resolves proteins in size range 21-100 kDa), using a mini-protean II apparatus [Bio-Rad Laboratories] at a constant voltage of 200v for 40 minutes.

Electroblotting: This was performed by placing the gel in contact with a Hybond™ PVDF membrane [GE Healthcare UK Ltd, Buckinghamshire]. The constituents of the electroblotting cassette were arranged as shown in Fig 2.9 and placed into a blotting tank (mini-protean II apparatus) [Bio-Rad Laboratories] with blotting buffer (Appendix II) and an ice pack. Blotting was performed over 1 hour at 350mA with stirring.

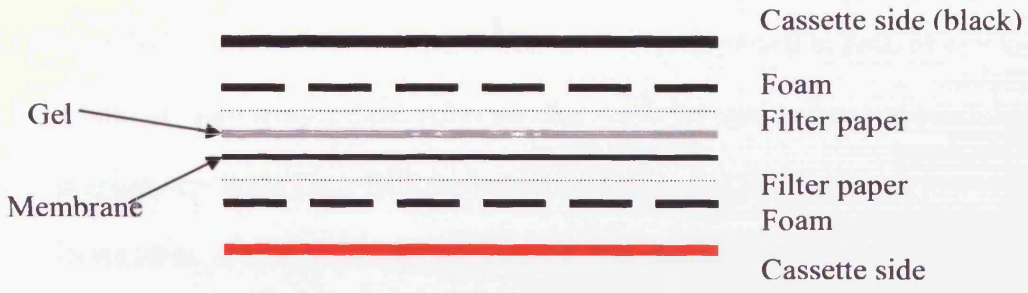



Fig 2.9 Preparation of cassette for electrolotting

Detection: The membrane was washed with TBS-T (appendix II) and placed in 50mL blocking buffer (appendix II) for 1 hour on an orbital mixer then incubated in primary antibody (4µL of phospho p44/42 MAPK mouse mAb, [Cell signaling Technologies]) dispersed in 8mL blocking buffer overnight at 4°C on an orbital mixer. The membrane was washed 3× with TBS-T for 5 minutes and incubated with the secondary antibody

(2μL ECL antimouse IgG horseradish peroxidase linked whole antibody (from sheep), [GE Healthcare UK Ltd]) dispersed in10mL blocking buffer for 1 hour at room temperature on an orbital mixer. The membrane underwent a number of TBS-T washes (2×30 seconds, 1×15 minutes, 3×5 minutes) and was then detected following darkroom protocol using ECL detection plus kit [GE Healthcare UK Ltd, Buckinghamshire, UK] according to the manufacturer instructions. The resulting photographic films were washed in water then dried in air before marking the standards by aligning the film with the membrane.

To reprobe the blot for total MAP kinase the membrane was incubated in pre-warmed (60°C) stripping buffer (appendix II) in a hybridisation oven at 60°C for 30 minutes with gentle mixing. The membrane was again washed with TBS-T then incubated with total MAP kinase targeted primary antibodies (2μL ERK1 rabbit polyclonal IgG and 2μL ERK2 rabbit polyclonal IgG, [Santa Cruz, Biotechnology]) dispersed in 8mL of blocking buffer for 1 hour at room temperature. After another wash the membrane was incubated with the secondary antibody (2μL ECL antirabbit IgG Horseradish peroxidase linked whole antibody (from donkey), [GE Healthcare UK Ltd, Buckinghamshire, UK]) dissolved in 10mLs blocking buffer to 1 hour at RT on an orbital mixer. The membrane was then washed and detected as above.

### 2.7.7 Adipogenesis protocol

3T3-L1 and PAZ6 cells were plated into 6 well plates and incubated in standard medium at 37°C until confluent at which point the medium was changed to a differentiation mix (Standard DMEM/F12 medium supplemented with 5% FCS, 17μM pantothenic acid*,

100nM hydrocortisone*, 33μM biotin*, 500nM insulin*, 1μM pioglitazone**, 0.2nM tri-iodothyronine*) [*Sigma Aldrich, ** kindly supplied by Takeda Pharmaceuticals, Osaka, Japan] with added DHEA ($10^{-7}$ molar) /DMSO 0.1% control. Incubation was continued for 12 (3T3-L1) and 15 days (PAZ6) with differentiation mix changes every 4 days in 3T3-L1 and every 5 days in PAZ6 coinciding with cell (for RNA extraction) and conditioned medium collection. For nucleoprotein complex dissociation 1mL of trizol was added to each well and left for 15 minutes at room temperature then transferred to 1.5mL tubes and stored at -80°C. At terminal differentiation photographs were taken and foci of differentiation counted.

**2.7.8 Oil red O staining**

The differentiation protocol was conducted as above in 24 well plates to terminal differentiation. 0.5g of oil red O was dissolved in 100mL of propan-2-ol prior to use and 12mLs of this solution was mixed with 8mLs of distilled water which was then immediately filtered.

Each well was washed with 1mL PBS then fixed with 500μL of 60% propan-2-ol. Following a further 1mL wash with PBS. 300μL of the filtered oil red O solution was added to each well for 15 minutes at room temperature then removed. This was followed by 3× washes with 1mL distilled water and photographs were taken. 200μL of 100% propan-2-ol was then added to each well then transferred to a 96 well plate which was in turn placed in an optical density reader [OpsysMR microplate reader, Dynex Technologies, Chantilly, USA] set to read at $OD_{490}$.

### 2.7.9 Markers of adipogenesis measured by QPCR

RNA extraction: 0.2mLs of chloroform was added to each 1mL sample collected for RNA extraction and then shaken vigorously and left at room temperature for 2 minutes prior to (cold) centrifugation at 4°C [Mikro 22R, Heittich Zentrifugen, Germany] at 12,000rpm for 15 minutes. This separates the sample into three phases; the uppermost aqueous phase contains the RNA, which is then transferred into a new 1.5mL tube, 0.5mL of propan-2-ol was added and mixed gently. Following 10 minutes at room temperature samples were cold centrifuged once more at 10,000rpm for 10 minutes. The resulting pellets were then washed with 1mL 75% ethanol which was then removed by cold centrifugation at 7500rpm for 5 minutes. The RNA pellet was then redissolved in 50μL of nuclease free water [Promega] by incubation at 60°C for 10 minutes on a heating block, then stored at -80°C. RNA concentration (ng/mL) and ratios were then calculated with a spectrophotometer [GeneQuant pro RNA/DNA calculator Biochrom Ltd, Cambridge, UK]. Sufficient sample quality was defined as a 260/280 ratio of greater than 1.7.

Reverse transcription: 1μg of RNA was used in each RT reaction and preheated to 60°C for 10 minutes on a heating block. The remaining RT reaction constituents are shown in table 2.1.

| RT reaction constituent | Amount per reaction |
|---|---|
| oligodT | 4μL (500ng/μL) |
| RNAse inhibitor (RNasin) | 1μL (40U/μL) |
| dNTP | 4μL (10mM) |
| MMLV reverse transcriptase | 1μL |
| 5× First-Strand buffer | 4μL |
| nuclease free water | To final volume of 20μL |

Table 2.1 RT reaction constituents

Samples in nuclease free 500μL microtubes underwent treatment at 37°C for 60 minutes followed by 95°C for 5 minutes in a Techne TC-512 standard PCR machine [Barloword Scientific Ltd, Staffordshire, UK] and the generated cDNA was stored at -20°C.

Standard Polymerase chain reaction: This was performed using 1μL input cDNA and PGK (phosphoglycerate kinase) housekeeper primers in nuclease free 500μL microtubes with reaction constituents described in table 2.2.

| **Standard PCR reaction constituent** | **Amount per reaction** |
|---|---|
| PGK primers (human for PAZ6, mouse for 3T3) | 1μL |
| Taq (DNA polymerase) | 0.5μL |
| dNTP | 1.0μL (10mM) |
| 10× PCR buffer | 2.5μL |
| nuclease free water | To a final volume of 25μL |

Table 2.2 Standard PCR reaction constituents.

The PCR machine generated the following temperature sequence to perform the reaction.

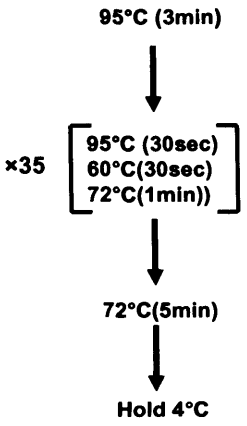



Fig 2.10 Temperature sequence in standard PCR reaction

The PCR product was separated on a 2% agarose gel (appendix II) at 150v for 25 minutes. The resulting gel was then visualised using ethidium bromide (0.5µg/mL) (Sigma Aldrich) under UV light (Alpha Innotech Corporation, MultiImage$^{TM}$ light cabinet) and compared to a 100bp ladder (Promega, Madison, WI, USA).

Quantitative polymerase chain reaction: Plasmid maxi preps for standard curves and optimised primers for mouse and human genes of interest were gifts from Dr Neera Agarwal and Dr Lei Zhang respectively, Centre of Endocrine and Diabetes Sciences, School of Medicine, Cardiff University. Primers were obtained from (tables 2.3 and 2.4) invitrogen.

| Gene | Forward primer | | | Reverse primer | | | Size |
|---|---|---|---|---|---|---|---|
| | Sequence (5'→3') | Exon | Tm (°C) | Sequence (5'→3') | Exon | Tm (°C) | base pairs |
| ARP | GAG GAA TCA GAT GAG GAT ATG GGA | 7 | 60 | AAG CAG GCT GAC TTG GTT GC | 7 | 60 | 172 |
| PPARγ | TTT TCA AGG GTG CCA GTT TC | 6 | 60 | AAT CCT TGG CCC TCT GAG AT | 6 | 60 | 220 |
| GPDH | ATG CTC GCC ACA GAA TCC ACA C | 8 | 60 | AAC CGG CAG CCC TTG ACT TG | 8 | 60 | 124 |

Table 2.3 Mouse primer sequence

| Gene | Forward primer | | | Reverse primer | | | Size |
|---|---|---|---|---|---|---|---|
| | Sequence (5'→3') | Exon | Tm (°C) | Sequence (5'→3') | Exon | Tm (°C) | base pairs |
| APRT | GCT GCG TGC TCA TCC GAA AG | 3 | 60 | CTT TAA GCG AGG TCA GCT GC | 5 | 60 | 247 |
| PPARγ | CAG TGG GGA TGT CTC ATA | 3 | 60 | CTT TTG GCA TAC TCT GTG AT | 5 | 60 | 390 |
| LPL | GAG ATT TCT CTG TAT GGA CC | 7 | 60 | CTG CAA ATG AGA CAC TTT CTC | 9 | 60 | 275 |
| UCP-1 | GGG GCT TCA GCG GCA AAT CAG | 2 | 60 | TAT AAG TCC CCG TGT AGC GAG GTT | 3 | 60 | 236 |
| PGC-1 | GAA GAG CGC CGT GTG ATT TA | 10-11 | 60 | CGC TGT CCC ATG AGG TAT TC | 13 | 60 | 433 |

Table 2.4 Human primer sequences

Real time Q-PCR was quantified relative to a housekeeper, for human samples APRT and for the murine samples ARP. Each Q-PCR reaction required 1μL of cDNA/standard, 12.5μL of SYBRgreen mastermix (Invitrogen) and appropriate quantities of forward and reverse target gene primers (Table 2.4) and nuclease free water [Promega] (to final volume of 25μL) that were analysed simultaneously with the diluted standard series ($10^7$ to$10^2$ transcript copy number).

Once placed into the PCR 96 well plate the solutions were centrifuged for 5 minutes at 10,000rpm. The plate was then transferred to the Stratagene mx3000P® thermocycler and analysed using the thermal profile below.

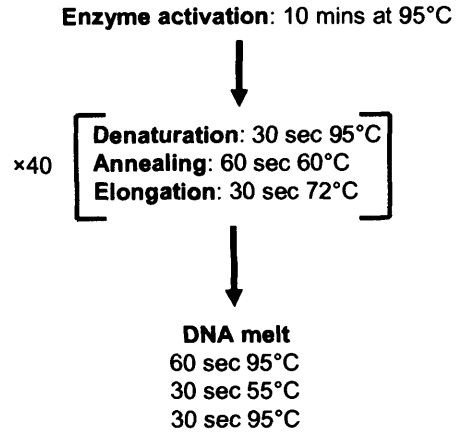



Fig 2.11 Thermocycling protocol for Q-PCR reactions

Q-PCR was performed on duplicate samples averaged for analysis. Results were expressed as fold change in target gene copy number relative to control corrected for the reference gene (APRT/ARP).

| Gene | Primer concentration (mouse) | | Primer Concentration (human) | |
|---|---|---|---|---|
| | Forward | Reverse | Forward | Reverse |
| ARP | 200nM | 200nM | - | - |
| APRT | - | - | 100nM | 100nM |
| PPARγ | 200nM | 200nM | 300nM | 500nM |
| GPDH | 500nM | 500nM | - | - |
| LPL | - | - | 300nM | 300nM |
| UCP-1 | - | - | 500nM | 300nM |
| PGC-1 | - | - | 500nM | 500nM |

Table 2.5 Optimised primer combinations

## 2.8 Statistical analysis

All statistical analysis was performed using SPSS for windows (Microsoft) version 12.0.1. Graphs for non-parametric data were also generated from SPSS version 12.0.1.

## 2.9 Results

Our hypothesis, that DHEA has a negative impact on adipose tissue, was examined using a preadipocyte experimental model such that inhibition of either proliferation of differentiation could be determined. We chose to begin our studies with an assessment of the former.

### 2.9.1 DHEA inhibits the proliferation of preadipoctye cell lines

In the absence of treatment 3T3-L1 and PAZ6 preadipocytes proliferate at specific rates which can be expressed in terms of doubling times. In our experimental model, doubling times were found to be approximately 22 hours for 3T3-L1 cells and 28 hours for the PAZ6 cells. DHEA appeared to produce a reduction in proliferation rates in both cell types. It should be noted however that within the same cell line some differences in proliferation rates were observed in different samples, the PAZ6 preadipocytes however, had uniformly slower proliferation rates and thus doubling times relative to the 3T3-L1 preadipocytes.

The generated data sets were not normally distributed and thus defined as non-parametric and analysed according to median and interquartile range (IQR) (Table 2.6). Friedman's test was performed on each experiment and Wilcoxon Signed Ranks test was performed on individual treatments relative to control. For ease of interpretation all 3T3-L1 (WAT) results have been represented graphically in light yellow and the PAZ6 (BAT) in brown.

Median and IQRs for 3T3-L1 preadipocytes treated with DHEA for 24 hours

| Cell Treatment | Median and IQR (number of cells per mL per well) |
|---|---|
| Non treatment | 4642 (3525-8231.75) |
| Control (DMSO 0.1%) | 5851 (3827-7824) |
| DHEA $10^{-8}$ M | 5121.5 (3404-7041) |
| DHEA $10^{-7}$ M | 5352.5 (3531-6976) |
| DHEA $10^{-6}$ M | 4811 (3729.75-6497) |
| DHEA $10^{-5}$ M | 3462 (3008.5-4128.25) |

Table 2.6 $1\times10^{3}$ 3T3-L1 cells were plated in 24 well plates (day 0) and trypsinised and counted (coulter counter) on days 1and 2. The results are the median and interquartile ranges for 3T3-L1 cells treated with varying concentrations of DHEA for 24 hours expressed as number of cells per mL

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of 3T3-L1 cells following 24 hours exposure

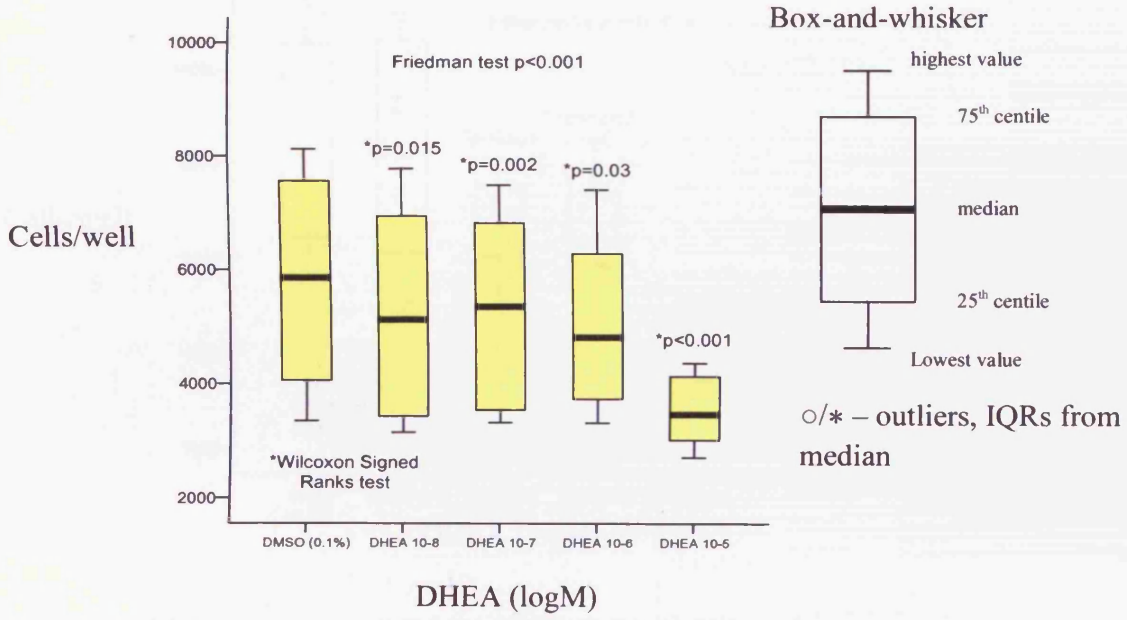



Fig 2.12 Box-and-whisker plot showing the effect on proliferation of 3T3-L1 cells following 24 hours exposure to various concentrations of DHEA. Results shown are a representative combination of two experiments performed in quadruplicate

Median and IQRs for 3T3-L1 cells treated with DHEA for 48 hours

| Cell Treatment | Median and IQR (cell number per mL per well) |
|---|---|
| Non treatment | 9695 (6054-10197.5) |
| Control (DMSO 0.1%) | 8024.5 (6258.25-10601.25) |
| DHEA $10^{-8}$ M | 6129.5 (5234.75-10925) |
| DHEA $10^{-7}$ M | 6034.5 (5488.5-7484.5) |
| DHEA $10^{-6}$ M | 5879.5 (5526-7281) |
| DHEA $10^{-5}$ M | 3490 (3165.25-4332.75) |

Table 2.7 $1\times10^{3}$ 3T3-L1 cells plated in 24 well plates as described above results are median and interquartile ranges following 48 hour treatment with varying concentrations of DHEA

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of 3T3-L1 cells following 48 hours exposure

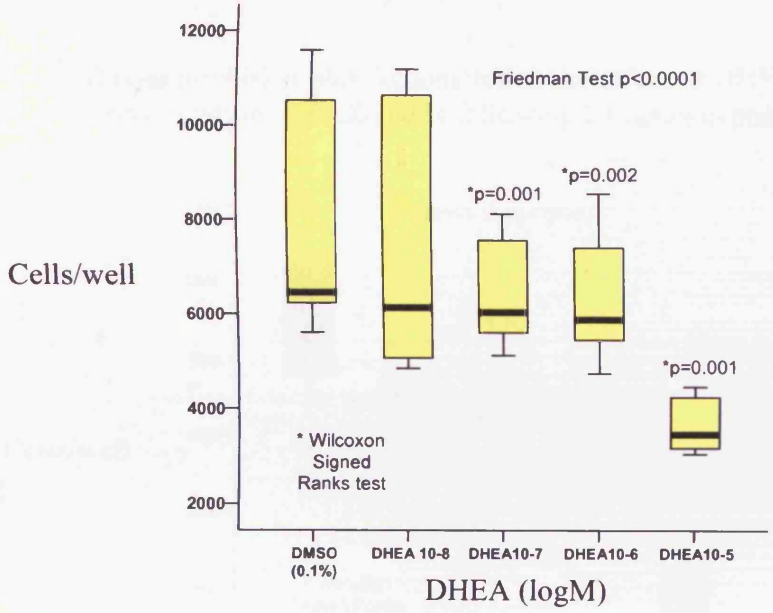



Fig 2.13 Box-and-whisker plot showing the effect on proliferation of 3T3-L1 cells following 48 hours exposure to varying concentrations of DHEA. Results are a representative combination of 2 experiments performed in quadruplicate

The tables and figures presented demonstrate that DHEA significantly inhibits proliferation at 24 and 48 hours in 3T3-L1 cells. The PAZ 6 cell line was then investigated.

Median and IQRs for PAZ6 cells treated with DHEA for 24 hours

| Cell Treatment | Median and IQR (Cell number per mL per well) |
|---|---|
| Non treatment | 3807 (3533.25-4010.75) |
| Control (DMSO 0.1%) | 3321 (3133-3684.5) |
| DHEA $10^{-8}$ M | 2630 (2440-2911) |
| DHEA $10^{-7}$ M | 2583 (2568-2910) |
| DHEA $10^{-6}$ M | 2388.5 (2229-2536.25) |
| DHEA $10^{-5}$ M | 1450 (1277-1698.5) |

Table 2.8 Experiments performed as with 3T3-L1 cell results are median and interquartile ranges for PAZ6 cell following 24 hours exposure to varying concentrations of DHEA

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of PAZ6 cells following 24 hours exposure

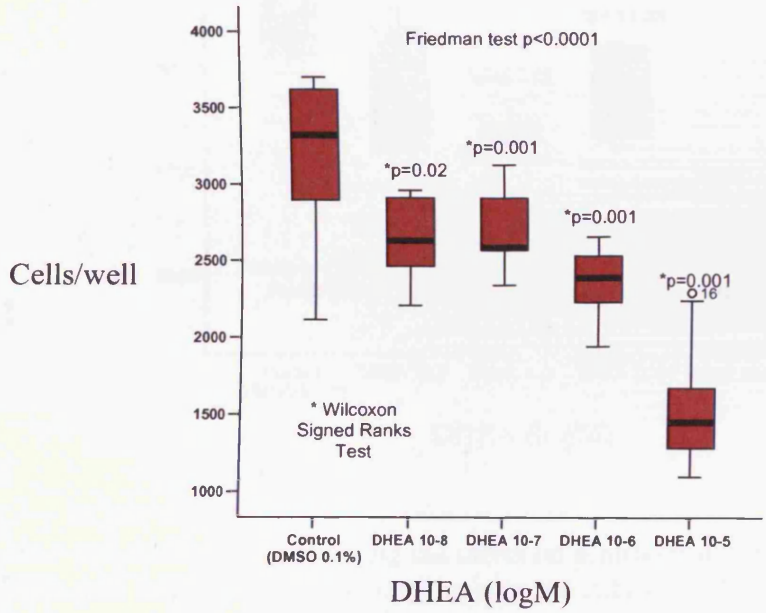



Fig 2.14 Box-and-whisker plot showing the effect on proliferation of PAZ6 preadipocytes following 24 hours exposure to varying concentrations of DHEA. Results are a representative combination of 2 experiments performed in quadruplicate

Median and IQRs for PAZ6 cells treated with DHEA for 48 hours

| Cell Treatment | Median and IQR (Cell number per mL per well) |
|---|---|
| Non treatment | 7049 (6435-8680.25) |
| Control (DMSO 0.1%) | 7852 (6370-9019) |
| DHEA $10^{-8}$ M | 5844 (4519-6719.75) |
| DHEA $10^{-7}$ M | 4892 (4633.75-6081.25) |
| DHEA $10^{-6}$ M | 5484 (4626.5) |
| DHEA $10^{-5}$ M | 1558 (1504.25-1700.25) |

Table 2.9 Experiments conducted as previous results are median and interquartile range for PAZ6 cells following 48 hours exposure to varying concentrations of DHEA

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of PAZ6 cells following 48 hours exposure

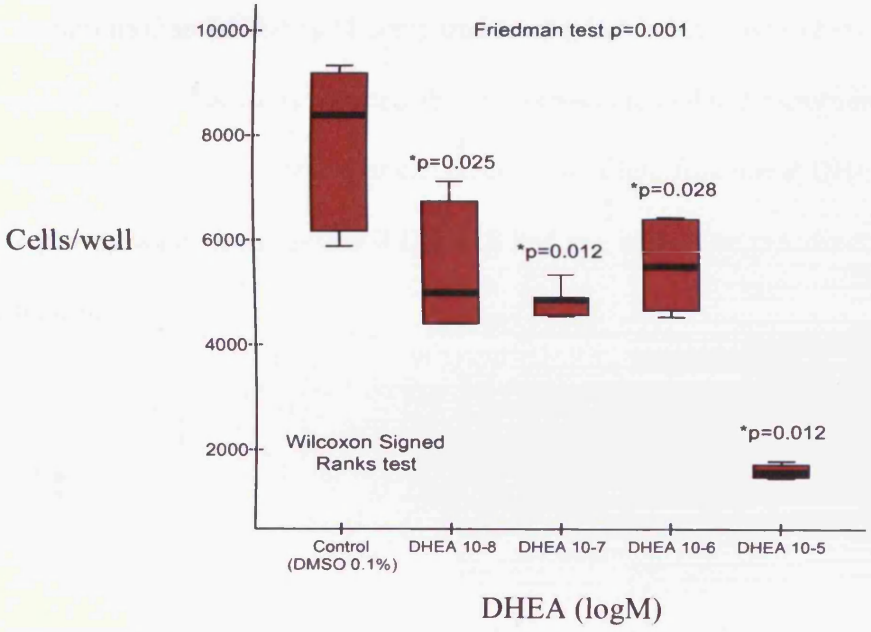



Fig 2.15 Box-and-whisker plot showing the effect on proliferation of PAZ6 preadipocytes following 48 hours exposure to varying concentrations of DHEA. Results are a representative combination of 2 experiments performed in quadruplicate

Our results show that DHEA at concentrations as low as $10^{-8}$ molar (excluding 48 hours in 3T3-L1 cells) had an inhibitory effect on proliferation in both cell types. Also, overall there was no significant difference in proliferation between the non treatment samples and the DMSO controls (Wilcoxon Signed Ranks test p=0.456). Circulating concentrations of DHEA are in the nM range but as previously discussed intra-adipose concentrations may be an order of magnitude higher still. Due to the differing nature of the cells lines utilised in standard culture conditions (PAZ6 cells have a slower proliferation rate/doubling time than 3T3-L1 cells) it is not possible to determine if one cell type is more proliferation-sensitive to DHEA than another.

DHEAS, the sulphated ester of DHEA, is present within the circulation at higher concentrations than DHEA (μM compared to nM) but is believed to have less in terms of biological activity due to its reduced ability to cross the cellular membrane. It is thought to behave as a circulating pool that can be converted into functional DHEA. Consequently we chose to assess if DHEAS had any impact on preadipocyte cell line proliferation.

Box-and-whisker plot demonstrating the effects of DHEAS on proliferation of 3T3-L1 cells following 48 hours exposure

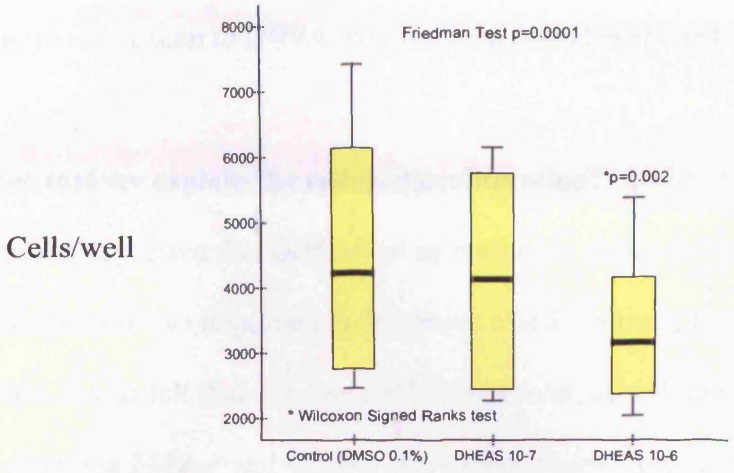



Fig 2.16 $1\times10^3$ per mL plated in 24 well plates (1mL per well) and counted on day 2 (coulter counter), results are the median and IQRs showing the effect on proliferation of 3T3-L1 preadipocytes following 48 hours exposure to DHEAS. Results shown are paired experiments performed in quadruplicate

Box-and-Whisker plot demonstrating the effect of DHEAS on proliferation of PAZ6 cells following 48 hours exposure

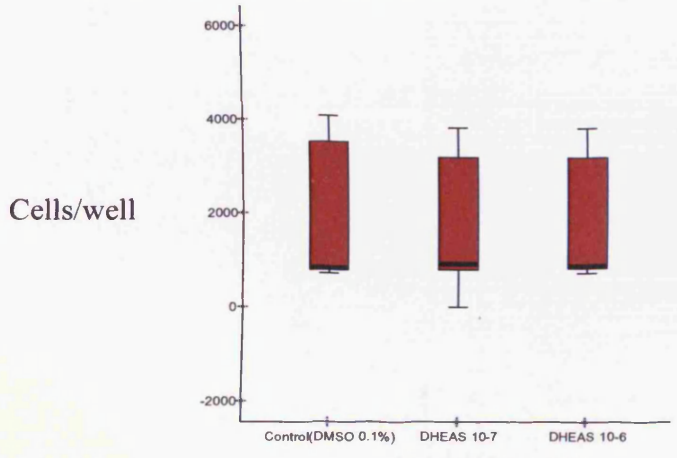



Fig 2.17 $0.5\times10^3$ per mL cells plated in 24 well plates and counted on day 2 (Coulter counter results are median and IQRs showing the effect on proliferation of PAZ6 preadipocytes following 48 hours exposure to DHEAS. Results shown are paired experiments performed in quadruplicate

In contrast to DHEA, DHEAS did not significantly inhibit the proliferation of 3T3-L1 or PAZ6 preadipocytes except at $10^{-6}$ molar in 3T3-L1 cells. It is possible that this was secondary to conversion to DHEA over the time period examined.

### 2.9.2 Does toxicity explain the reduced proliferation?

As our results suggested that DHEA had an inhibitory effect on both the 3T3-L1 and PAZ6 cells lines it was important to determine whether simple toxicity provided an explanation. It was felt that this was unlikely however, as cell numbers continued to increase from the 24 hour and 48 hour assessment points. Nevertheless trypan blue assays were carried out on both cell lines after 48 hours exposure to DHEA $10^{-5}$ molar and $10^{-9}$ molar and control (DMSO 0.1%) as well as on treatment naïve samples. Results were determined by direct visual assessment.

3T3-L1 cells stained with trypan blue following 48 hours exposure to DHEA and Control (DMSO 0.1%)

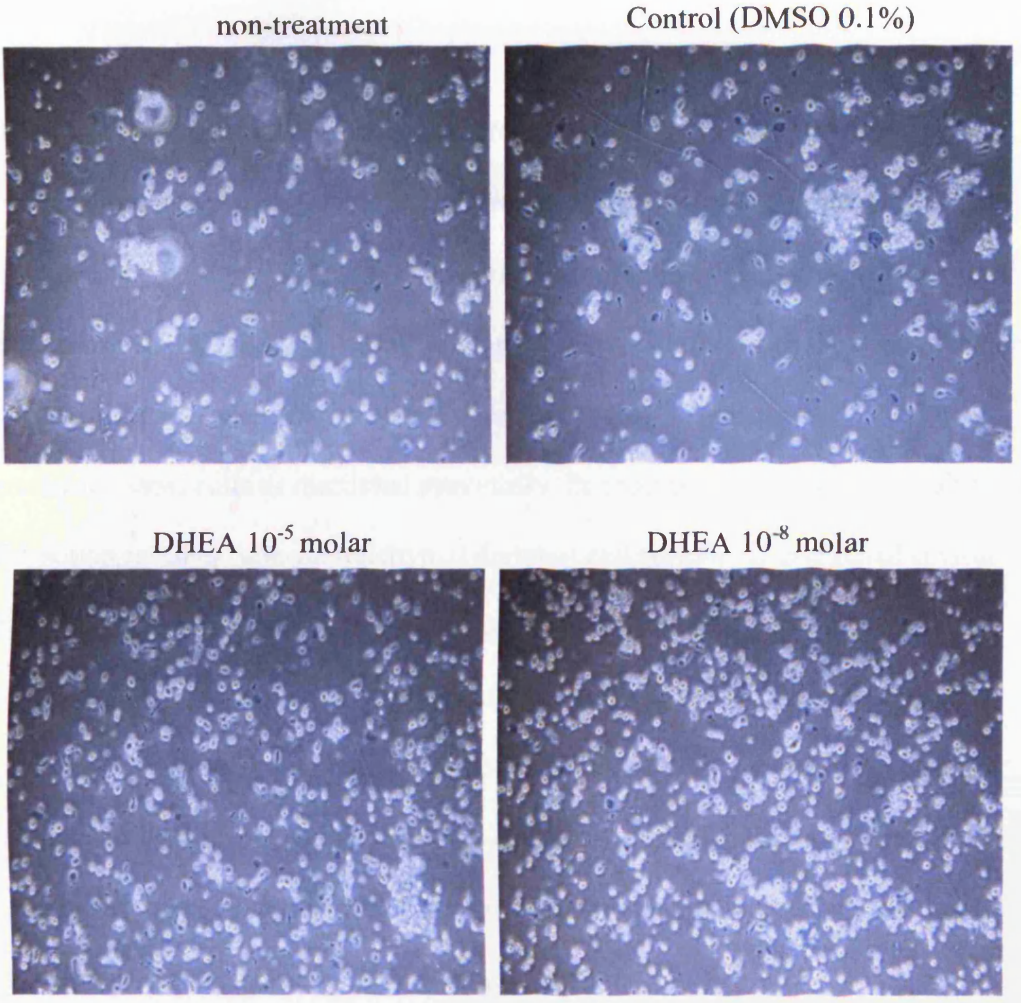



Fig 2.18 3T3-L1 cells from confluent $75cm^2$ flask plated in 24 well plate (1mL per well) treated with trypan blue following 48 hours exposure to DHEA. Results are a single experiment performed in quadruplicate

We estimated toxicity to be less than 1% in all samples assessed. The photographs in Fig 2.18 do demonstrate some dye uptake into cell fragments that are likely to be a result of the trypsinisation/centrifugation as opposed to any toxicity related cell damage. The results seen in the PAZ6 cells were similar to those seen in the 3T3-L1 cells although the

cell number generated per experiment was less as demonstrated in the proliferation experiments.

### 2.9.3 Is the observed anti-proliferation effect specific to preadipoctyes?

The widespread distribution of both the androgen and oestrogen receptor means that determination of cell type specific DHEA effect is potentially difficult. Previous studies have demonstrated DHEA exhibits effects on bone, vascular smooth muscle and adipose tissue. All these cell types have a common embryological origin being derived from mesenchymal stem cells as discussed previously. In order to evaluate the possibility of DHEA action in other (non-mesenchymal derived) cell types a differentiated thyroid cell line (FRTL5) and an undifferentiated skin keratinocyte cell line (HACAT) were employed. It is acknowledged that skin as an organ can be potentially influenced by DHEA secondary to androgenic action on the glandular tissue present. Both acne and greasy skin are well recognized side effects of DHEA therapy particularly in females, most likely secondary to the minimal increase in serum testosterone observed, but the effect on keratinocytes specifically was examined here.

FRTL-5 differentiated thyroid cell line

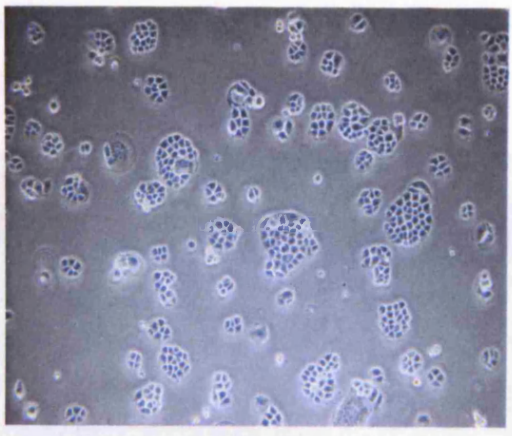


Fig 2.19 Untreated FRTL-5 differentiated thyrocytes on day 2 of culture

Box-and-whisker plot demonstrating the effect of DHEA on Proliferation of FRTL-5 cells following 48 hours exposure

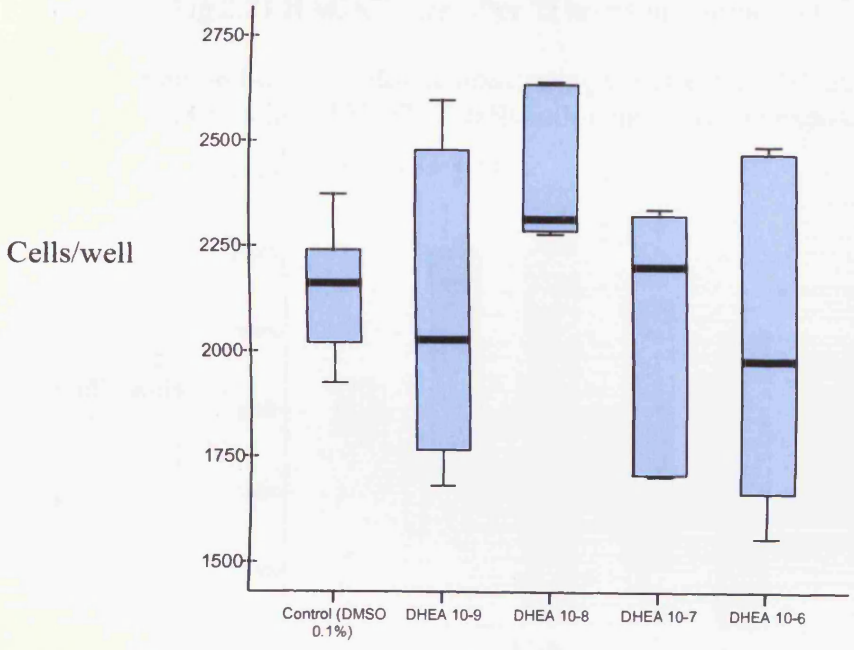



Fig 2.20 Following 4 days in treatment free conditions FRTL-5 cells from 75cm$^2$ were trypsinised and plated in 24 well plate (1mL per well) and counted on day 2. Results are median and IQRs showing the effect of DHEA on proliferation of FRTL5 cells. Results presented are from a single experiment performed in quadruplicate

DHEA did not significantly effect the proliferation of FRTL5 cells (Friedman test p=0.225) in culture conditions (for FRTL5 culture medium see appendix II).

HACAT cells

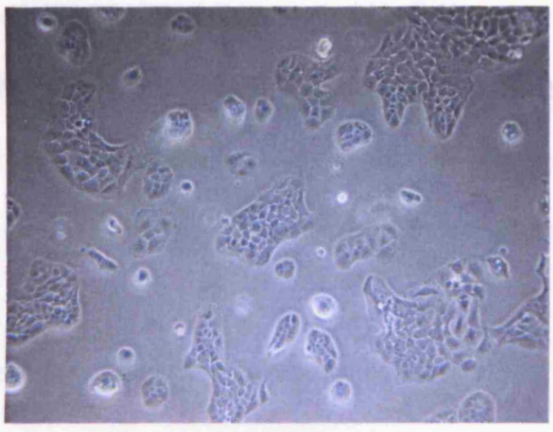


Fig 2.21 HACAT cells after 72 hours in routine culture

Box-and whisker plot demonstrating the effect of DHEA on proliferation of HACAT cells following 48 hours exposure

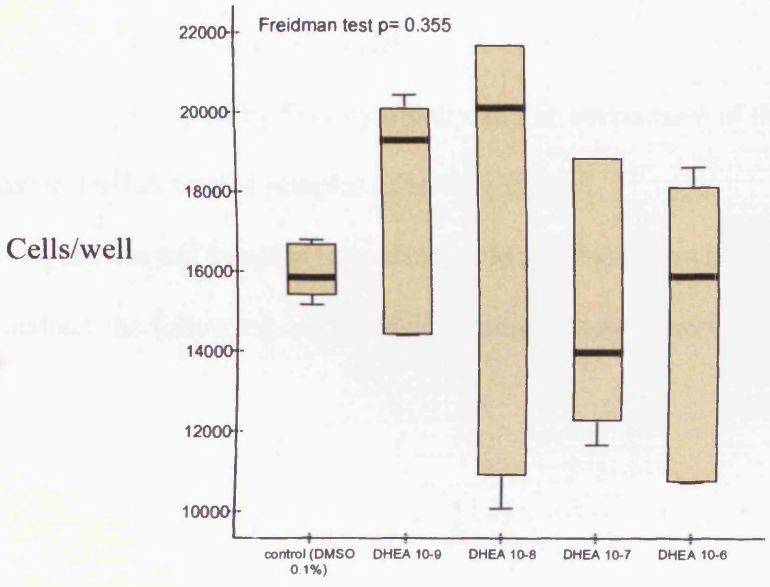



Fig 2.22 Confluent HACAT cells from 75cm$^2$ flask trypsinised and plated in 24 well plated with control (DMSO 0.1%) and varying concentrations of DHEA for 48 hours. Results are median and IQRs. Data are from a single experiment performed in quadruplicate

In HACAT cells a reduction in cell proliferation at higher DHEA concentrations ($10^{-6}$ and $10^{-7}$ molar) was observed but this was not significant (for HACAT cell culture medium see appendix II).

Despite using a differentiated and an undifferentiated cell line we were unable to demonstrate statistically significant anti-proliferative DHEA effects. These data, combined with those from previous studies, suggest that the inhibitory outcomes of DHEA on cell proliferation may be specific for cells of mesenchymal origin.

### 2.9.4 Does DHEA affect the cell cycle in preadipocyte cell lines?

Previous experiments indicated that inhibition of proliferation was not due to toxicity. The possible mechanisms that explain this include:

i) Increased apoptosis

ii) Interruption of the cell cycle

Both of these can be tested by flow cytometry and an assessment of the cell cycle was undertaken in DHEA treated samples relative to control.

In untreated and control samples (containing DMSO 1%) following 48 hours in standard culture medium the following cell cycle distributions were observed:

Scatter plot and histogram showing cell cycle distribution in untreated 3T3-L1 cells

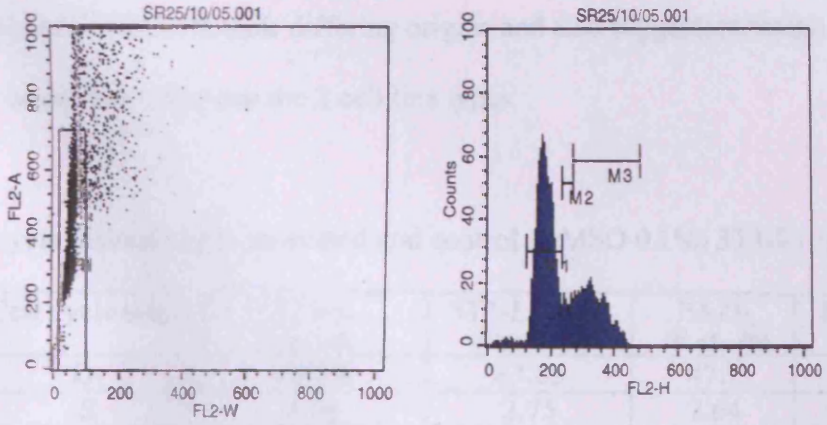



Fig 2.23 Confluent 3T3-L1 cells in 6 well plates were collected and treated with DNA stain (propidium iodide) and analysed by flow cytometry. Data shown are from a representative experiment performed in duplicate

Scatter plot and histogram demonstrating cell cycle distribution in untreated PAZ6 cells

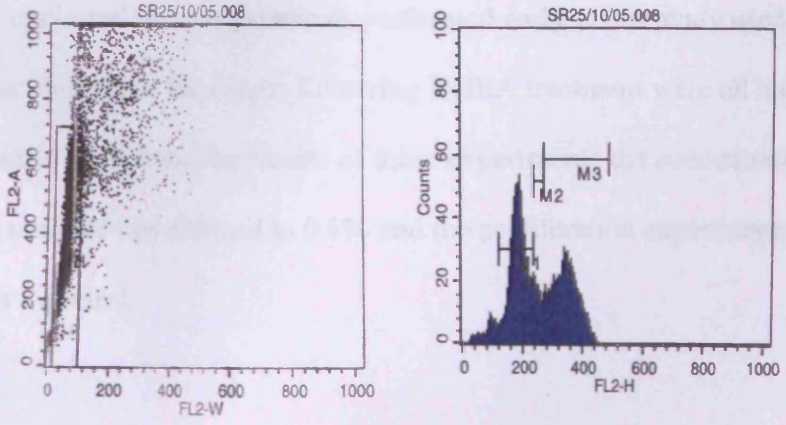



Fig 2.24 Confluent PAZ6 cells treated as in previous experiment. Data presented are from a representative experiment performed in duplicate

These data and the table below (table 2.10) outline the cell cycle distributions in untreated cells. It is clear that there are differences between the 2 cell types from the outset that could be consistent with their differing origins and also suggests potential difficulty in direct comparisons between the 2 cell line types.

Cell cycle distribution in untreated and control (DMSO 0.1%) 3T3-L1 and PAZ6 cells

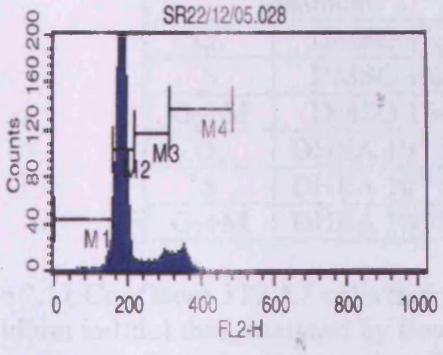
| Cell cycle stage | 3T3-L (% of cells) | 3T3-L1 control (DMSO 1%) | PAZ6 (% of cells) | PAZ6 control (DMSO 1%) |
|---|---|---|---|---|
| $G_1$ | 65.65 | 83.85 | 47.7 | 60.56 |
| S | 6.06 | 2.75 | 7.64 | 6.54 |
| $G_2$+M | 28.21 | 13.3 | 41.80 | 28.57 |

Table 2.10 $1\times10^3$ cells per mL plated in 6 well plates (3mL per well). Cells collected on day 2 under went flow cytometry (propidium iodide staining). Results are percentages of cells in each cell cycle stage following non treatment and treatment with DMSO (1%) control. Results presented are for a single experiment performed in duplicate. A repeat experiment performed with sets of 6 samples gave similar results.

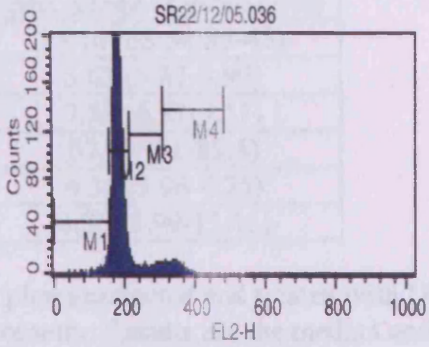
The results presented in table 2.10 clearly indicate that the diluent DMSO had an impact on the cell cycle and these experiments performed early in our study used a 1% DMSO control. For this reason the results following DHEA treatment were all compared to the DMSO control. Following the results of these experiments the concentration of DMSO in all control samples was reduced to 0.1% and the proliferation experiments were repeated with this as a control.

Histograms demonstrating the effect of DHEA compared to control on the cell cycle in 3T3L1 and PAZ6 cells following 48 hours exposure

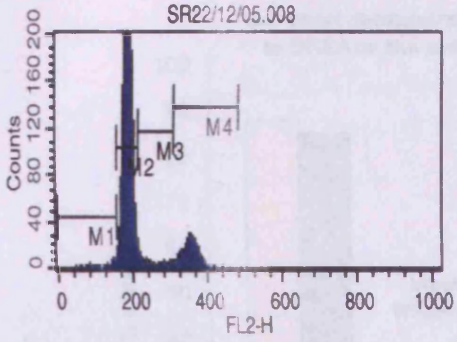
Control (DMSO 1%)




DHEA $10^{-6}$ molar




Control (DMSO 1%)




DHEA $10^{-6}$ molar

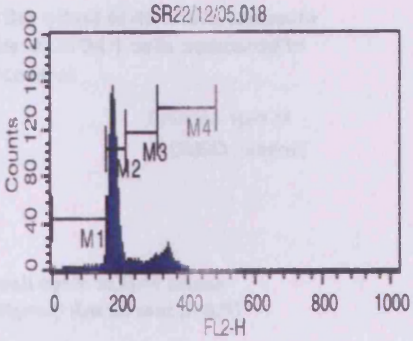



Fig 2.25 Cells exposed to DHEA $10^{-6}$ molar and control (DMSO) in 6 well plates for 48 hours with treated with DNA strain (propidium iodide) and assessed for cell cycle stage by flow cytometry. Results are histograms for PAZ6 and 3T3-L1 cells and controls (DMSO 1%). Data presented are from a single experiment performed in replicates of six that provided similar results to a repeat experiment

Results from the above experiments were combined with all flow cytometry results and are presented below.

Median and IQR for cell cycle stage as percentages of cells gated for 3T3-L1 preadipocytes following 48 hours exposure to DHEA and control

| Cell cycle stage and treatment | | Median and IQRs (percentage of gated cells) |
|---|---|---|
| $G_1$ | DMSO 1% | 85.16 (68.54-85-45) |
| S | DMSO 1% | 5.63 (3.37-5.99) |
| $G_2$+M | DMSO 1% | 7.53 (6.82-9.51) |
| $G_1$ | DHEA $10^{-6}$ M | 87.98 (81-89.5) |
| S | DHEA $10^{-6}$ M | 4.36 (3.96-4.75) |
| $G_2$+M | DHEA $10^{-6}$ M | 4.36 (3.99-13.76) |

Table 2.11 Confluent 3T3-L1 cells in 6 well plates collected and treated with DNA stain (propidium iodide) then analysed by flow cytometry. Results are the median and IQRs for the percentage of gated cells in each cell cycle stage. Data presented are a combination of 2 experiments the first performed in duplicate and the second in replicates of 6

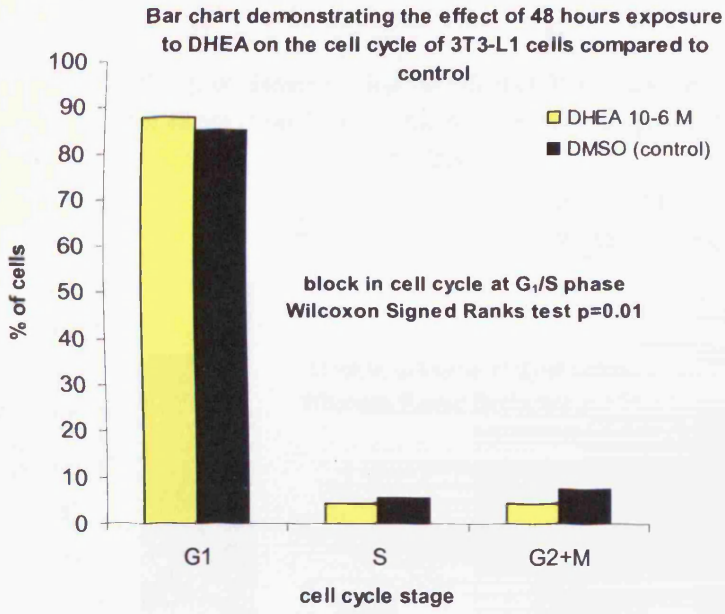



Fig 2.26 Experiment performed as summarised in table 2.11. Results are median values of percentages of gated controls for each cell cycle stage in 3T3-L1 preadipocytes following 48 hours exposure to DHEA $10^{-6}$ molar and control (DMSO 1%)

Median and IQR for cell cycle stage as percentages of cells gated for PAZ6 preadipocytes following 48 hours exposure to DHEA and control

| Cell cycle stage and treatment | | Median and IQRs (percentage of gated cells) |
|---|---|---|
| $G_1$ | DMSO 1% | 85.76 (66.56-86.51) |
| S | DMSO 1% | 2.86 (2.43-5.65) |
| $G_2$+M | DMSO 1% | 10.74 (10.08-24.17) |
| $G_1$ | DHEA $10^{-6}$ M | 64.46 (58.53-72.88) |
| S | DHEA $10^{-6}$ M | 12.25 (8.41-13.57) |
| $G_2$+M | DHEA $10^{-6}$ M | 15.8 (11.98-27.75) |

Table 2.12 Confluent PAZ6 cells in 6 well plates collected and treated with DNA stain (propidium iodide) then analysed by flow cytometry. Results are the median and IQRs for the percentage of gated cells in each cell cycle stage. Data presented are a combination of 2 experiments the first performed in duplicate and the second in replicates of 6

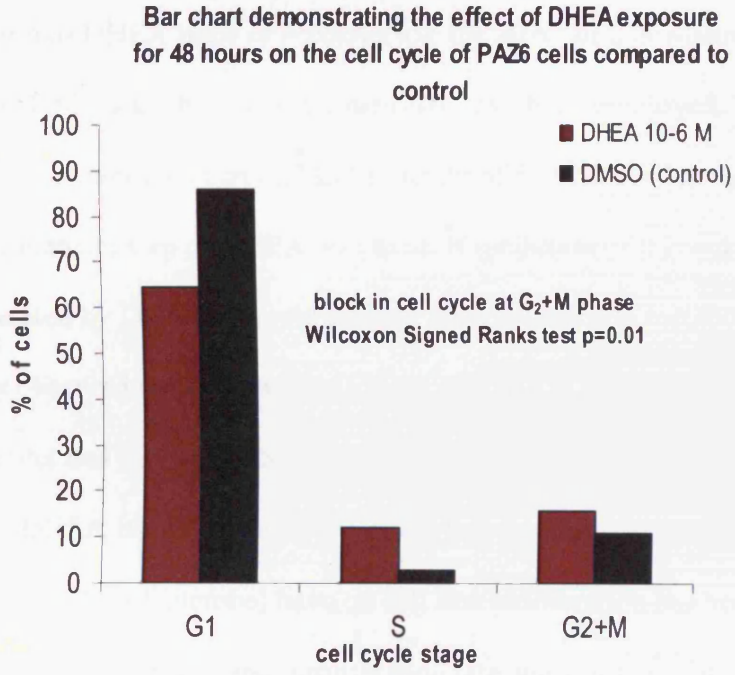



Fig. 2.27 Experiment performed as summarised in table 2.12. Results are median values of percentages of gated controls for each cell cycle stage in PAZ6 preadipocytes following 48 hours exposure to DHEA $10^{-6}$ molar and control (DMSO 1%)

DHEA at a concentration of $10^{-6}$ molar caused a block in the cell cycle of both 3T3-L1 and PAZ6 cells but at different points within the cell cycle (3T3-L1 G1/S, PAZ6 G2+M). Furthermore analysis of the flow cytometry histograms, for both cell types, revealed no change at the sub-$G_1$ peak suggesting that DHEA treatment was not an apoptotic stimulus.

**2.9.5 Are effects directly due to DHEA or secondary to a downstream metabolite?**

DHEA is a prohormone that has the potential to be converted to either androgenic or oestrogenic terminal hormones (Fig 1.5) and adipose tissue contains the necessary enzymes to do this. Therefore any effects that are observed in adipose tissue could be as a result of the action of DHEA itself or secondary to the effect of a downstream metabolite. In order to address this issue, two separate methods have been employed. Firstly an inhibitor of DHEA metabolism that blocks the action of 3-βHSD which catalyses the first biosynthetic downstream step of DHEA was used. If inhibition of this enzyme terminated any effects generated by DHEA it would suggest that downstream metabolites had precipitated any observed response whereas if the addition of this enzyme to the experimental model had no impact the suggestion would be that any effects were originating from DHEA itself. Secondly the effect that varying concentrations of DHEA metabolites (oestradiol, testosterone) have on cell line proliferation has been examined. The 3T3-L1 cell line has a more rapid proliferation rate and was therefore chosen for use in these experiments.

Box-and-whisker plot demonstrating the effect of trilostane on proliferation of 3T3-L1 cells

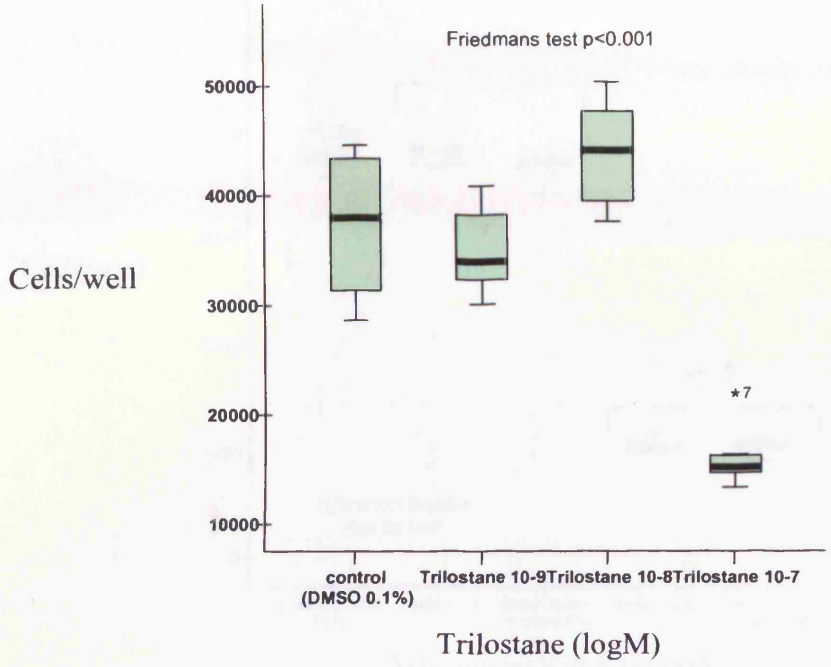



Fig 2.28 $1\times10^3$ cells per mL were plated in 24 well plates (1mL per well) and counted on day 2 (coulter counter) results are expressed as median and IQRs showing the effect of trilostane on proliferation of 3T3-L1 preadipocytes

From the box plot (Fig 2.28) it is clear that at a concentration of $10^{-7}$ molar trilostane itself had a negative impact on cell number in our experimental model although it was not determined whether this was secondary to toxicity. At a concentration of $10^{-8}$ molar however, trilostane did not have a negative effect on cell number and at this concentration did not influence the previously demonstrated DHEA influence on proliferation (Fig 2.29).

Box-and-whisker plot demonstrating the effect of DHEA and trilostane on proliferation of 3T3-L1 cells following 48 hours exposure

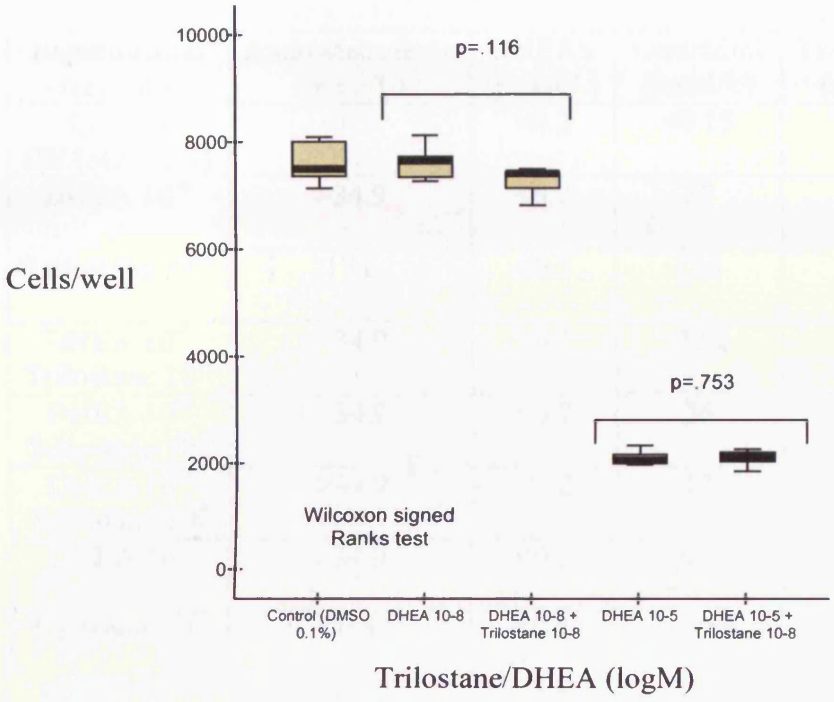



Fig 2.29 Experiment performed as previous, results are expressed as median and IQRs showing the effect of trilostane $10^{-8}$ molar on the antiproliferative effect of DHEA in 3T3-L1 preadipocytes following 48 hours exposure. Results presented are a single experiment performed in quadruplicate

From these data it was initially concluded that 3β-HSD inhibition (by trilostane $10^{-8}$ molar) did not reduce the effect of DHEA on proliferation thus suggesting a direct action, however it was not possible to confirm if this concentration of inhibitor was sufficient to block all (or any) downstream metabolite production.

To examine the ability of trilostane to inhibit downstream metabolite production, conditioned medium from completed experiments using approximate adipose tissue DHEA concentrations ($10^{-7}$ molar and $10^{-6}$ molar) was assayed for testosterone, oestradiol, androstendione and DHEAS (assays kindly performed by Dr Carol Evans, Medical Biochemistry department, University Hospital of Wales, Cardiff).

Conditioned medium metabolite concentrations following exposure of 3T3-L1 cells to DHEA and/or trilostane for 48 hours

| Experimental reagent/s | Androstenedione (nmol/L) | DHEAS (μmol/L) | Oestradiol (nmol/L) | Testosterone (nmol/L) |
|---|---|---|---|---|
| Control (DMSO 0.1%) | <0.5 | <0.2 | <0.15 | <0.35 |
| DHEA $10^{-6}$ | >34.9 | <0.2 | 27 | 15.5 |
| Trilostane $10^{-7}$ | 17.6 | <0.2 | 136 | 42.4 |
| DHEA $10^{-6}$ Trilostane $10^{-7}$ | >34.9 | <0.2 | 290 | 50.1 |
| DHEA $10^{-6}$ Trilostane $10^{-8}$ | >34.9 | <0.2 | 36 | 20.8 |
| DHEA $10^{-6}$ Trilostane $10^{-9}$ | >34.9 | <0.2 | 12 | 13.7 |
| DHEA $10^{-7}$ | >34.9 | <0.2 | <0.15 | 1.7 |
| Trilostane $10^{-8}$ | <0.5 | <0.2 | <0.15 | 13.7 |
| DHEA $10^{-7}$ Trilostane $10^{-7}$ | >34.9 | <0.2 | 15 | 33.4 |
| DHEA $10^{-7}$ Trilostane $10^{-8}$ | >34.9 | <0.2 | <0.15 | 11.8 |
| DHEA $10^{-7}$ Trilostane $10^{-9}$ | >34.9 | <0.2 | <0.15 | 3.2 |

Table 2.13 $1\times10^3$ per mL cells plated in 24 well plates for 2 days. Conditioned medium (1mL) collected and analysed for metabolites. Results are concentrations of DHEAS, androstenedione, oestradiol and testosterone in conditioned medium following exposure of 3T3-L1 cells to different concentrations of DHEA and trilostane. Data presented are from single experiment repeated with similar results

The results shown in table 2.13 revealed that the addition of DHEA to the experimental model increased androstenedione, testosterone and oestradiol concentrations as expected; the androstenedione levels are high, consistent with the use of supraphysiological serum levels (the assay is designed for the assessment of human serum). Interestingly, despite using these DHEA concentrations, no increase in DHEAS was observed and this could

suggest that, in our *in vitro* model at least, there is no significant sulphotransferase activity (Fig 1.3) catalysing the conversion of DHEA to DHEAS. Whether this is true of all adipose tissue is unclear, although a previous study using human subcutaneous adipose tissue was unable to demonstrate expression of sulphotransferase despite confirming the presence of steroid sulphatase [87]. Another possible explanation is that the assay system employed (designed for use with human serum) was unable to detect the murine DHEAS; against this theory however was the fact that other hormones were readily detected despite species differences.

Somewhat unexpectedly however, our results demonstrate that trilostane itself increased the amount of metabolite production and to a greater extent than DHEA alone; further, when combined with DHEA the effect was additive. As the concentration of the inhibitor was reduced the metabolite concentration also declined, when the opposite was anticipated.

These unexpected results with trilostane revealed that it had activity that was the converse to that anticipated in our experimental model, therefore the observation previously made that the effect on proliferation was as a direct result of DHEA could not be corroborated. However to further investigate the continuing question concerning direct versus indirect DHEA action I undertook an assessment of terminal metabolites with known metabolic activity.

Box-and-Whisker plot demonstrating the effect of testosterone on proliferation of 3T3-L1 cells following 48 hours exposure

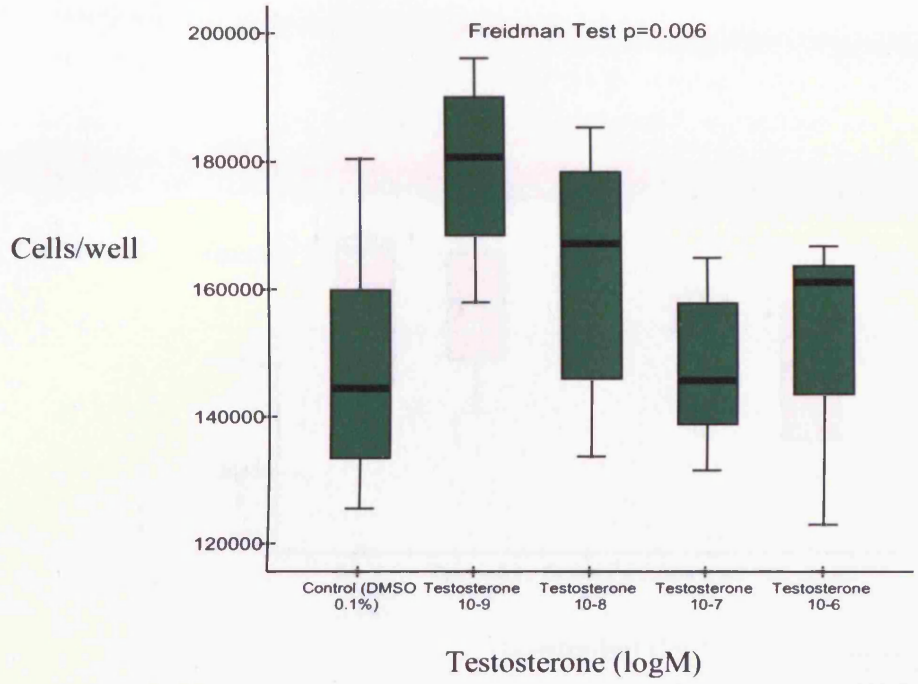



Fig 2.30 Confluent 75cm$^2$ flask containing 3T3-L1 cells was trypsinised and plated into a 24 well plate. Cell were counted (coulter counter) on day 2 following the addition of varying concentrations of testosterone. Results are the median and IQRs showing the effect on proliferation of 3T3-L1 preadipocytes following 48 hours exposure to varying concentrations of testosterone. Data presented are from a single experiment performed in quadruplicate

Box-and-whisker plot demonstrating the effect of β-oestradiol on Proliferation of 3T3-L1 cells following 48 hours exposure

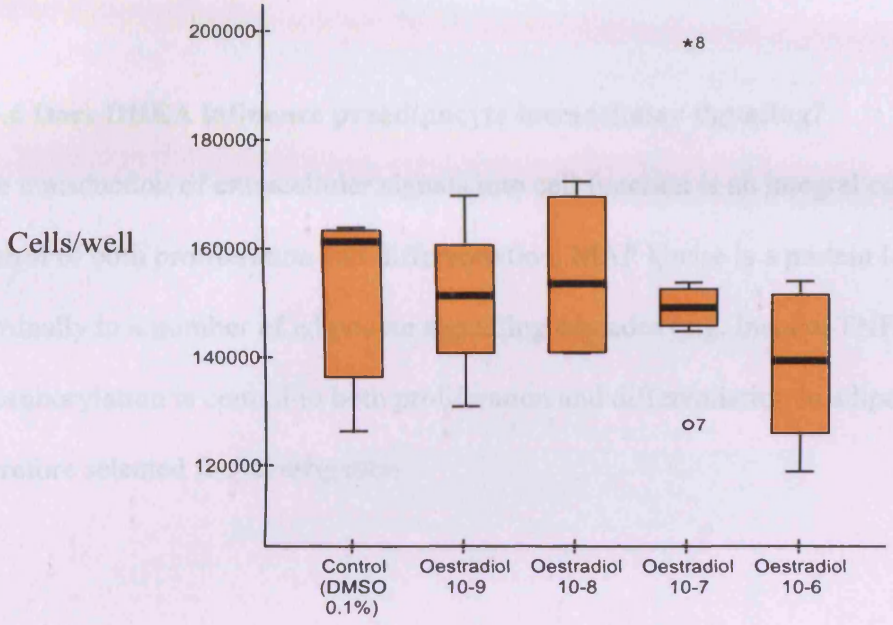



Fig 2.31 Experiment performed as previous. Results are the median and IQRs showing the effect on proliferation of 3T3-L1 preadipocytes following 48 hours exposure to varying concentrations of β-oestradiol. Data presented are a single experiment performed in quadruplicate.

Neither testosterone nor β-oestradiol exhibited a significant inhibitory effect on proliferation of 3T3-L1 preadipocytes although testosterone did significantly increase the amount of proliferation, however further investigation of this observation was not undertaken.

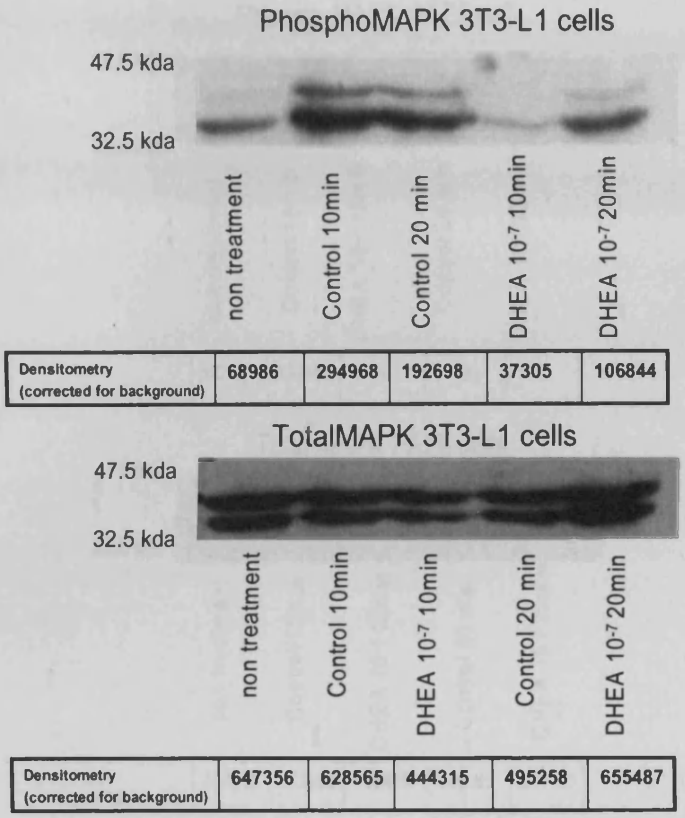
Our investigation into direct versus indirect DHEA effect, despite the failings of the trilostane experiment, do still favour a direct effect. It is acknowledged however that a number of metabolites other than testosterone and oestradiol (Fig 1.3) are generated by

DHEA metabolism and that these were not investigated as our intention was to focus on metabolites with known biological activity.

### 2.9.6 Does DHEA influence preadipocyte intracellular signaling?

The transduction of extracellular signals into cell function is an integral component in the control of both proliferation and differentiation. MAP kinase is a protein located terminally in a number of adipocyte signalling cascades (e.g. Insulin, TNFα) and its phosphorylation is central to both proliferation and differentiation in adipocytes and was therefore selected for investigation.

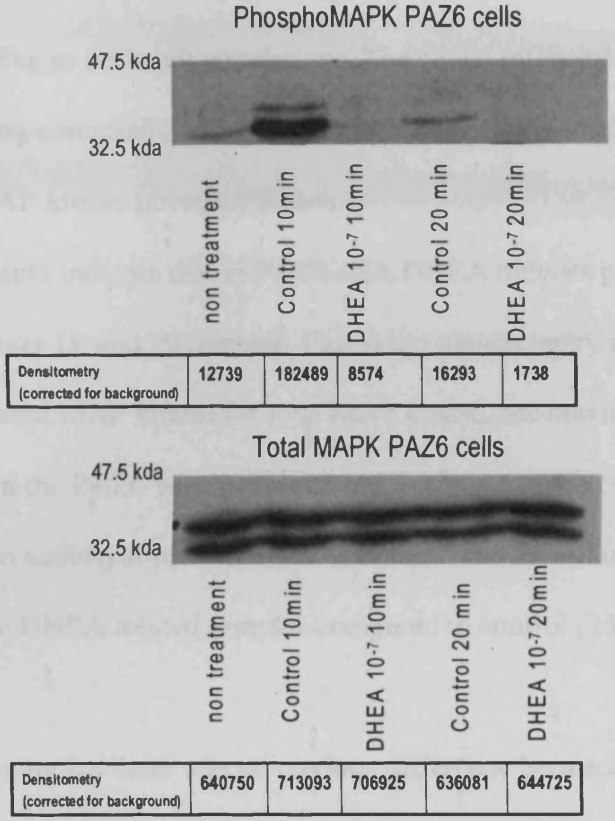
Western blots demonstrating short-term effects of DHEA ($10^{-7}$ molar) on phosphorylated levels of MAP kinase activity in 3T3-L1 preadipocytes




| 3T3-L1 sample | PhosphoMAK densitometry corrected for totalMAPK densitometry |
|---|---|
| Non treatment | 0.11 |
| Control 10 minutes | 0.47 |
| DHEA $10^{-7}$ molar 10 minutes | 0.43 |
| Control 20 minutes | 0.07 |
| DHEA $10^{-7}$ molar 20 minutes | 0.16 |

Fig 2.32/Table 2.14 Cells grown to confluence in 6 well plates in standard medium followed by 2×2 hours in serum free medium (DMEM) had DHEA $10^{-7}$ molar (containing 0.01% FCS) and DMSO 0.1% control (containing 0.01%FCS) added for 10 and 20 minutes. Cells were then homogenised, blotted and detected following standard Western blotting protocol for phosphorylated and total MAP kinase. The blots then underwent densitomery assessment and correction for total MAP kinase measurement. Experiments repeated at least 3 times

Western blots demonstrating short-term effects of DHEA ($10^{-7}$ molar) on phosphorylated levels of MAP kinase activity in PAZ6 preadipocytes




| PAZ6 sample | PhosphoMAK densitometry corrected for totalMAPK densitometry |
|---|---|
| Non treatment | 0.019 |
| Control 10 minutes | 0.256 |
| DHEA $10^{-7}$ molar 10 minutes | 0.012 |
| Control 20 minutes | 0.026 |
| DHEA $10^{-7}$ molar 20 minutes | 0.003 |

Fig 2.33/table 2.15 Cells grown to confluence in 6 well plates in standard medium followed by 2×2 hours in serum free medium (DMEM) had DHEA $10^{-7}$ molar (containing 0.01% FCS) and DMSO 0.1% control (containing 0.01%FCS) control added for 10 and 20 minutes. Cells were then homogenised, blotted and detected following standard Western blotting protocol for phosphorylated and total MAP kinase. The generated blots then underwent densitometry assessment and correction for total MAP kinase measurement. Experiments repeated at least 3 times

As can be observed in Fig 2.32 and Fig. 2.33 addition of control (containing 0.1% DMSO and 0.01% FCS) precipitated significant phosphorylation of MAP kinase and this was a consistent finding in both cell populations. The signal intensity for total MAP kinase, whilst indicating essentially equal loading in all lanes, provided a method of quantifying the level of MAP kinase phosphorylation.

The Western blots indicate that in PAZ6 cells DHEA reduces phosphorylated MAP kinase levels over 10 and 20 minutes. Following densitometry assessment and correction of phosphorylated MAP kinase for total MAP kinase, the maximum phosphorylated MAP kinase levels in the PAZ6 were observed at 10 minutes in the control sample with a 13.5 fold increase in activity from base line. At both 10 and 20 minutes less activity was observed in the DHEA treated samples compared to control (25 and 8.6 fold respectively).

In the 3T3-L1 samples there was no obvious difference between DHEA and control samples at 10 minutes when corrected for total MAP kinase, and at 20 minutes the control sample revealed reduced activity as did the DHEA treated sample but to a lesser extent. The data for total MAP kinase in 3T3-L1 cells do however demonstrate some variability in lane loading. Following densitometry and correction of phosphorylated MAP kinase for total MAP kinase levels it was apparent that maximal phosphorylated MAP kinase activity was observed in the control sample at 10 minutes but this was very similar to that seen in the 10 minute DHEA treated sample (4.2 and 3.9 fold increase from baseline respectively). At 20 minutes marginally more activity was observed in the DHEA treated sample compared to control (1.4 and 2 fold respectively). The 3T3-L1 preadipocyte results may be interpreted as demonstrating essentially no difference in

levels of phosphorylated MAP kinase in DHEA and control samples. These experiments were repeated at least 3 times with similar results.

As MAP kinase activation and deactivation is necessary for cell signal transduction all that can be interpreted from the data generated is that DHEA does have an effect on intracellular signalling and that this involves MAP kinase, however it is not possible to conclude if the cell signalling effect is one of inhibition or stimulation.

In the majority of cases the above technique is used to confirm or refute the activation of a cell signalling protein (not the inactivation). In our experiments, the use of standard culture medium (containing 10% FCS) as a diluent as well as DMSO, precipitated phosphorylated MAP kinase activation though it is not clear which of these agents brought about this effect. This was somewhat fortuitous and it enabled us to demonstrate an inactivation effect caused by DHEA in the PAZ6 cells.

Protein assay was not performed prior to sample loading as each sample within an individual experiment underwent precisely the same procedure and the short exposure periods used were unlikely to affect the relative amounts of generated samples the same amounts of which were loaded into each well.

The Western blotting experiments were also complicated by a difficultly in obtaining a 'zero' blot for the background level of activation in untreated samples.

### 2.9.7 Does DHEA affect preadipocyte cell line differentiation?

Placed in the appropriate environment both PAZ6 and 3T3-L1 cells will differentiate into mature adipocytes characterised, as previously discussed, by morphological change with an increase in size, a more rounded appearance and the accumulation of triacylglyceride

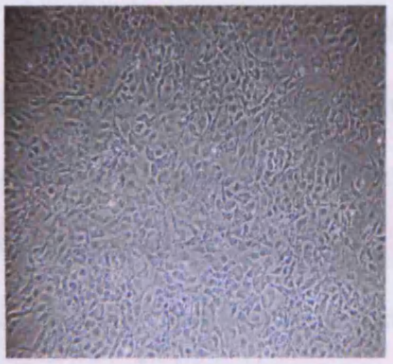
rich droplets. From experience it is clear that the 3T3-L1 preadipocytes possess a greater ability to differentiate when compared to PAZ6 cells with up to 60% of cells in 3T3-L1 cultures undergoing differentiation. Also the 3T3-L1 cells undergo significant differentiation over a shorter time period when again compared to PAZ6. The differentiation protocol in the 3T3-L1 cells achieved terminal differentiation in 9-12 days whereas in PAZ6 the cells required 15 days or longer to differentiate, and invariably did so to a lesser degree. The differential adipogenic capacity impinged on our first chosen method of assessing the extent of differentiation in DHEA treated and control samples. The number of differentiation foci in PAZ6 cells was easy to ascertain as they tended to occur in isolated pockets conducive to counting whereas in the 3T3-L1 cultures the amount of differentiation could be so great that distinguishing between the various foci at terminal differentiation became difficult.

#### 2.9.7.1 Foci of differentiation

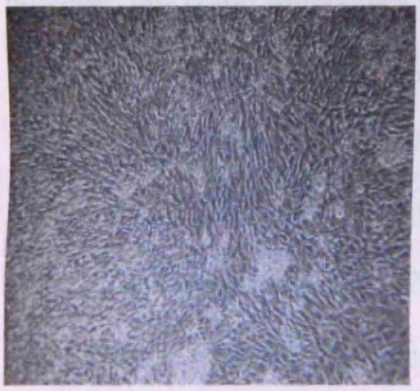
Assessing differentiation can be performed visually. What follows are photographic time lines for both 3T3-L1 and PAZ6 cells as they proceed through the differentiation protocol. At the final points, foci of differentiation were counted.

3T3-L1 cells differentiation day 0

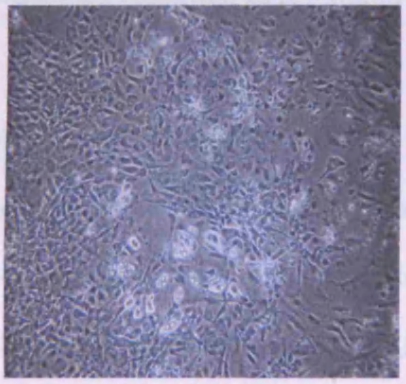
PAZ6 cells differentiation day 0



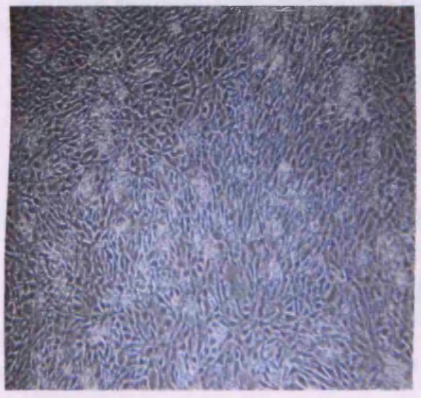
3T3-L1 cells in differentiation mix + DHEA $10^{-7}$ molar day 9



PAZ6 cells in differentiation mix + DHEA $10^{-7}$ molar day 15



3T3-L1 cells in differentiation mix + DMSO 0.1% day 9



PAZ6 cells in differentiation mix + DMSO 0.1% day 15

Fig 2.34 Confluent 3T3-L1/PAZ6 cells in 6 well plates cultured in differentiation mix with either control (DMSO 0.1%) or DHEA $10^{-7}$ molar for 9/15 days. Results are photographs taken on day 0 and day 9/15. Data presented are from a single representative experiment. Magnification ×100

The photographic data demonstrate how readily 3T3-L1 cells undergo differentiation relative to PAZ6 cells and also that some differentiation will occur in the absence of differentiation mix as evident in the day 0 photograph, which showns cell cultures in normal culture medium plus DMSO (0.1%) for 4 days. The photographs taken at day 9 highlights the difficulty in counting the differentiation foci in this cell type as differentiation could be so widespread that identifying individual foci became difficult. These data would suggest that more differentiation occurs in the absence of DHEA; however, this could simply be a function of the demonstrated reduction in proliferation affecting the extent of clonal expansion.

The presented photographs also highlight the reduced differentiation potential of the PAZ6 cells relative to the 3T3-L1 cells and the easily identifiable foci present at the end of the differentiation protocol. There are a number of potential explanations for these differences between the 2 cell lines. It may be related to the species difference (3T3-L1 mouse and PAZ6 human, it could also be that brown preadipocytes in general have a reduced tendency to differentiate relative to the white equivalent and finally it could be as a result of the treatments that were used to generate the cell lines in that the PAZ6 cells underwent SV40 transformation whereas the 3T3-L1 cells did not.

The following foci of differentiation data refer to the PAZ6 cells only because of the difficulty identifying individual foci in the 3T3-L1 cells as discussed. My general opinion and one that had been noted on a number of occasions, was that there did appear to be a reduction in differentiation observed in the DHEA treated 3T3-L1 preadipocytes.

Box-and-whisker plot demonstrating the effect of DHEA $10^{-7}$ molar on the number of foci of differentiation per field observed in PAZ6 cells following 15 days in differentiation medium

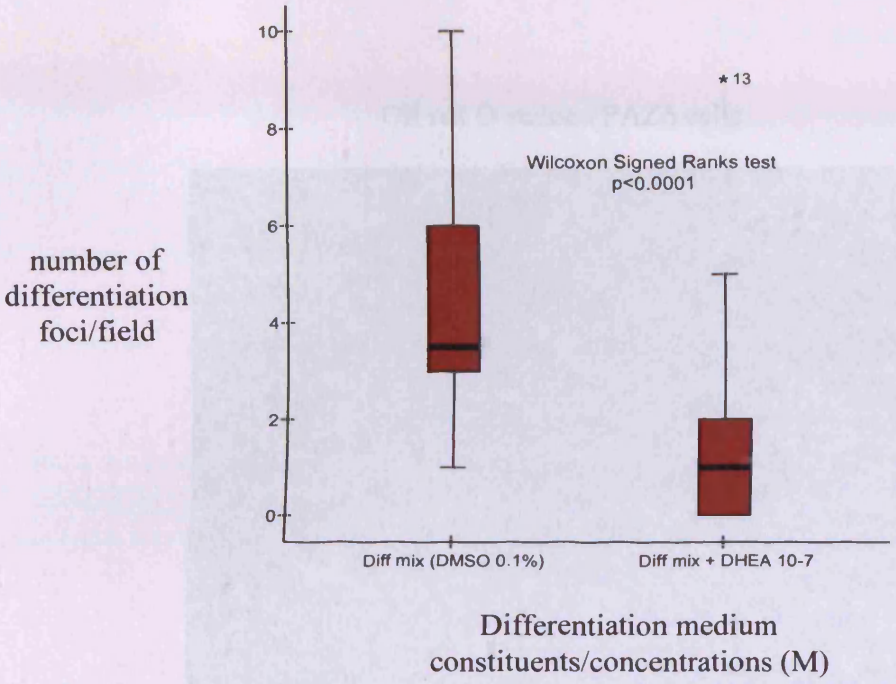



Fig 2.35 PAZ6 cells grown to confluence in 6 well plates followed by addition of differentiation medium with DHEA $10^{-7}$ molar or control (DMSO 0.1%). Medium and reagents were changed every 5 days. At 15 days the number of differentiation foci was counted. Results are median and IQRs. Presented data are from a single representative experiment in which each well underwent 10 randomly chosen field counts using a light microscope

The presented data clearly illustrate the reduction in differentiation foci observed in the DHEA treated samples. This result however may not consider any reduction in clonal expansion that may have occurred.

### 2.9.7.2 Oil red O staining

The oil red O stain taken up by the lipid component in the differentiated adipocytes could be dissolved out and assessed per sample via direct colourimetry; this was performed in

both the 3T3-L1 and PAZ6 preadipocytes. Once more, when interpreting results from these experiments, one must consider the impact of the previously demonstrated proliferation inhibition for which this methodology was more conducive.

Oil red O stained PAZ6 cells

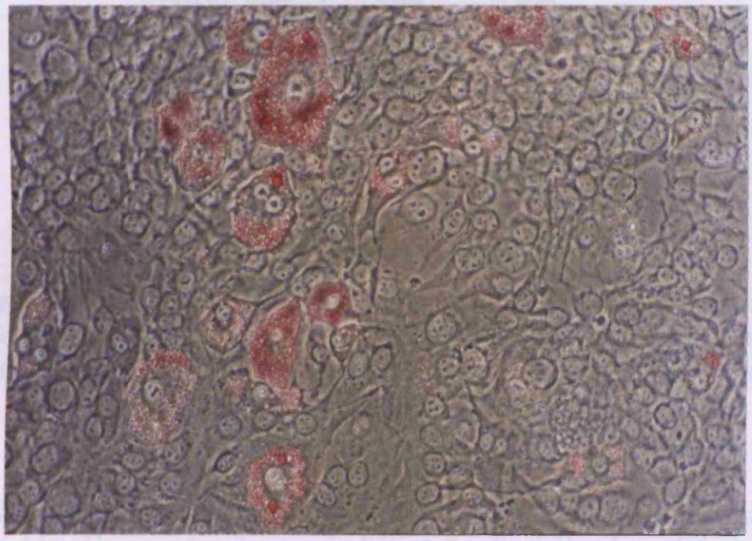


Fig 2.36 Confluent PAZ6 cells in 24 well plates treated in differentiation mix (with DHEA or DMSO control) for 15 days then stained with oil red O
Magnification ×200

Box-and-whisker plot demonstrating the effect of DHEA on amount of oil red O stain measured by direct colourimetry in PAZ6 cells following differentiation

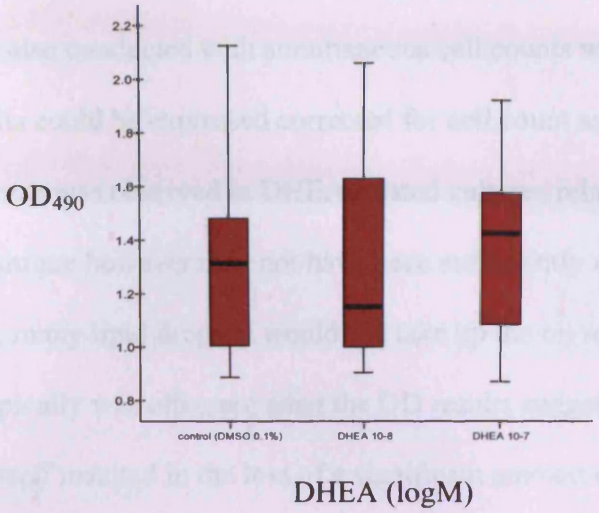



Fig 2.37 Confluent PAZ6 cells in a 24 well plate exposed to differentiation mix with DHEA or control for 15 days then stained (oil red O), the amount of stain was then assessed by direct colourimetry. Results are the median and IQRs expressed as $OD_{490}$. Data presented are from a single representative experiment

Box-and-whisker plot demonstrating the effect of DHEA on amount of oil red Ostain measured by direct colourimetry in 3T3-L1 cells following differentiation

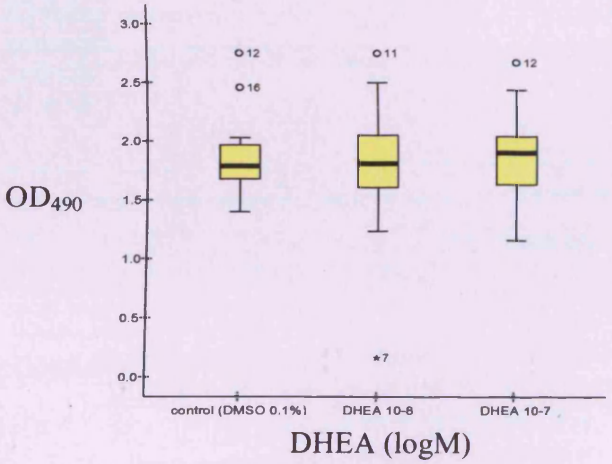



Fig 2.38 Confluent 3T3-L1 cells in a 24 well plate exposed to differentiation mix with DHEA or control for 9 days then stained (oil red O), the amount of stain was then assessed by direct colourimetry. Results are the median and IQRs expressed as $OD_{490}$. Data presented are from a single representative experiment

These results indicate that there was no difference between control and DHEA treated samples in terms of differentiation despite not considering proliferation rates. These experiments were also conducted with simultaneous cell counts with DHEA $10^{-7}$ molar such that the results could be expressed corrected for cell count and once again no significant difference was observed in DHEA treated cultures relative to control.

The oil red O technique however may not have been sufficiently accurate in that, for unknown reasons, many lipid droplets would not take up the oil red O stain and what was viewed macroscopically was often not what the OD results suggested. Also the oil red O staining process itself resulted in the loss of a significant amount of cellular material despite reducing the amount of washes used in the protocol. Secondary to the concerns relating to this technique a more sensitive method for assessing the extent of differentiation was required. Quantitative PCR was selected; this would facilitate measurements of transcripts of adipogenesis markers, such as GPDH and LPL. These would be compared between control and DHEA treated cells.

### 2.9.7.3 PCR/QPCR

Data on RNA quality, standard and dissociation curves and gene expression rates expressed as copy numbers corrected for housekeeper genes follow.

#### 2.9.7.3.1 RNA quality

To confirm effective RNA extraction and RNA quality in 3T3-L1 and PAZ6, visualisation was performed using 2% ethidium bromide loaded gels. Tanks were cleaned with 0.5% SDS (in distilled $H_20$) for 1 hour prior to gel analysis.

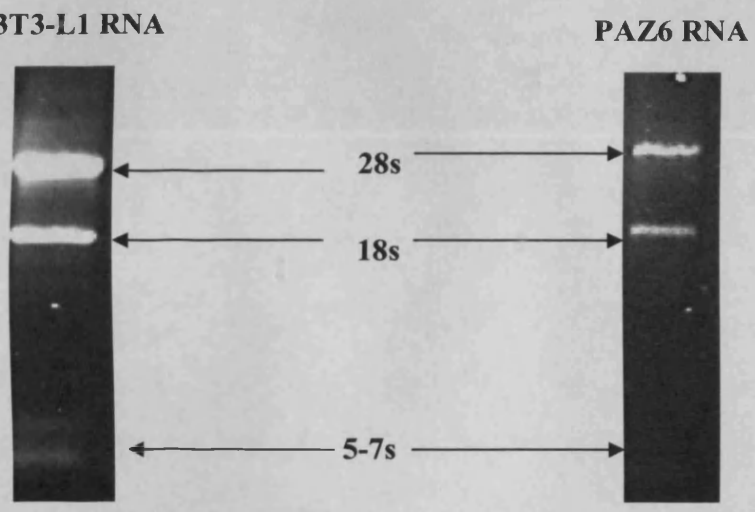



Fig 2.39 5µg of 6×dye with 1µg of extracted RNA from PAZ6 and 3T3-L1 cells at day 0 of the differentiation protocol visualised on a 2% ethidium bromide agarose gel. Data presented are from a single experiment

The presence of bands at 28s and 18s confirms RNA has not degraded and is thus suitable for RT.

### 2.9.7.3.2 Standard curve

To allow for the estimation of copy number of a gene of interest in unknown samples firstly a standard curve was generated from samples of known copy number quantity. Serial dilutions from stock standard solutions ($10^9$ copies) analysed using SYBR green dye and opitimised primer concentrations generated standard curves for ARP/APRT (housekeeper) and GPDH/LPL (terminal adipogenesis markers).

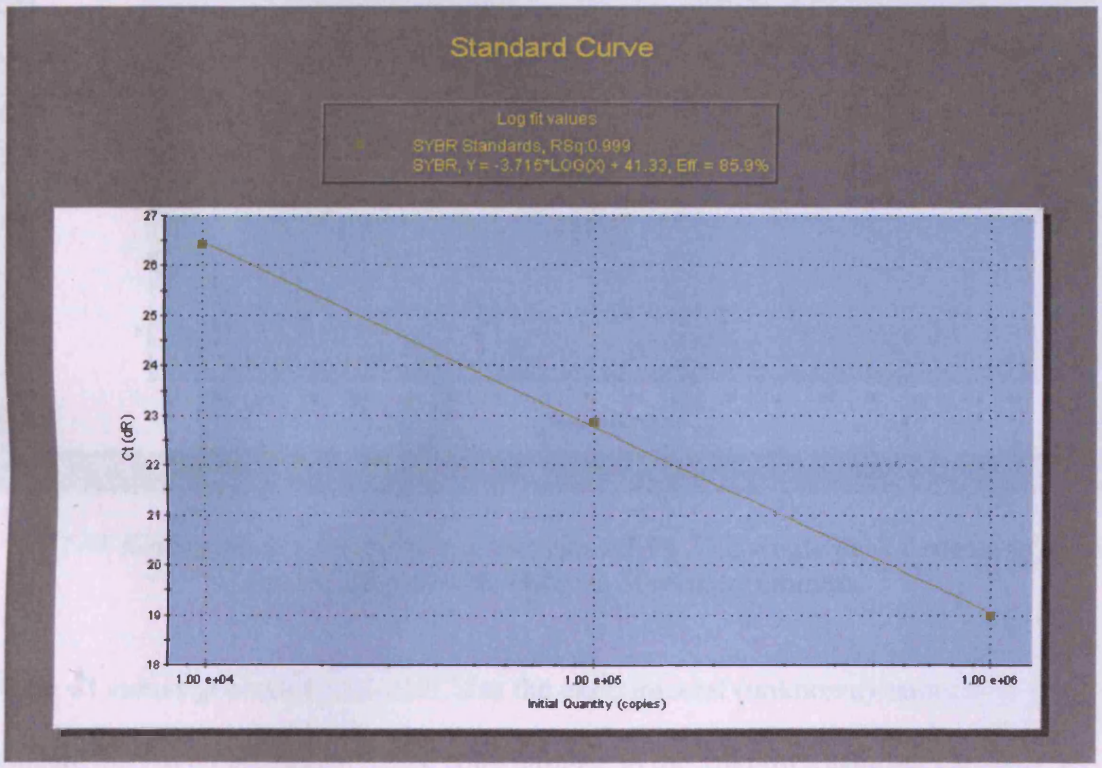



Fig 2.40 Representative standard curve (for ARP housekeeper gene) generated by SYBR green QPCR analysis

### 2.9.7.3.3 Melting point analysis/dissociation curve

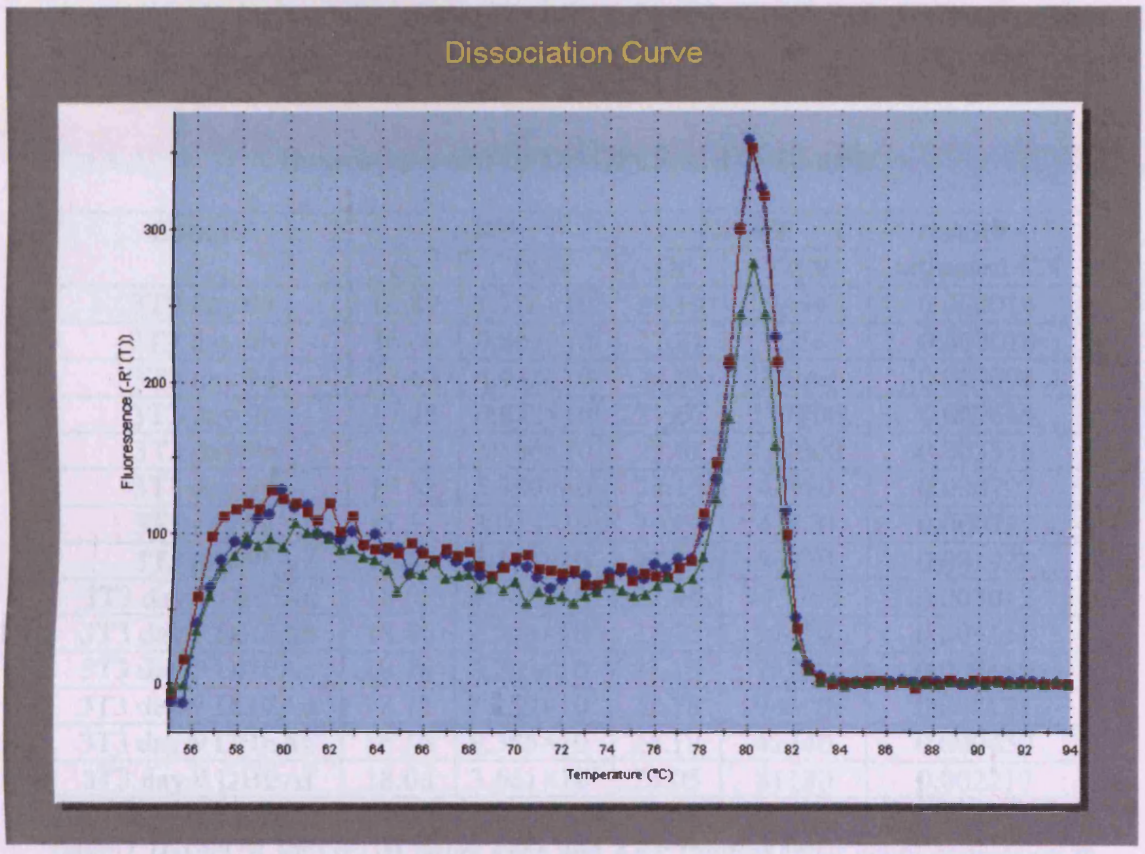



Fig 2.41 Representative dissociation curve (for ARP). The single peak denotes a single gene product and the absence of primer dimmers.

Using Ct values generated from QPCR in the experimental (unknown) samples and the standard curves an estimation of the copy number in the unknown samples can be made in day 0 and in non-treatment and DHEA treated samples at later time points.

### 2.9.7.3.4 3T3-L1 QPCR results

Analysis was performed on samples collected on day 0 and on final day of differentiation protocol (day 12) for housekeeper gene (ARP) and terminal differentiation marker gene (GPDH).

Housekeeper and GPDH QPCR in 3T3-L1 cells

| Sample | ARP | | GPDH | | ARP |
|---|---|---|---|---|---|
| | Ct | TCN | Ct | TCN | adjusted CN |
| 3T3 day 0a | 15.89 | $1.756\times10^{8}$ | 29.19 | 2454 | 0.000014 |
| 3T3 day 0b | 16.69 | $9.867\times10^{7}$ | 27.21 | 7558 | 0.000078 |
| 3T3 day 9a | 17.64 | $4.934\times10^{7}$ | 24.56 | 34340 | 0.000696 |
| 3T3 day 9b | 17.23 | $6.672\times10^{7}$ | 21.67 | 177800 | 0.002665 |
| 3T3 day 9c | 18.27 | $3.136\times10^{7}$ | 22.61 | 104000 | 0.003316 |
| 3T3 day 9d | 17.52 | $5.390\times10^{7}$ | 24.16 | 42980 | 0.000797 |
| 3T3 day 9e | 18.31 | $3.053\times10^{7}$ | 24.21 | 41870 | 0.000777 |
| 3T3 day 9f | 16.69 | $8.133\times10^{7}$ | 22.74 | 96970 | 0.001728 |
| 3T3 day 9 DHEAa | 18.02 | $3.775\times10^{7}$ | 22.46 | 113700 | 0.003012 |
| 3T3 day 9 DHEAb | 18.46 | $2.743\times10^{7}$ | 22.73 | 97410 | 0.003551 |
| 3T3 day 9 DHEAc | 18.76 | $2.203\times10^{7}$ | 23.15 | 76780 | 0.003485 |
| 3T3 day 9 DHEAd | 18.73 | $2.259\times10^{7}$ | 22.78 | 94670 | 0.004191 |
| 3T3 day 9 DHEAe | 16.76 | $9.385\times10^{7}$ | 24.19 | 42240 | 0.000450 |
| 3T3 day 9 DHEAf | 18.06 | $3.661\times10^{7}$ | 23.05 | 81180 | 0.002217 |

Table 2.16 QPCR for GPDH target gene and ARP (housekeeper gene) expression in 3T3-L1 cells following 9 days exposure to differentiation mix (DMSO 0.1%) and differentiation mix + DHEA $10^{-7}$ molar. Data shown are Ct value, total copy number (TCN) and copy number (CN) adjusted for housekeeper. Results demonstrated are an amalgam of 2 experiments performed in triplicate repeated a third time with similar results. Each QPCR reaction was performed in duplicate

When corrected for housekeeper gene (ARP) expression the difference in GPDH copy number from day 0 to day 9 in both non-treatment and DHEA treated samples was increased consistent with the occurrence of differentiation, though the magnitude of increase was smaller than anticipated.

These results demonstrate after 9 days in ×1 differentiation medium, there was a 31-53 fold increase in GPDH gene expression (corrected for ARP) in 3T3-L1 cells that was not significantly different in the presence of DHEA (Wilcoxon Signed Ranks test p=0.075).

### 2.9.7.3.5 PAZ6 QPCR results

Analysis was performed on samples for day 0 and at differentiation protocol terminus (day 15) for housekeeper (APRT) and LPL.

Housekeeper and LPL QPCR in PAZ6 cells

| Sample | APRT | | LPL | | APRT adjusted CN |
|---|---|---|---|---|---|
| | Ct | TCN | Ct | TCN | |
| PAZ6 day 0 | 34.65 | 56.82 | 34.04 | 0.7621 | 0.0134 |
| PAZ6 day 0 | 29.52 | 117.4 | 29.73 | 25.44 | 0.2167 |
| PAZ6 day 15 | 29.95 | 91.89 | 26.85 | 232.2 | 2.5269 |
| PAZ6 day 15 | 31.14 | 46.2 | 24.91 | 1029 | 22.2727 |
| PAZ6 day 15 | 30.33 | 73.6 | 25.55 | 625.8 | 8.5027 |
| PAZ6 day 15 DHEA | 29.80 | 100.2 | 27.07 | 151 | 1.5070 |
| PAZ6 day 15 DHEA | 28.94 | 163.8 | 28.07 | 91.13 | 0.5563 |
| PAZ6 day 15 DHEA | 29.99 | 89.43 | 28.61 | 59.97 | 0.6705 |

Table 2.17 QPCR for LPL target gene and APRT (housekeeper gene) expression in PAZ6 cells following 15 days exposure to differentiation mix (DMSO 0.1%) and differentiation mix + DHEA $10^{-7}$ molar. Data shown are Ct value, total copy number (TCN) and copy number (CN) adjusted for housekeeper. Results demonstrated are an amalgam of 2 experiments performed in triplicate and each QPCR reaction was performed in triplicate

The results demonstrate an increase in LPL expression from baseline in both DHEA and non-treatment samples consistent with the occurrence of differentiation, though once again not with the extent that was anticipated. No difference was observed between DHEA and non-treatment samples. (Wilcoxon Signed Ranks p=0.173) thus concluding

that DHEA does not influence differentiation in PAZ6 preadipocytes when generic adipocyte gene expression is evaluated.

In PAZ6 cell differentiation and also in BAT in general, it is possible that the expression of alternate genes may better reflect the degree with which differentiation has occurred (or not). The PAZ6 experimental samples were also analysed therefore for BAT specific genes following differentiation with and without exposure to DHEA. Interestingly there was a significant increase in UCP-1 expression (Wilcoxon Signed Ranks test $p=0.028$ corrected for input RNA) in the DHEA treated samples albeit with the caveat that high Ct values were observed (35-36).

**2.10 Discussion**

The experiments described in this chapter demonstrate that DHEA, at intra-adipose tissue relevant concentrations has an inhibitory, non-toxic, effect on proliferation in both white (3T3-L1) and brown (PAZ6) cell line preadipoctyes and that this effect, as far as this body of work can ascertain, is preadipocyte specific. The data suggests, although not conclusively, that this effect may be due to a direct action of DHEA. The results demonstrate that the anti-proliferative action is likely to be due, at least in part, to cell-cycle blockade and that MAP kinase pathway modulation may be involved. The rapidity of the response observed on phosphorylated MAP kinase activity is such that a direct effect on a cell membrane receptor is likely to have been the mediator. Previously, investigators have demonstrated an anti-proliferative action on both the 3T3-L1 cell line [139] and other mesenchymal derived cell types [212] and the data presented here would be supportive of these findings .

An anti-proliferation effect could be considered to be pro-adipogenic as the cessation of proliferation is a differentiation prerequisite however no appreciable difference in differentiation following treatment with DHEA was seen in either the 3T3-L1 or PAZ6 cells.

Investigators have however demonstrated that DHEA has an inhibitory action on cell line preadipoctye differentiation [139, 257]. The discrepancy between the results generated here and these previous studies may be due to a number of factors such as the use of different DHEA concentrations and methods of assessment. In the paper by Shantz and Colleagues [257] DHEA at a concentration of $250 \times 10^{-6}$ molar caused a reduction in differentiation of 3T3-L1 cells as measured by GPDH levels in supernatant fluid following centrifugation at 20,000 g for 40 minutes. The DHEA concentration is therefore far greater than that used in the experiments presented here and the methodology used also differs.

The findings presented relating to the PAZ6 BAT preadipocyte cell line are novel. The inhibitory effect on proliferation followed by the possible increase in BAT specific genes following DHEA exposure demonstrates a pro-adipogenic effect. Also the Western blotting data, as well as demonstrating activation of MAP kinase by 0.1% DMSO/0.01% FCS, clearly demonstrates involvement of the MAP kinase signalling system in the PAZ6 although though not as obviously in the WAT cell line. The possibility that DHEA may be supportive in some way of BAT differentiation is consistent with the temporal association between DHEA and BAT in the foetal state.

A further interesting observation is that there was no detectable sulphotransferase activity in the 3T3-L1 preadipocytes as demonstrated by the lack of DHEAS production in culture

conditions, despite the addition of supra-physiological serum DHEA concentrations; this in the light of confirmed generation of terminal metabolites (testosterone, oestradiol). This observation is in support of a previous study as discussed [87] and may suggest that adipose tissue is, in terms of DHEA, pro-metabolic and does not posses the capacity to either inactivate it or add to the circulating pool.

These studies demonstate some interesting results though one must acknowledge a number of limitations apparent within the various experiments. When interpreting data derived from lower order mammals relating to the effects of DHEA, one must consider the issue of concentration. The cell line utilised in many of the experiments is murine in origin and as such will be exposed to a grossly supraphysiological DHEA environment as it is only humans (and higher order mammals) that generate such high and fluctuating concentrations [56]. Furthermore direct extrapolation of data derived from different species may be misleading and will require confirmation using adipose tissues from the same origin. Results generated from transformed cell lines (SV 40 or spontaneous) also need to be evaluated with caution particularly if one (as in this case) is assessing effects on proliferation and linked modalities (cell cycle, apoptosis). Transformed cell lines by definition are altered such that they are encouraged to remain within the cell cycle and to undergo repeated cycles of division. This, although conducive to laboratory based studies, is far removed from the *in vivo* setting. Furthermore, cell lines are removed form their native environment and as such are protected from any stimulatory or inhibitory stimuli that may be present *in vivo*. It is therefore imperative to confirm any cell line data using primary cultures *in vitro* and preferably *in vivo*.

Although demonstrating an antiproliferative effect of DHEA, these studies have only utilised a single method, namely cell counting. Other techniques are however available that can determine the effect of a reagent on cell proliferation. Thymidine incorporation has been discussed earlier in this chapter and relies on radioactive isotope uptake into dividing cells such that a substance augmenting proliferation will generate a sample containing higher levels of isotope and *vice versa*. The relative amounts of isotope present can then be quantified by scintigraphy. There are obvious advantages in confirming results using different techniques and were such an alternate method to demonstrate similar findings to my cell counting data the generated data would certainly be more robust.

The data generated from the flow cytometry experiments suggests that there is no increase in apoptosis following exposure to DHEA. Since the process of apoptosis can occur rapidly, applying a single method can not exclude the possibility of stimulated cell death with confidence. Consequently no single universally accepted method of determining apoptotic stimuli exists and thus it is important to confirm ones finding using different approaches. The TUNEL method has been described earlier in this chapter and relies on labeling damaged sections of DNA of cells about to undergo programmed cell death. Other methods are also available namely Annexin 5 staining which probes for the presence of the apoptotic cell surface marker phosphatidyl serine. Apoptotic regulatory protein (Bcl/Bax ratio) analysis is a further possible method available. Therefore, although my data does not suggest that DHEA stimulates apoptosis, in order to state this with confidence experiments using these alternate methods would be required.

I have attempted to address the possibility of oestrogen and testosterone effects in this experimental model. However an admitted limitation to this body of work is the absence of experiments utilising androgen and oestrogen receptor blockade and certainly these should be included in any future studies. This would have obvious benefits when attempting to establish the origin of the observed effects (DHEA or a downstream metabolite). Other authors have also employed albumin bound DHEA in order to investigate genomic *versus* non-genomic actions, the albumin bound form being unable to penetrate the cell membrane such that any observed actions must therefore be directed through cell surface receptors [81, 211].

Exactly what role the demonstrated possible anti-proliferative effect of DHEA on preadipocytes may play in human physiology is not clear; moreover it may not be possible to extrapolate with confidence from these cell line data directly to the human model. Nevertheless these effects may point to a modulating action of DHEA on adipose tissue in humans although further investigation is paramount.

# Chapter 3

# Effects of DHEA on proliferation and differentiation of human primary preadipocytes

### 3.1 Overview

In the previous chapter the effects of DHEA on preadipocyte cell lines has been examined. 3T3-L1 is a well established *in vitro* model for evaluating adipogenesis and PAZ6 is one of the few openly available BAT cell lines. However studies utilising cell lines are associated with a number of short comings. Firstly and most obviously many cell lines, such as 3T3-L1, are non-human and as such will tend to generate questions concerning the extrapolation of derived data to the human model. The observation that clonal expansion occurs during cell line differentiation, but most probably not in primary cell differentiation illustrates one potential difference. There is a commercially available immortalised human preadipocyte with augmented differentiating capacity termed Chub-S7. The differentiating ability in this cell type is preserved following SV40 T-antigen transformation by co-expression of human telomerase reverse transcriptase (hTERT) and papillomavirus E7 oncoprotein (HPV-E&7) genes [282]. As attractive a cell type as this may be its acquisition was beyond the financial capacity of our unit.

Cell lines are designed purposely with exaggerated proliferation potential, the curtailment of which is a requirement to initiate differentiation, and this innate tendency to remain within the cell cycle may impair differentiation capacity. Furthermore cell line studies do not allow depot specific differences to be assessed which is of particular importance with the emergence of centrally distributed fat as a cardiometabolic risk factor. Also, cell lines by definition are developed out of their normal environment and are not exposed to other factors that could prime or protect against proliferation/differentiation. There are also specific considerations concerning DHEA itself in that there is a stronger body of evidence to support the notion that it can precipitate beneficial effects, relating either to

adipose tissue or the vasculature, from *in vitro* and animal models when compared to the data available from human studies. There is a possible explanation for this that has been discussed in chapter 1 centering around the fact that it is only in higher primates that high levels of DHEA/DHEAS are generated. Furthermore only higher primates demonstrate the dramatic reduction in circulating concentrations after early middle age; thus the concentrations of DHEA/DHEAS that have been used, particularly in animal studies or those using non-human cell lines, are potentially non-physiological for that particular model. In order to address some of these issues it was therefore necessary to repeat some of the previous experiments with human primary preadipocyte cultures.

Our hypothesised link between the adipocyte and any observed vascular and/or metabolic effects is modification of the adipocytokine profile. Signs of a pro-metabolic health effect could be determined for example by observed increases in adiponectin production or a reduction in levels of TNFα, leptin or IL-6. These adipocytokines have been discussed in greater detail in chapter 1. Once a preadipocyte approaches maturity it will gain the ability to synthesise and secrete these apidocytokines in response to alterations in the external environment so their identification could also provide a measure of effective differentiation, and to that end whether a particular reagent impinges upon it. The adipocytokine adiponectin was chosen for examination in this study for a number of reasons. It has well known metabolic actions as illustrated by the inverse correlation with obesity. Furthermore, in the context of the cell line data demonstrating proliferation inhibition, any observed increase in production would have to overcome any potential reduction in cell number and therefore be of greater significance. As briefly discussed in chapter 1 adiponectin is found in low and high molecular weight forms, the high

molecular weight form potentially being more metabolically important. However, most commercially available adiponectin assays measure total adiponectin, as was chosen for use in this study.

Conditioned medium collected at the end of the differentiation protocol (21 days exposure to ×1 differentiation mix) formed the raw material for the adipocytokine assays utilising the enzyme-linked immunosorbent assay (ELISA) technique.

ELISA methodology (Fig 3.1) uses a sequence of linked antibodies and is termed a sandwich technique. A plate surface coated with a capture antibody directed against the analyte of interest (e.g. adiponectin) is blocked to prevent non-specific binding and exposed to samples containing the analyte that then binds to the capture antibody. Primary antibodies specific for the target substance are then added followed by secondary antibodies that are specific to the primary antibody and are conjugated to an enzyme able to generate a colour change following substrate addition. The reaction is terminated and the colour intensity is determined using a plate reader that measures optical density.

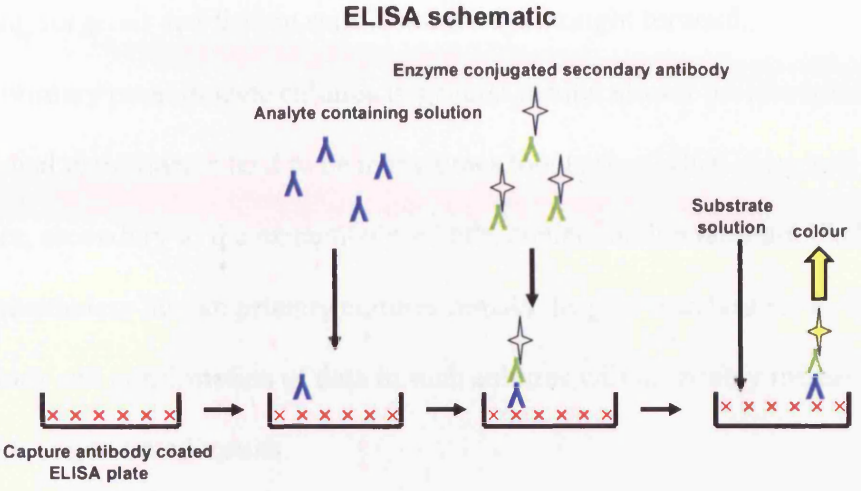



Fig 3.1 ELISA method schematic

### 3.1.1 Human primary cultures

The use of primary cultures is attractive for a number of reasons, not least for the proximity to human physiology however a number of problems are involved with their usage. Any such study will require approval from both the local ethics committee, and hospital and/or trust Research and Development departments. This involves a not inconsiderable amount of bureaucracy and can therefore take a prolonged period of time. Consequently, our consent forms and information sheets (appendix III) not only encompass this study but a number of other current and possible future projects. Due to the internalised location of adipose tissue a surgical procedure will invariably be required for retrieval and so consent from individuals concerned as well as assistance from those performing the surgical procedures is paramount. Obtaining consent for research

purposes from individuals before what is often a major surgical procedure and coordinating surgeons and theatre staff is not always straight forward.

Extracted primary preadipocyte cultures in general exhibit slower proliferation rates such that individual experiments tend to be many times longer than when using cell lines and furthermore, secondary to the extraction methods, contamination rates are likely to be higher. Nevertheless human primary cultures remain the gold standard for *in vitro* investigations and confirmation of data in such cultures will invariably increase the significance of generated results.

### 3.1.2 Visceral fat depots

Region specific fat depots have differing metabolic effects and abdominally distributed adipose tissue has been demonstrated to generate a greater metabolic derangement than that located subcutaneously. The metabolic abnormalities associated with an increased visceral fat depot include insulin resistance [283] and dyslipidaemia [284], which, in conjunction with hypertension and a predilection to cardiovascular disease [285], have been termed the metabolic syndrome (Fig 3.2 and table 3.1). Possible explanations as to why this particular distribution of fat has a greater associated health risk include an exaggerated lipolytic activity of omental fat cells and the portal circulation drainage pattern that potentially exposes hepatocytes to higher levels of free fatty acids resulting in dyslipidaemia and hepatic insulin resistance [283]. As to whether the metabolic syndrome is simply a reflection of dietary habits in today's society or whether the laying down of central fat deposits during times of excess had past survival benefits remains

unclear. What is clear however is that the metabolic syndrome, be it a separate disease entity or a collection of risk factors, predisposes to cardiovascular risk [286-288].

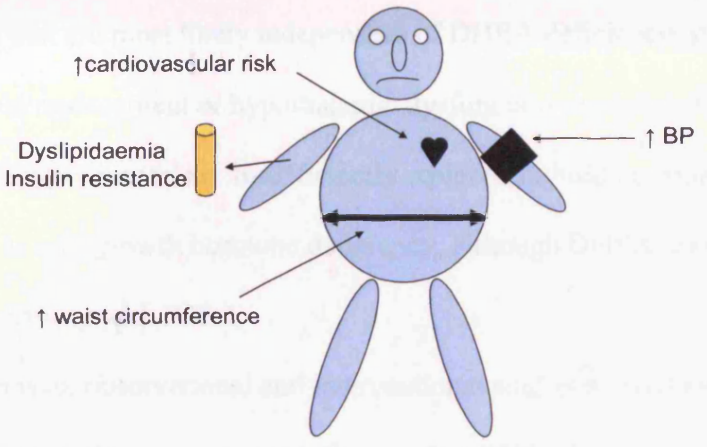



Fig 3.2 Features of the metabolic syndrome

| Diagnostic criterion | Measurement |
|---|---|
| ↑ Waist circumference<br>+ any 2 of the following | ♂ ≥ 94cm<br>♀ ≥ 80cm |
| ↑ triacylgyceride<br>(or treatment for) | ≥ 1.7mmol/L |
| ↓ HDL cholesterol<br>(or treatment for) | ♂ < 1.03mmol/L<br>♀ < 1.29mmol/L |
| ↑ BP (or treatment for) | ≥138/≥85 mmHg |
| ↑ fasting glucose (or diabetes) | ≥ 5.6 mmol/L |

Table 3.1 International Diabetes Federation diagnostic criteria (Caucasian) for the metabolic syndrome 2005 (www.idf.org)

### 3.1.3 Previous human adipose tissue studies

There have been no studies to date that have specifically compared the body composition of individuals with a pathological DHEA deficiency to matched controls. Individuals with either primary of secondary adrenal insufficiency may be overweight due to mechanisms that are most likely independent of DHEA deficiency, such as excessive glucocorticoid replacement or hypothalamic dysfunction secondary to disease and/or treatment for hypopituitarism. Insufficiently replaced thyroid hormone will precipitate weight gain as will growth hormone deficiency, although DHEA may exhibit co-stimulatory activity with IGF-1.

Numerous *in vivo*, observational and interventional studies as well as work performed *in vitro* have attempted to evaluate the influence that DHEA has on adiposity and adipocytes and these have been reviewed in chapter 1. Overall, however, the results have not been conclusive, although in some studies in men with physiological DHEA deficiency there is some evidence of reduction in measures of body fat [169]. Many of the *in vivo* studies have been performed on individuals with a physiological rather than pathological DHEA deficiency presumably due to the low incidence of pathological deficiency and possibly due to the greater potential reward that demonstrating anti-aging DHEA related effects would generate.

There is a paucity of *in vitro* studies examining the effect of DHEA on primary preadipocyte cultures. However one study by McIntosh and colleagues [258], having previously demonstrated inhibition of both proliferation and differentiation after DHEA treatment in both 3T3-L1 preadipocytes [139] and rat and pig derived preadipocytes [289], concluded that DHEA reduces proliferation of preadipocytes isolated from

subcutaneous adipose tissue but stimulated the differentiation of these cells. DHEA has also been shown to reduce the proliferation of human vascular smooth muscle cells which, like preadipocytes, are of mesenchymal origin [212]. A further study in human adipose tissues demonstrated upregulation of adiponectin in omental adipocytes following exposure to DHEAS [146].

### 3.1.4 Aims

Following the results from the cell line experiments the aims were firstly to compare these results to the effect that DHEA has on proliferation and differentiation on human primary preadipocytes and also to compare effects observed in subcutaneous and visceral fat depots.

## 3.2 Materials and Methods

Once primary samples had been obtained essentially the same experimental techniques were applied to this section of the study that were used in the cell line experiments described in chapter 2. However, modifications were required taking account the differing nature of the primary culture cells that became apparent during the course of the study.

### 3.2.1 Study design

Cardiff and Vale NHS trust Research and Development office (reference 06/CMC/3598) and Local Ethics committee (South East Wales Research Ethics Committee (reference 06/WSE/03/37)) approval was obtained to approach individuals undergoing routine open

abdominal surgery at the University Hospital of Wales, Cardiff. The gastrointestinal surgeons based at this site (Mr M. Puntis, Mr B. Rees and Mr N. Kumar) kindly agreed to provide possible participants for the study and also performed the adipose tissue harvest. Initially it was intended that all participants would be undergoing surgical procedures for non-malignant conditions, however it became apparent that the number of non-malignancy related open abdominal surgical cases were limited to such a degree that malignancy related cases were eventually included. Potential study subjects were approached by a member of the research team following admission to the surgical wards and were then provided with a patient information sheet (appendix III) that outlined the study. Subjects were then given the opportunity to discuss their inclusion with the research team member. On agreement, subjects were asked to co-sign an informed consent form with the research team member (appendix III). Information on subject age, sex, past medical history and prescription medications was collected followed by measurement of height, weight, waist and hip circumference and percentage body fat [Tanita Body fat monitor/scale TBF-538, Tanita Corporation, Tokyo, Japan]. During surgery paired subcutaneous and omental samples of approximately $1cm^3$ in size were obtained and then collected by the investigating team. Samples were stored at 4°C until processed which in most cases occurred within hours of collection. Occasionally samples were kept overnight and processed the next day in which case 1-2mLs of standard 3T3-L1 culture medium was added to the samples to prevent sample dehydration.

### 3.2.2 Reagents and materials

Identical materials and reagents to those used in the cell line experiments described in detail in chapter 2 were utilised for human primary preadipocyte maintenance, storage and experimentation.

### 3.2.3 Preadipocyte extraction

2 different preadipocyte extraction methods were trialled during the study with varying degrees of success.

Explants: samples were scalpel dissected into small pieces (0.25-0.5 millimeters diameter) then placed directly (4-5 explants per well) into 6 well plates with sufficient standard 3T3-L1 culture medium to surround the base of each explant (1-2mls). Fresh medium was added every 3$^{rd}$ day. Preadipocytes potentially migrate out of the explant and attach to the surrounding plate.

Collagenase digest: collected adipose tissue samples were placed in 20ml universal containers (approximately 1cm$^3$ of tissue per container) to which 7ml of Hank's balanced salt solution, 2ml of 7.5% BSA (7.5g Bovine serum albumin in 100ml distilled $H_2O$) and 1ml of collagenase type II (300mg collagenase type II in 10mls of 3T3-L1 culture medium) were added. This was then incubated for 1 hour in a water bath at 37°C, shaking every 5 minutes. The resultant solution was centrifuged at 4°C for 5 minutes at 1500rpm. Supernatant was removed leaving a cell pellet that was resuspended in 3T3-L1 culture medium and placed in a 75cm$^2$ flask containing 10ml of 3T3-L1 culture medium at 37°C. Following 24 hours the cells were washed twice with 5ml of Hanks balanced salt solution removing any remaining red blood cells.

### 3.2.4 Human primary preadipocyte culture

All cells were cultured in 75cm$^2$ flasks at 37°C in standard 3T3-L1 medium changed every 4$^{th}$-5$^{th}$ day. Trypsinisation, freezing and thawing procedures were identical to those used in cell line culture experiments described in chapter 2.

### 3.2.5 Assessment of proliferation

Approximately $10^3$ preadipocytes (per well) were plated into 24 well plates in standard 3T3-L1 culture medium for 24 hours to allow optimal cell-to-plate adhesion. Following this, varying concentrations of DHEA ($10^{-5}$ - $10^{-8}$ molar) and control (DMSO 0.1%) were introduced in a similar manner to that described in chapter 2. Cells were counted in duplicate with a Coulter cell/particle counter at 72 and 168 hours with a medium/reagent change at 72 hours. Each experiment was performed in quadruplicate.

### 3.2.6. Primary culture cell signalling

Confluent preadipocytes in 6 well plates following PBS wash underwent 2×2 hour incubations in serum free medium (DMEM only). Exposure to DHEA $10^{-7}$ molar and control (DMSO 0.1%) was then performed over 10 and 20 minute periods. Resulting samples then underwent homogenisation as described in detail in chapter 2 followed by standard protocol Western blotting to determine phosphorylated and total MAP kinase activity.

### 3.2.7 Primary culture differentiation protocol

Preadipoctyes in 6 well plates were grown to confluence in standard 3T3-L1 medium. Culture medium was then changed to a ×1 differentiation mixture (standard medium + ×2 differentiation medium ratio 1:1) containing DHEA $10^{-7}$ molar or control (DMSO 0.1%) and cultured for 21 days with medium/reagent change every 7 days. RNA was extracted using trizol reagent (as described in chapter 2); conditioned medium was collected on days 0, 7, 14 and 21. Rates of differentiation relative to control were assessed via foci counting and by transcription rates of differentiation markers in control and DHEA ($10^{-7}$ molar) treated cells relative to the APRT housekeeper as described in chapter 2. Due to the slower rate of metabolic events observed in both subcutaneous and omental preadipocytes the differentiation protocol time frame was duly increased. Furthermore to obtain sufficient RNA for QPCR, RNA from 3 wells for each conditon was amalgamated such that each experiment generated only a single data set.
Human primer sequences and optimised concentrations have been described in chapter 2.

### 3.2.8 Adiponectin ELISA

The Human adiponectin Duoset® ELISA development system was employed [R&D systems, Oxford, UK]. Costar ELI 96 well plates [R&D systems] were coated with 100µL of capture antibody (55µL/mL in PBS) the day prior to experimentation. All reagents were brought to room temperature before use. Following the wash protocol, wells were blocked with reagent diluent (1% bovine serum albumin [Sigma-Aldrich] in PBS) for 2 hours then 100µL of prepared standard/neat conditioned medium was added to each well and incubated for 2 hours at room temperature. 100µL detection antibody

(55μL/mL reagent diluent), 100μL Strepavidin-HSP (50μL/mL reagent diluent), and 100 μL substrate solution (1:1 colour reagent A and B [R&D systems]) were then sequentially added interspersed by wash protocols as per the manufacturer's instructions. Experiments were terminated by addition of 50μL of stop solution (10% sulphuric acid) and optical density immediately determined using a microplate reader [OpsysMR microplate reader, Dynex Technologies, Chantilly, USA] set to 450nm.

### 3.2.9 Statistical analysis

Data sets generated were deemed not parametric and examined as such using SPSS version 12.0.1 for Windows (Microsoft).

### 3.2.10 Demographic data of study subjects

The demographic and brief medical history data from study subjects who provided samples of sufficient quality for preadipocyte extraction and culture are described below.

| Number | Age | Sex | Diagnosis | Height (cm) | Weight (Kg) | Waist/hip Circumference (cm) | % fat | BMI Weight (kg)/Height ($m^2$) |
|---|---|---|---|---|---|---|---|---|
| 1 | 64 | F | Carcinoma Pancreas | 163 | 74 | Waist 92 Hip 111 | 31 | 27.9 |
| 2 | 74 | F | Diverticular Abcess | 155 | 48 | Waist 91 Hip 91 | 27 | 20.0 |
| 3 | 74 | F | Rectal Carcinoma | 163 | 59.5 | Waist 83 Hip 98 | 30 | 22.4 |
| 4 | 55 | M | Crohn's Disease | 178 | 69.9 | Waist 91 Hip 89 | 10 | 22.1 |
| 5 | 76 | M | Rectal Carcinoma | 175 | 62.2 | Waist 100 Hip 106 | 8.5 | 20.3 |
| 6 | 75 | M | Pancreatic neuroendocrine Tumor | 178 | 82 | Waist 94 Hip 107 | 26 | 25.9 |
| 7 | 30 | F | Insulinoma | 155 | 60 | not measured | not measured | 25.0 |
| 8 | 66 | F | Liver lesion (possible metastasis) | 152 | 57 | Waist 79 Hip 102 | 35 | 24.7 |

Table 3.2 Demographic data from subjects providing adipose tissue samples for preadipocyte extraction

## 3.3 Results

Data has been presented in the same manner as in chapter 2 as median and interquartile ranges of DHEA treated samples versus DMSO 0.1 % controls. Human primary preadipocyte experimentation began with an assessment of the effect on proliferation.

### 3.3.1 Primary preadipocyte extraction

Explants: following previous success extracting preadipocytes from synovial and orbital fat [269] it was anticipated that this would be a successful mode of extraction however despite several attempts no identifiable preadipoctyes migrated out of the explant culture such that this technique was abandoned.

Collagenase digest: Preadipocytes were derived form this procedure but often in very small numbers such that up to a month in standard culture conditions was necessary to generate sufficient cell number for experimentation.

### 3.3.2 Primary preadipocyte cell culture

No macroscopic differences were observed between omental and subcutaneous samples during routine culture although in the majority of omental samples a second cell type (mesothelial cells) was seen; these were smaller relative to the adipocytes, more elliptical in shape and also more granulated. This contaminating cell type was present to a varying extent in most of the omental samples and exhibited a greater proliferation rate than preadipocytes which were larger, more rounded and less granulated (Fig 3.3). Wells containing a high concentration of these contaminating cells failed to demonstrate the

typical macroscopic features normally associated with differentiation following standard protocol suggesting that the contaminating cells were not preadipocytes.

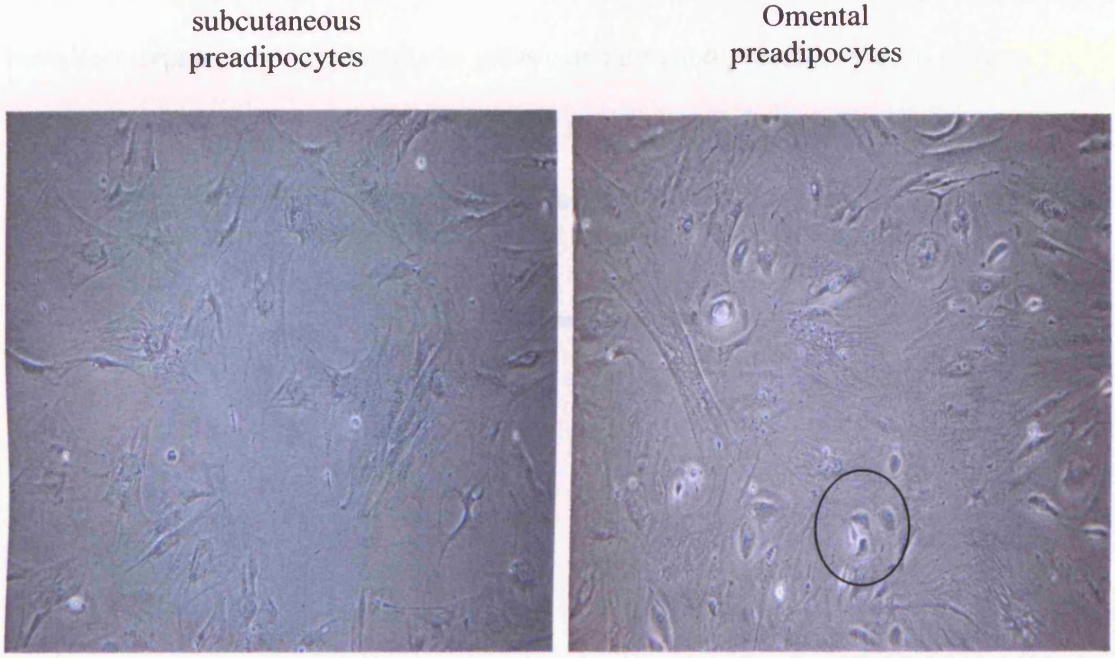



Fig 3.3 Subcutaneous and omental preadioctye in standard culture conditions. Note the presence of atypical cells in the omental sample located in the circled area Magnification ×100

The primary preadipocytes were invariably larger than the cell line preadipocytes and so working cell number was reduced. It was also apparent the preadipocytes became less biologically active with increasing passage number entering replication senescence invariably after passage 3. It therefore became my intention to perform all experiments at as low a passage number as possible, particularly when attempting to stimulate differentiation.

### 3.3.3 Does DHEA effect proliferation in human primary preadipocytes?

Proliferation was determined in omental and subcutaneous preadipocytes. Presentation of the results begins with the latter. For ease of interpretation subcutaneous preadipocytes have been represented graphically in yellow and omental preadipocytes in orange.

Box-and-whisker plot demonstrating the effects of DHEA on proliferation of human subcutaneous preadipocytes following 72 hours exposure

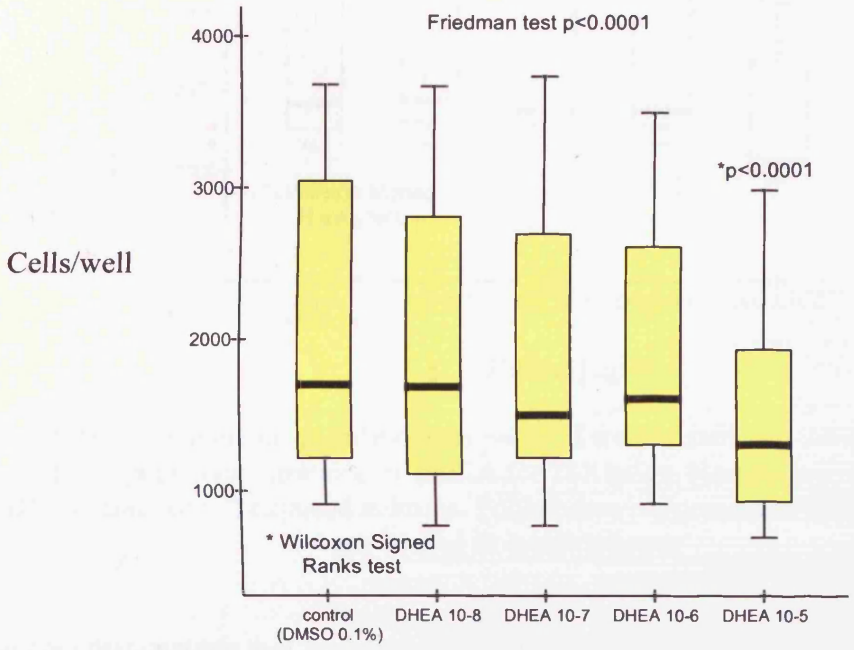



Fig 3.4 $10^3$ subcutaneous preadipocytes per well were plated into 24 well plates and exposed to varying concentrations of DHEA for 72 hours. Results are medians and IQRs for DHEA treated cells and control. Pooled data are presented from 4 experiments performed in quadruplicate

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of subcutaneous primary preadipocytes following 168 hours exposure

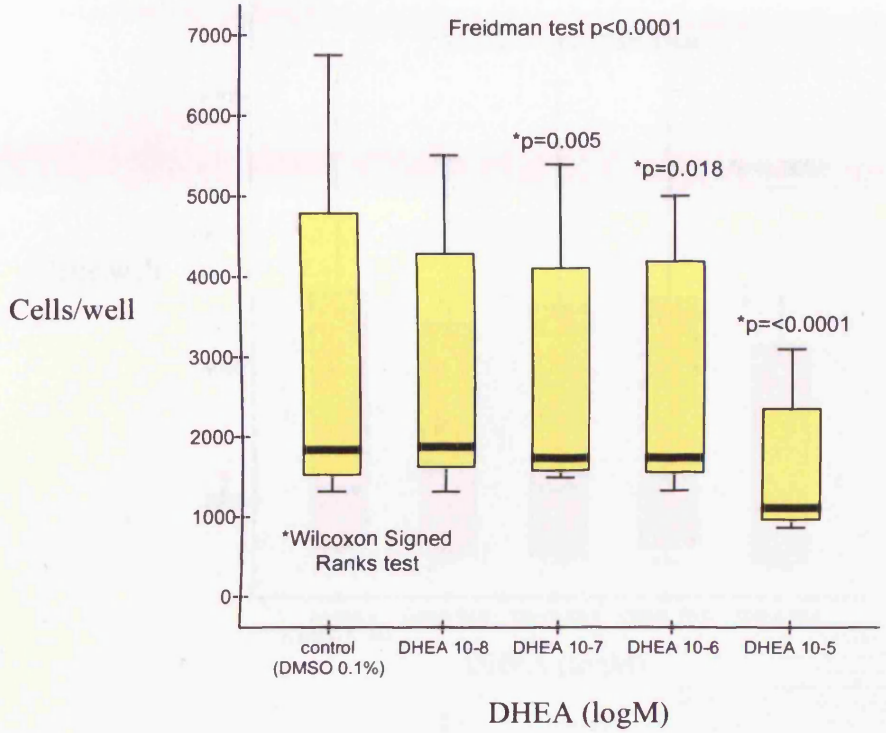



Fig 3.5 $10^3$ subcutaneous preadipocytes per well were plated into 24 well plates and exposed to varying concentrations of DHEA for 168 hours. Results are medians and IQRs for DHEA and control exposed cultures. Pooled data are presented from 4 experiments performed in quadruplicate

These data demonstrate that in subcutaneous preadipocytes DHEA inhibits proliferation at both 72 and 168 hours although no effect was seen at the longer incubation with DHEA $10^{-8}$ molar, and at 72 hours only the highest concentration caused a significant effect. Nevertheless our results demonstrate that following prolonged exposure particularly, DHEA inhibited proliferation at physiological, intra-adipose tissue concentrations.

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of omental preadipocytes following 72 hours exposure

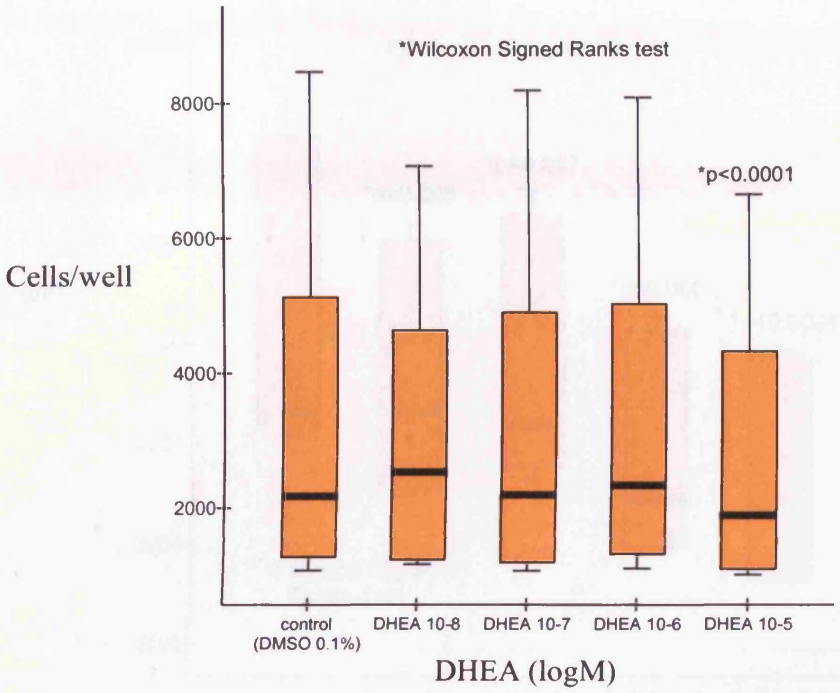



Fig 3.6 $10^3$ omental preadipocytes per well were plated in 24 well plates and exposed to control and varying concentrations of DHEA for 72 hours. Results are medians and IQRs representing the number of cells in DHEA and control treated samples. Pooled data are presented from 4 experiments performed in quadruplicate

Although an inhibitory effect on proliferation was demonstrated this occurred at a supraphysiological concentration. These data suggest that over a short time period at least DHEA has no effect on omental preadipocyte proliferation at both serum and intra-adipose relevant concentrations.

Box-and-whisker plot demonstrating the effect of DHEA on proliferation of omental preadipocytes following 168 hours exposure

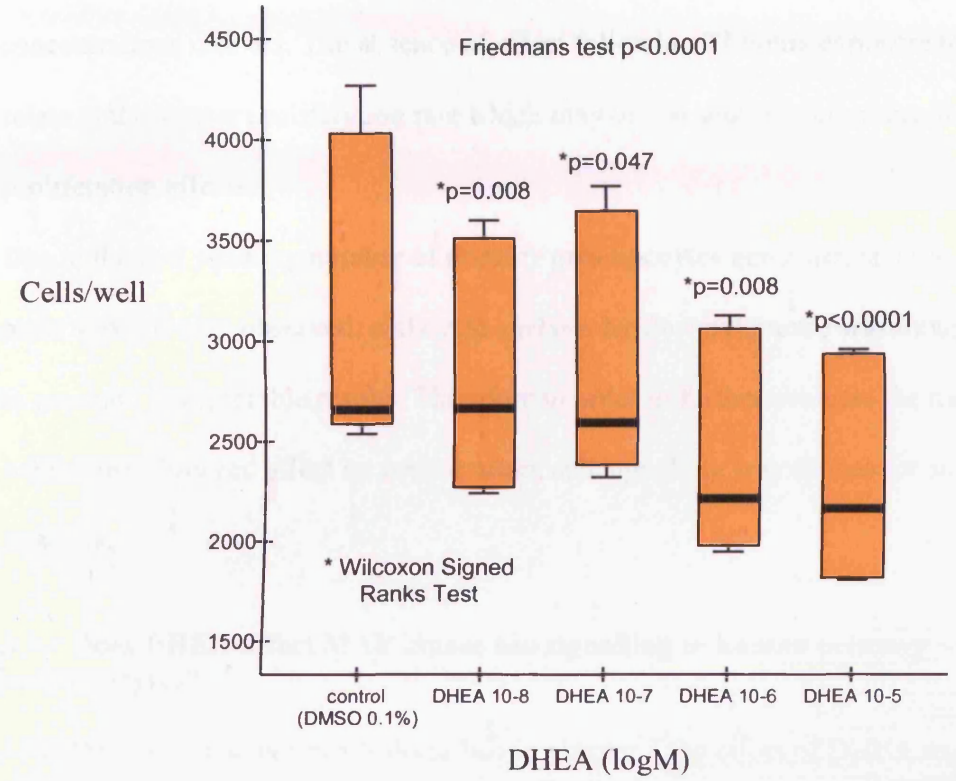



Fig 3.7 $10^3$ omental preadipocytes were plated in 24 well plates and exposed to control and varying concentrations of DHEA for 168 hours. Results are medians and IQRs representing the number of cells in DHEA and control treated samples. Pooled data are presented from 2 experiments performed in quadruplicate

The data from experiments in omental preadipocytes suggest that 72 hour exposure to DHEA does not affect proliferation at physiological concentrations, however following more prolonged exposure inhibition is demonstrated at relevant concentrations. Also the dose related effect is more pronounced in the omental relative to the subcutaneous preadipocytes.

Overall the effects in the primary cells are less dramatic than those observed in the cell line experiments described in chapter 2 and this possibly relates to the fact that primary

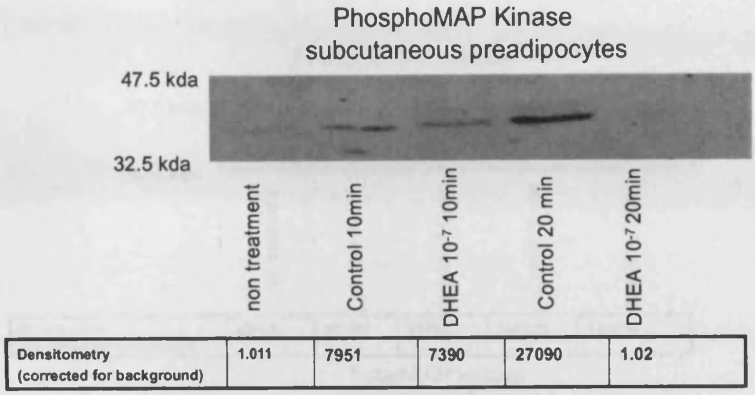
cultures are likely to have been exposed to high DHEA concentrations previously, when their donors were in middle age, and thus are less sensitive to the experimental concentrations utilised. The absence of effect following 72 hours exposure to DHEA may relate to the slower proliferation rate which may be too short to determine any anti-proliferation affects.

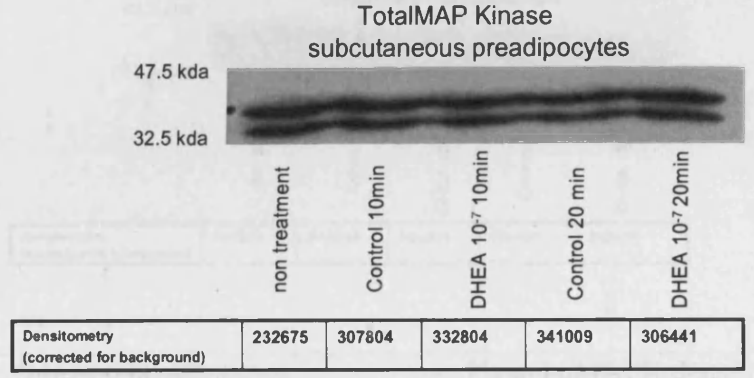
Due to the low working number of primary preadipocytes generated and the larger and more variable size observed, cell cycle analysis by flow cytometry was thought unlikely to generate interpretable results. Therefore in order to further evaluate the mechanisms behind the observed affect on proliferation, cell signalling was chosen for subsequent analysis.

### 3.3.4 Does DHEA affect MAP kinase cell signalling in human primary preadipocytes?

As in the cell line experiments described in chapter 2 the effect of DHEA exposure on P42/44 MAP kinase phosphorylation was used to determine any effect on cell signalling.

Western blots demonstrating the effect of DHEA on phosphorylated MAP kinase activity over 10 and 20 minutes in subcutaneous preadipocytes

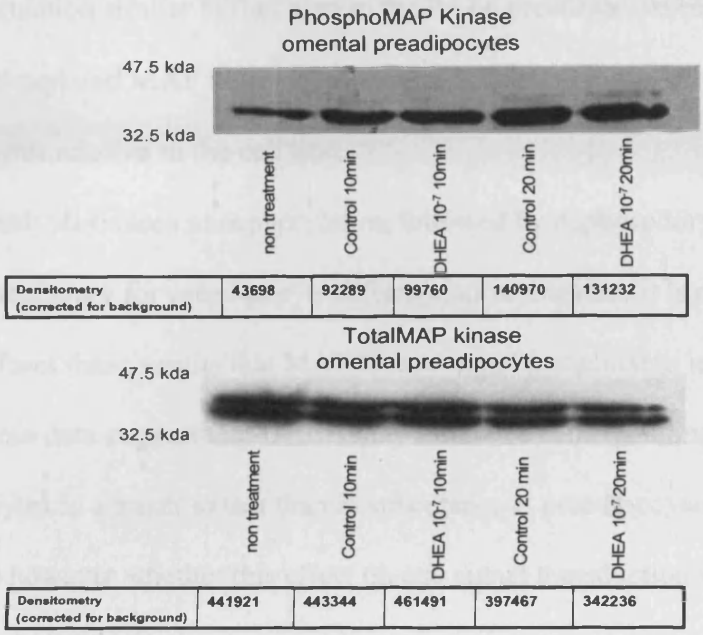



| Subcutaneous preadipocyte Samples | PhosphoMAPK densitomtry corrected for totalMAPK densitometry |
|---|---|
| Non treatment | $4.3 \times 10^{-6}$ |
| Control 10 minutes | 0.026 |
| DHEA $10^{-7}$ molar 10 minutes | 0.022 |
| Control 20 minutes | 0.079 |
| DHEA $10^{-7}$ molar 20 minutes | $3.3 \times 10^{-6}$ |

Fig 3.8/Table 3.3 Cells grown to confluence in 6 well plates in standard medium followed by 2×2 hours in serum free medium (DMEM) had DHEA $10^{-7}$ molar (containing 0.01% FCS) and DMSO 0.1% control (containing 0.01%FCS) control added for 10 and 20 minutes. Cells were then homogenised, blotted and detected following standard Western blotting protocol for phosphorylated and total MAP kinase. The blots then underwent densitomery assessment and correction for total MAP kinase measurement. Data shown are a representative blot of duplicate experiments

Western blots demonstrating the effect of DHEA on phosphorylated MAP kinase activity over 10 and 20 minutes in omental preadipocytes




| Omental preadipocyte Sample | PhosphoMAPK densitomtry corrected for totalMAPK densitometry |
|---|---|
| Non treatment | 0.09 |
| Control 10 minutes | 0.21 |
| DHEA $10^{-7}$ molar 10 minutes | 0.22 |
| Control 20 minutes | 0.35 |
| DHEA $10^{-7}$ molar 20 minutes | 0.38 |

Fig 3.9/Table 3.4 Cells grown to confluence in 6 well plates in standard medium followed by 2×2 hours in serum free medium (DMEM) had DHEA $10^{-7}$ molar (containing 0.01% FCS) and DMSO 0.1% control (containing 0.01%FCS) control added for 10 and 20 minutes. Cells were then homogenised, blotted and detected following standard Western blotting protocol for phosphorylated and total MAP kinase. The blots then underwent densitomery assessment and correction for total MAP kinase measurement
Data shown are a representative blot of duplicate experiments

These data demonstrate that both control and DHEA treatment increase phosphorylated

MAP kinase levels. DHEA exposure had no effect relative to control on phosphorylated

MAP kinase levels in omental preadipocytes. In subcutaneous preadipoctes however at both time points less activity is observed in the DHEA treated samples relative to control. This is a situation similar to that seen in the PAZ6 preadipoctye cell line. It is also noted that phosphorylated MAP kinase deactivation is delayed in the subcutaneous preadipocytes relative to the cell line.

As previously discussed phosphorylation followed by dephosphorylation of MAPK kinase is necessary for preadipocyte differentiation therefore it is only possible to conclude from these results that MAP kinase signal transduction is influenced by DHEA, though these data suggest that DHEA may influence cell signalling in omental preadipocytes to a lesser extent than in subcutaneous preadipocytes. It is not possible to determine however whether this effect on cell signal transduction would influence proliferation/differentiation in a positive or negative manner.

### 3.3.5 Does DHEA affect differentiation in human primary preadipocytes?

A number of independent end-points were used to evaluate the effect of DHEA on adipogenesis in primary preadipocyte differentiation. As described in chapter 2 foci of differentiation were counted at terminus of differentiation protocol (21 days) in DHEA exposed and control samples. Greater amounts of differentiation were observed macroscopically at early passage (1 or 2) and differentiation capacity varied between samples. QPCR evaluation of a terminal differentiation marker (LPL) was also utilised. Finally an assessment of the degree of the generation of the adipocytokine adiponectin was also utilised as a potential sign of the occurrence (or not) of adipogenesis. Presentation of the results commences with the foci of differentiation data.

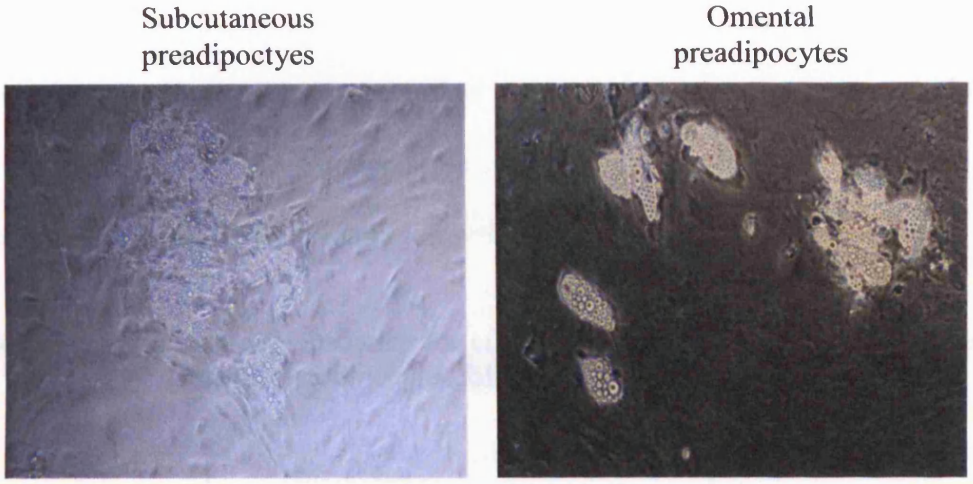



Fig 3.10 Confluent omental and subcutaneous preadipocytes in differentiation mix (+ 0.1% DMSO) for 21 days. Photographs presented are representative of a number of experiments and clearly demonstrate differentiation foci
Magnification ×100

Box-and whisker plot demonstrating the effect of DHEA on foci of differentiation in subcutaneous preadipocytes following 21 days exposure

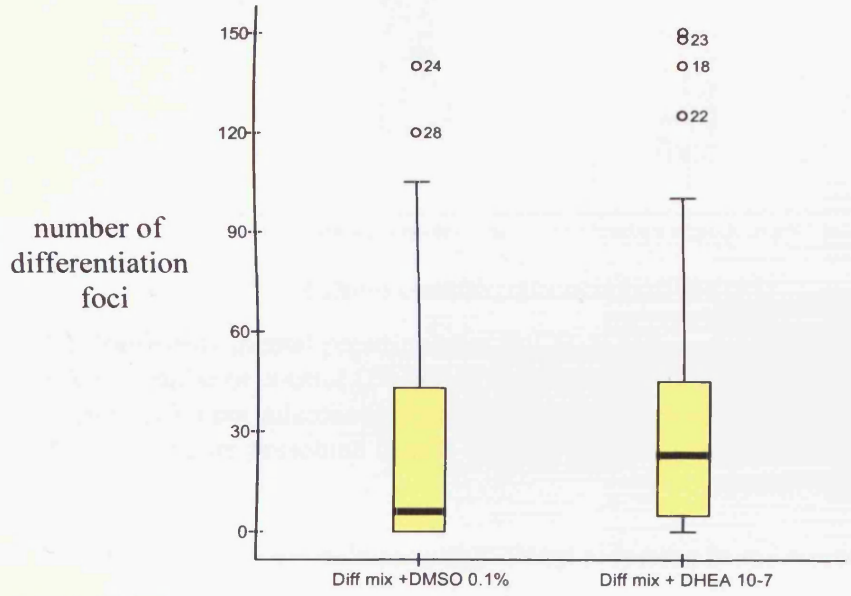



Fig 3.11 Confluent subcutaneous preadipocytes in 6 well plates cultured in differentiation mix + DHEA $10^{-7}$ molar or control (DMSO 0.1%) for 21 days. Results are the number of foci of differentiation per microscopic field (10 random fields examined per well). Pooled data are presented from 3 experiments performed in triplicate

In subcutaneous cultures no reduction in foci number was demonstrated, conversely more foci were observed in DHEA exposed samples and the difference just achieved statistical significance (Wilcoxon Signed Ranks test p=0.034).

Box-and-whisker plot demonstrating the effect of DHEA on foci of differentiation in omental preadipocytes following 21 days exposure

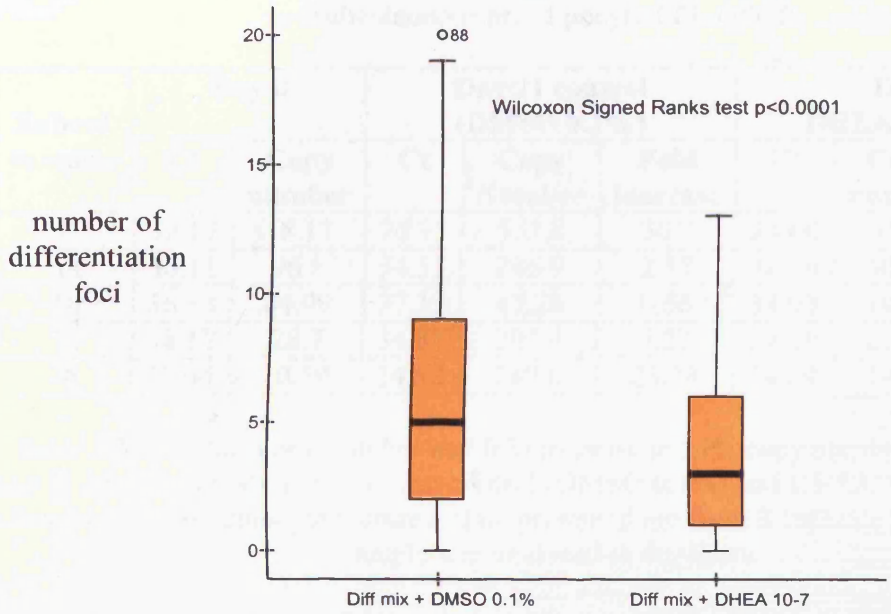



Fig 3.12 Confluent omental preadipocytes in 6 well plates cultured in differentiation mix + DHEA $10^{-7}$ molar or control (DMSO 0.1%) for 21 days. Results are the number of foci of differentiation per microscopic field (10 random fields examined per well). Pooled data are presented from 3 experiments performed in triplicate

In the omental preadipocyte cultures a significant reduction in the number of foci of differentiation was demonstrated. These data overall could suggest that DHEA may be inhibitory in omental but not in subcutaneous preadipocyte differentiation. The potential failings of this method of assessment have been discussed in chapter 2 and a more robust

technique is required to confirm these results; as in the cell line experiments QPCR assessment of terminal differentiation marker (LPL) expression rate was chosen.

### 3.3.5.1. QPCR – subcutaneous preadipocytes

QPCR assessment of the terminal differentiation marker (LPL) was performed.

Subcutaneous preadipocyte LPL QPCR

| Subcut samples | Day 0 | | Day 21 control (DMSO 0.1%) | | | Day 21 DHEA $10^{-7}$ molar | | |
|---|---|---|---|---|---|---|---|---|
| | Ct | Copy number | Ct | Copy Number | Fold increase | Ct | Copy number | Fold increase |
| 8 | 32.17 | 18.17 | 26.75 | 557.8 | 30.7 | 24.00 | 3163 | 174.1 |
| 1a | 36.11 | 96.8 | 34.53 | 246.9 | 2.57 | 34.16 | 306.7 | 3.19 |
| 1b | 36.33 | 84.99 | 37.33 | 47.26 | 0.56 | 34.93 | 195.2 | 2.3 |
| 7a | 38.17 | 28.7 | 34.82 | 207.4 | 7.22 | 34.49 | 252.4 | 8.79 |
| 7b | 37.04 | 10.59 | 34.52 | 247.6 | 23.38 | 34.89 | 199.0 | 18.26 |

Table 3.5 Ct value, copy number and fold increase in LPL copy number from day 0 to day 21 of differentiation protocol in control (DMSO 0.1%) and DHEA $10^{-7}$ molar treated subcutaneous preadipocyte cultures. Data presented are from 3 separate patients and each sample was analysed in duplicate

These data suggest an overall increase in LPL copy number following the differentiation protocol (mean fold increase in control samples 12.89 and in DHEA treated samples 41.31) consistent with the occurrence of adipogenesis. However the various samples demonstrate considerable variability in adipogenesis potential hence the amount of differentiation occurring in DHEA and control samples was not significantly different (Wilcoxon Signed Ranks test p=0.138). On interpretation of these data one must also consider the high Ct values generated. Furthermore the results are not corrected for housekeeper gene expression due to the formation of primer dimers following housekeeper QPCR.

### 3.3.5.2 QPCR – omental preadipocytes

The tendency for the omental preadipocyte samples to be affected by a contaminating cell type reduced the number of samples available for QPCR analysis however samples from patients 2, 2a (2nd passage sample 2), 1, 5 and 8 were analysed although samples from 1 and 5 did contain the non-preadipocyte population. QPCR assessment of the terminal differentiation marker (LPL) was performed.

Omental preadipocyte LPL QPCR

| Omental samples | Day 0 | | Day 21 control (DMSO 0.1%) | | | Day 21 DHEA $10^{-7}$ molar | | |
|---|---|---|---|---|---|---|---|---|
| | Ct | Copy number | Ct | Copy number | Fold increase | Ct | Copy number | Fold increase |
| 2 | 29.57 | 185.0 | 24.22 | 4918 | 26.58 | 25.29 | 2546 | 13.76 |
| 2a | 30.29 | 207.4 | 27.72 | 994.1 | 4.8 | 29.54 | 327.0 | 1.6 |
| 8 | 32.01 | 139.0 | 21.78 | 70130 | 504 | 22.42 | 47500 | 341 |
| 5* | 29.13 | 399.4 | 30.19 | 208.6 | 0.52 | 31.00 | 127.3 | 0.31 |
| 1* | 33.60 | 122.9 | 33.45 | 134.2 | 1.09 | 33.92 | 102.6 | 0.83 |

Table 3.6 Ct value, copy number and fold increase in LPL copy number from day 0 to day 21 of differentiation protocol in control (DMSO 0.1%) and DHEA $10^{-7}$ molar treated omental preadipocyte cultures. Data presented are from 4 separate patients analysed simultaneously in duplicate (* denotes mesothelial cell presence)

These results confirm that in general, differentiation was occurring although not in samples 5 and 1 (mesothelial cells). It is also apparent that a large degree of variation in extent of differentiation potential existed between samples and also, as anticipated the extent of differentiation in the second passage sample (2a) was reduced relative to the initial, passage 1 sample (2). In all experiments with evident differentiation there was a tendency for reduced adipogenesis following treatment with DHEA in keeping with the data on foci of differentiation and this observation reached statistical significance

(Wilcoxon Signed Ranks test p=0.043). These data are corrected for input cDNA (1μL cDNA per QPCR reaction generated from 1μg input RNA RT) as the housekeeper gene (APRT) data generated by QPCR tended to develop primer dimers.

### 3.3.6 Does DHEA affect adiponectin production in human primary preadipocytes?

From the generated ELISA data it was clear that not all samples synthesised sufficient adiponectin for assay detection. This observation is likely to be a function of the variable amounts of differentiation occurring within each sample despite the use of identical experimental conditions. Also, no sample beyond the first passage generated sufficient adiponectin for assay detection, suggesting the occurrence of senescence. Alternatively the ELISA kit chosen may not have been sensitive enough to detect lower levels of differentiation. However, 4 omental and 5 subcutaneous samples underwent sufficient increases in adiponectin levels following a standard 21 day differentiation protocol with and without DHEA exposure for analysis.

#### 3.3.6.1 Adiponectin standard curve

From the standard solution provided in the human adiponectin ELISA kit [Duoset® ELISA, R&D Systems, Oxford, UK] a standard curve was generated in order to estimate sample adiponectin concentration.

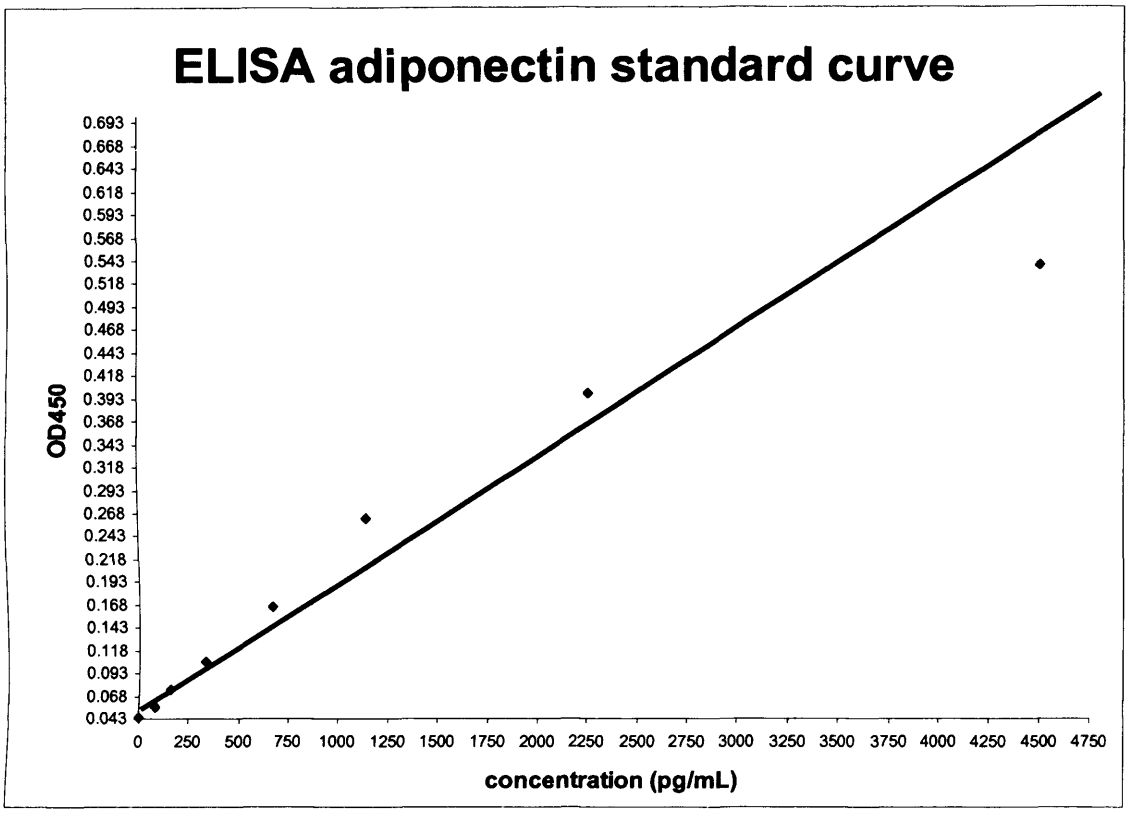



Fig 3.13 Human adiponectin standard curve

### 3.3.6.2 Does adiponectin production increase following the differentiation protocol in subcutaneous and omental preadipocytes?

The adiponectin assay data demonstrate a statistically significant overall increase in concentration (fig 3.14) from day 0 to differentiation protocol terminus (day 21) in omental and subcutaneous preadipocyte samples and in DHEA treated and untreated samples. Considerable variability was however observed in different samples, which is likely to be a reflection of the heterogeneity of adipose tissue as well as the originating subjects.

Box-and-whisker plot demonstrating the change in conditioned medium adiponectin concentration from omental and subcutaneous preadipocyte cultures following 21 days exposure to differentiation mix (± DHEA/control)

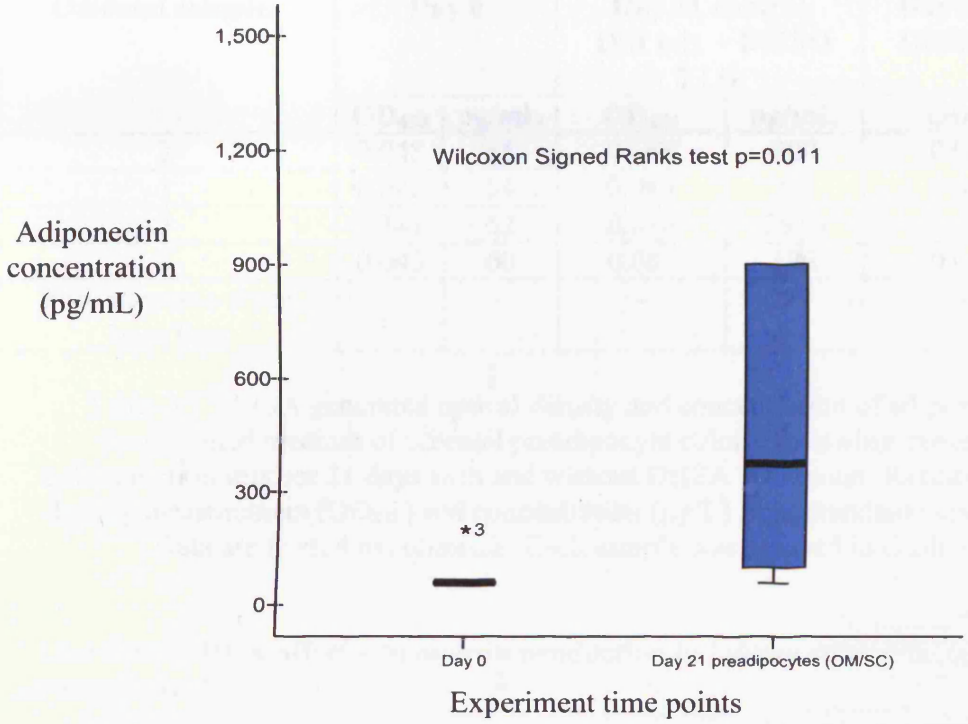



Fig 3.14 Conditioned medium was collected from omental and subcutaneous preadipocyte cultures following 21 days exposure to differentiation mix and assayed for adiponectin concentration using a standard ELISA method. Data presented are from day 0 and day 21 for both cell types using control and DHEA treated samples

### 3.3.6.3 Does DHEA affect adiponectin production in human omental preadipocytes?

Adiponectin generation in omental preadipocytes

| Omental samples | Day 0 | | Day 21 control Diff mix + DMSO 0.1% | | Day 21 Diff mix + DHEA $10^{-7}$ molar | |
|---|---|---|---|---|---|---|
| | $OD_{450}$ | pg/mL | $OD_{450}$ | pg/mL | $OD_{450}$ | µg/L |
| 2 | 0.048 | 65 | 0.188 | 900 | 0.046 | 62 |
| 1 | 0.042 | 54 | 0.044 | 58 | 0.053 | 100 |
| 3 | 0.046 | 62 | 0.616 | 3950 | 0.689 | 4450 |
| 4 | 0.045 | 60 | 0.067 | 125 | 0.048 | 65 |
| **Fold increase from base line** | - | - | - | **21** | - | **19.5** |

Table 3.7 ELISA generated optical density and concentration of adiponectin in conditioned medium of omental preadipocyte culture following exposure to differentiation mix for 21 days with and without DHEA $10^{-7}$ molar. Results are optical density measurement ($OD_{450}$) and concentration (µg/L) from standard curve analysis. Data are from 4 experiments. Each sample was assayed in duplicate

### 3.3.6.4 Does DHEA affect adiponectin production in human subcutaneous preadipocytes?

Adiponectin generation in subcutaneous preadipocytes

| Subcutaneous sample | Day 0 | | Day 21 control Diff mix + DMSO 0.1% | | Day 21 Diff mix + DHEA $10^{-7}$ molar | |
|---|---|---|---|---|---|---|
| | $OD_{450}$ | pg/mL | $OD_{450}$ | pg/mL | $OD_{450}$ | µg/L |
| 5 | 0.042 | 56 | 0.103 | 375 | 0.049 | 72 |
| 1 | 0.045 | 60 | 0.058 | 115 | 0.188 | 900 |
| 4 | 0.088 | 200 | 0.683 | 4440 | 0.624 | 3825 |
| 2 | 0.043 | 56 | 0.123 | 460 | 0.113 | 450 |
| 3 | 0.044 | 58 | 0.043 | 56 | 0.059 | 120 |
| **Fold increase from base line** | - | - | - | **12.7** | - | **12.5** |

Table 3.8 ELISA generated optical density and concentration of adiponectin in conditioned medium of subcutaneous preadipocyte following exposure to differentiation mix for 21 days with and without DHEA $10^{-7}$ molar. Results are optical density measurement ($OD_{450}$) and concentration (µg/L) from standard curve analysis. Data are from 4 experiments. Each sample was assayed in duplicate.

The omental preadipocytes tended to generate a greater increase in adiponectin concentration than the subcutaneous preadipocytes following differentiation though this was not statistically significant (Wilcoxon Signed Ranks test p=0.889). Furthermore there was no difference observed between the control and DHEA treated samples in either the omental or subcutaneous cell types (Wilcoxon Signed Ranks test p=1.0 and p=0.5 respectively).

### 3.4 Discussion

In support of the findings presented in chapter 2, DHEA inhibited the proliferation of both subcutaneous and omental primary preadipocytes and at similar concentrations to those shown to be effective in the cell line experiments. The absence of effect at 72 hours in the primary cells is most likely a reflection of the overall slower proliferation rate in these cell types. The relative absence of effect on proliferation in the primary preadipocytes at 72 hours compared to 168 hours suggests possible implications to the cell line data presented in chapter 2. If the cell line experiments had been continued over further time points (terminated at 48 hours currently) a greater effect may have been observed. Antiproliferative activity may also have been seen to be greater at physiological concentrations had I continued the experiments to further time points and certainly this should be a part of any future experiments.

MAP kinase primary preadipocyte cell signalling is also shown to be influenced by DHEA though differential effects are seen in the subcutaneous and omental cell types whereby DHEA reduces the level of phosphorylated MAP kinase in the subcutaneous cells but not in the omental cells over a short time period (20 minutes). As in the cell lines

it is not possible to conclude if these effects are adipogenesis supporting or inhibiting as first phosphorylation then dephosphorylation are required for adipogenesis progression as discussed in chapter 1.

Two methods of assessing the extent of preadipocyte differentiation have been employed; foci counts and QPCR. These confirm the occurrence of differentiation in response to the adipogensis protocol employed and also interestingly show that DHEA inhibits differentiation in omental but not subcutaneous preadipocytes. This is of potential importance given the adverse metabolic effects associated with omental fat accumulations relative to subcutaneous adipose tissue deposits. These experiments were however performed on a limited number of samples that exhibited a wide degree of differentiation potential. Furthermore RNA concentrations, and copy number generated were often low with associated high Ct values at QPCR, despite attempts to increase sample yield. The omental samples were also complicated by a contaminating cell line which, when present in larger enough quantities prevented the occurrence of differentiation which is likely secondary to the contaminating cell effectively 'taking-over' the plate. In order to combat the heterogeneity observed within the primary cell cultures it would be necessary to either perform these experiments in many more samples or, if possible to identify a sample with optimal differentiation potential and perform a number of experiments with this.

Although the data from these QPCR experiments demonstrates that DHEA may inhibit adipogenesis in omental but not subcutaneous primary preadipocytes the results have been generated without the use of a reference (housekeeper) gene. Thus the data have been exposed to potentially excessive experimental variability and this in turn increases

the risk of type I statistical errors. The results must therefore be interpreted with this in mind, although the fact that results obtained by counting foci of differentiation produced a similar and statistically significant effect lend weight to the conclusions drawn. Adipogenesis in both omental and subcutaneous preadipocytes resulted in the generation of the adipocytokine adiponectin and thus represents a further marker of the occurrence of differentiation though no difference was observed between DHEA treated and control samples. Once more a large degree of variability in the production of adiponectin was observed and it is likely that only with an increased number of experimental samples would potential differences between omental, subcutaneous, DHEA and control samples be observed. QPCR assessment of adipogenesis markers remains however a more sensitive method of determining the extent of differentiation.

The anti-adipogenic effect of DHEA on human omental preadipoctyes is a novel finding with potential significance in the clinical setting, particularly in individuals with pathological DHEA deficiency in whom there is known to be augmented cardiometabolic risk. To further investigate and also to add weight to these findings a clinical trial supplementing DHEA in this population group is required.

# Chapter 4

## A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function and body composition in patients with primary and secondary adrenal insufficiency – Study design

## 4.1 Overview

From the *in vitro* studies described in chapters 2 and 3 it is evident that in the laboratory setting at least, DHEA exerts a negative influence over some biological actions of precursor fat cells. In the clinical setting however, the role of DHEA supplementation in either physiological or pathological DHEA deficiency remains unclear. In order to determine the impact of DHEA replacement on adipocyte biology *in vivo*, a clinical trial was designed to investigate direct measures of body fat as well as markers of vascular health. The populations chosen for the study comprised individuals with pathological DHEA deficiency arising as a result of primary or secondary adrenal insufficiency. Various adipose and vascular parameters were examined before and after a period of DHEA therapy thus enabling correlation of any beneficial vascular effects to alterations in adiposity.

The clinical trial consisted of a randomised, double blind, placebo-controlled cross-over design, thereby adhering to gold standard clinical trial methodology as well as augmenting study power, which was of particular importance when considering the relative rarity of the chosen study population and limited sample size.

Supplementation of DHEA in either pathological or physiological deficiency has been a topic of previous investigations and studies have attempted to address the potential benefits of DHEA replacement in a variety of settings, in particular in adrenal insufficiency and mood disorders. In the normal elderly population, treatment with DHEA improves psychological well-being, lean body mass and bone turnover, and increases circulating IGF-1 levels [182, 290]. More recently, studies have reported the effects of DHEA replacement in patients with Addison's disease (primary adrenal

insufficiency) who were on standard glucocorticoid and mineralocorticoid replacement [12, 13]. These patients are generally young, have severe DHEA/DHEAS deficiency at an age when serum DHEA/DHEAS concentrations would normally be high, and have few of the confounding variables associated with ageing. In randomised, double blind, placebo-controlled studies, DHEA therapy has been shown to increase DHEAS concentrations in both sexes from subnormal to within the young adult physiological range [12, 13]. In women, total testosterone similarly increases, though as testicular Leydig cell function is normal in men with Addison's disease, testosterone levels are already normal at baseline and do not rise further with treatment. Improvements were also evident in self-esteem and mood, with reduced fatigue and variable improvement in sexual function [12, 13], leading to suggestions that DHEA therapy should be considered as part of the standard replacement therapy in all patients with Addison's disease.

However, comparatively little attention has been paid to the vascular effects of DHEA therapy in this population, despite cross-sectional and prospective epidemiological data in elderly populations demonstrating an inverse correlation between DHEAS concentration and vascular mortality. Furthermore, the evidence suggests that this relationship may be gender-specific. In a prospective cohort study of 622 elderly subjects, lower DHEAS levels were significantly associated with increased total mortality in men but not women [9]. A separate larger study has confirmed this relationship and the sexually dimorphic nature of this effect but also has shown a clear association with cardiovascular in addition to all-cause mortality [8]. These studies point to a gender difference in correlation between DHEAS levels and disease which could be explained, at least in part, by the sex-specific differences in the bioconversion of DHEA, whereby DHEA administration

increases androgens in women [51] and oestrogens in men [52]. It is possible, however, that this association is merely an epiphenomenon as chronic disease induces a shift of intra-adrenal steroid biosynthesis that favours cortisol over DHEA production [71], raising the possibility that low DHEAS levels merely represent a surrogate marker of an unrelated illness. It thus remains unclear whether the relationship between DHEAS concentration and vascular risk is a causative one or simply an association.

To address some of these questions Kawano and colleagues prospectively examined the effects of DHEA administration on endothelial function in a cohort of middle-aged men with hypercholesterolaemia [180]. Endothelial dysfunction contributes to the pathogenesis of atherosclerosis and cardiovascular disease, preceding the development of overt complications. DHEA supplementation in this cohort improved flow-mediated dilation of the brachial artery, an endothelium-dependent process. The authors were also able to demonstrate a reduction in PAI-1 and steady-state glucose concentrations without alteration in fasting insulin, demonstrating improved insulin sensitivity. Similar prospective analyses on vascular risk have not been conducted in women and it remains unclear whether or not these beneficial effects of DHEA administration are confined to men, or whether indeed these improvements are restricted to a normal ageing population or are also apparent in disease states such as adrenal insufficiency. Furthermore, the mechanisms of these effects are not yet fully apparent though animal studies have demonstrated an antiatherosclerotic effect of DHEAS [210, 219], possibly by prevention of platelet aggregation [214] and uptake of cholesterol [180], and decreasing vascular smooth muscle cell proliferation [212]. However, little is known about the direct effects of DHEA on the vasculature. DHEA has no known cellular nuclear receptors though

recently nongenomic plasma membrane receptors for DHEA have been identified in endothelial cell cultures which couple to eNOS [211]. Thus, DHEA has potential to stimulate NO generation. In addition, DHEA may be converted *in vivo* to oestradiol which itself increases NO synthase activity and NO production by either genomic or nongenomic mechanisms within the vascular endothelium [291]. It is entirely possible therefore that oestradiol converted from DHEA may have been responsible for the improvement in endothelial function observed in the study by Kawano *et al* [180].

The critical importance of oestrogens to endothelial health in males is highlighted by data from studies in an individual with an oestrogen receptor mutation and in men with aromatase deficiency. The patient with a mutation in the oestrogen receptor gene, and thus oestrogen insensitivity, was shown to have premature coronary artery disease [215]. His serum oestradiol and oestrone, FSH and LH concentrations were elevated, though serum testosterone concentrations were normal [216]. Computed tomography scanning showed premature calcification of a coronary artery and brachial artery studies demonstrated absence of flow-mediated dilation in response to ischaemic cuff occlusion despite preserved nitroglycerin and oestradiol-induced vasodilation [215]. Of the few male individuals described with aromatase deficiency to date [217, 292], one displayed elevated LDL, triglycerides and insulin levels, indicative of insulin resistance. Hormone analysis demonstrated undetectable oestrogens and very high circulating androgens in one of these individuals, together with elevated FSH and LH, indicative of an important role of oestrogens in the negative feedback regulation of gonadotrophins in males as in females. No cardiovascular studies have been performed to date in either of these individuals though there is evidence of impaired endothelium-dependent vasodilation in

the aorta of aromatase knockout mice, suggesting a role for endogenous oestrogens in the regulation of endothelial function in this model [293]. A recent randomised, double-blind, placebo-controlled study in healthy young men treated with the aromatase inhibitor anastrozole showed an impairment in endothelial function independent of changes in well-established cardiovascular risk factors such as high-sensitivity C-reactive protein (HSCRP), homocysteine or lipoprotein(a) [218], supporting the hypothesis that physiological levels of oestrogen play an important role in the male cardiovascular system.

The data from these studies suggest that DHEA could improve endothelial function and in turn contribute to an improved vascular risk profile in patients with low circulating DHEAS levels, though it is conceivable that these effects may be gender-specific and mediated indirectly via conversion of this steroid to oestrogens. I sought to test this postulate in two cohorts of patients with primary and secondary adrenal insufficiency.

### 4.1.1 Study approval and funding

Prior to study commencement approval to conduct this clinical trial was obtained from Cardiff University (Sponsor; reference number SPON CU 086), Cardiff and Vale NHS trust Research and Development department (reference number 05/CMC/3294E), the South East Wales Research Ethics Committee (reference number 05/WSE04/42) and the Medicines and Healthcare Products Regulatory Authority (EudraCT number 2005-000115-10) who also, during the course of the study undertook an inspection of the trial as part of their routine statutory inspection of clinical trial activity at Cardiff and Vale NHS Trust (December 2007). The study was registered prospectively on the

International Standard Randomised Controlled Trial Number register and ascribed the registration number ISRCTN 46268487. Grant funding was obtained principally from the Wales Office for Research and Development, supplemented by support from the Cardiff and Vale NHS trust small grant scheme and a one-year Clinical Fellowship awarded to me from the Royal College of Physicians (Lewis Thomas Gibbon Jenkins of Briton Ferry Fellowship). The pre-initiation approval process began in January 2005 and final approval was obtained in August 2006 following which patient recruitment commenced. The delay in approval was mainly related to the need to accrue data, as part of the final approval process by the MHRA, to support the proposed shelf-life of the study drug.

### 4.1.2 Overall study design

This was a 32 week randomised, double-blind, placebo-controlled, cross-over study of the effects of DHEA on arterial stiffness and endothelial function in patients with primary (PAI; n=20) and secondary (PAI; n=20) adrenal insufficiency. Patients were randomly assigned to consecutive 3-month treatment periods of either DHEA 50mg followed by placebo capsules of identical appearance or placebo followed by DHEA. A wash-out interval of 2 months separated the two treatment phases. Randomisation was performed prospectively by an independent source, with half receiving DHEA first and the other half receiving placebo; this was carried out by St Mary's Pharmaceuticals Unit. Arterial stiffness and endothelial function were assessed non-invasively by means of pulse wave analysis and pulse wave velocity measurements at baseline and after each treatment phase.

### 4.1.3 Discussion of design

Primary adrenal insufficiency is an uncommon disorder and the total number of subjects available for study is limited. A double-blind, placebo-controlled, cross-over design is therefore justified in order to enhance the likelihood of detecting true change in outcome measures after DHEA treatment. A dose of 50mg was used as this has been shown from previous studies to increase DHEAS levels to young adult values. A washout interval of 2 months was used to separate the two treatment phases to minimise the chance of 'carry over' into the next treatment phase; DHEA has a short circulatory half-life and 1 month washout intervals have usually been employed on this basis in previous studies.

### 4.1.4 Study participants, inclusion and exclusion criteria

Patients were recruited from the Endocrine clinic at the University Hospital of Wales. The diagnosis of primary or secondary adrenal insufficiency was based on the following criteria. Addison's disease was confirmed by documented hypocortisolaemia (subnormal ACTH stimulation test) associated with raised serum ACTH and, where available, positive adrenal autoantibodies. A minimum of 1 year duration of Addison's disease was an inclusion criterion. Patients with a history of current or previous DHEA replacement were excluded from participation in the study. All participants had a stable glucocorticoid and/or mineralocorticoid replacement dose for at least six months prior to study commencement.

Secondary adrenal insufficiency was defined by documented hypocortisolaemia on an insulin stress test or short ACTH stimulation test associated with inappropriately low ACTH where available. To minimise confounding, subjects in this group comprised

patients with a history of non-functioning pituitary adenoma or craniopharyngioma only (as opposed to hormone-secreting adenomas) who were treated surgically with or without concomitant radiotherapy, and were panhypopituitary on full hormone replacement therapy including growth hormone.

Exclusion criteria for both study groups were age less than 18 years or greater than 70 years, pregnancy, breastfeeding and any intercurrent significant medical or psychiatric condition. All patients took their usual glucocorticoid and mineralocorticoid hormone replacement, with dosage and timing being kept unaltered before and during the study, though brief increases in glucocorticoid therapy were permitted in cases of intercurrent illness. Similarly, patients with secondary adrenal insufficiency took their usual hormone replacement therapy with no alteration in dose or drug timing prior to or during the study.

### 4.1.5 Recruitment

Potential participants were initially identified from the Endocrinology database at the University Hospital of Wales. Eligible subjects were invited to participate through a letter (patient information sheet see appendix IV) from the clinician responsible for their care (Dr J. S. Davies, Professor M. F. Scanlon or Dr D. A. Rees). Potential participants were also identified as they passed through the Endocrine clinic systems at UHW.

Following provisional agreement an appointment was made for the initial visit. At this visit participants were first given the opportunity to ask any questions regarding the study and were shown the study techniques involved. If subjects were still in agreement informed consent was obtained using a participant-investigator signed consent form (appendix IV). All study visits took place in the temperature controlled ward area of the

Clinical Research Facility at the University Hospital of Wales, Cardiff. Subjects attended fasted on weekday mornings, each visit lasting 2-3 hours.

### 4.1.6 Interruption or discontinuation of treatment

The term discontinuation refers to a patient's non-completion of the study. Information about discontinuation was collected in this study. Patients who discontinued from the study prematurely completed the final safety evaluations at the time of their discontinuation. An explanation of why the patient was withdrawn from the study was recorded, even if the patient refused to return for a final visit. Patients who discontinued prematurely due to significant adverse events were followed until resolution of the adverse event. Patients who discontinued due to clinically significant abnormalities in clinical laboratory results continued to be evaluated until the abnormality resolved or until it was judged to be permanent.

If for any patient either study treatment or observations were discontinued the reason was recorded. Reasons that a patient could discontinue participation were considered to constitute one of the following:

1. adverse event(s)
2. abnormal laboratory value(s)
3. abnormal test procedure result(s)
4. unsatisfactory therapeutic effect
5. subject's condition no longer requires any study treatment
6. protocol violation

7. subject withdrew consent

8. lost to follow-up

9. administrative problems

10. death

### 4.1.7 Treatments/Interventions

The two treatment arms were DHEA and placebo. The DHEA and placebo were sourced and packaged by St Mary's Pharmaceutical Unit, Cardiff. During the study phase, patients received consecutive daily 50mg doses of DHEA for 12 weeks (84 days) followed by placebo or placebo followed by 50mg DHEA, separated by an 8 week wash-out period.

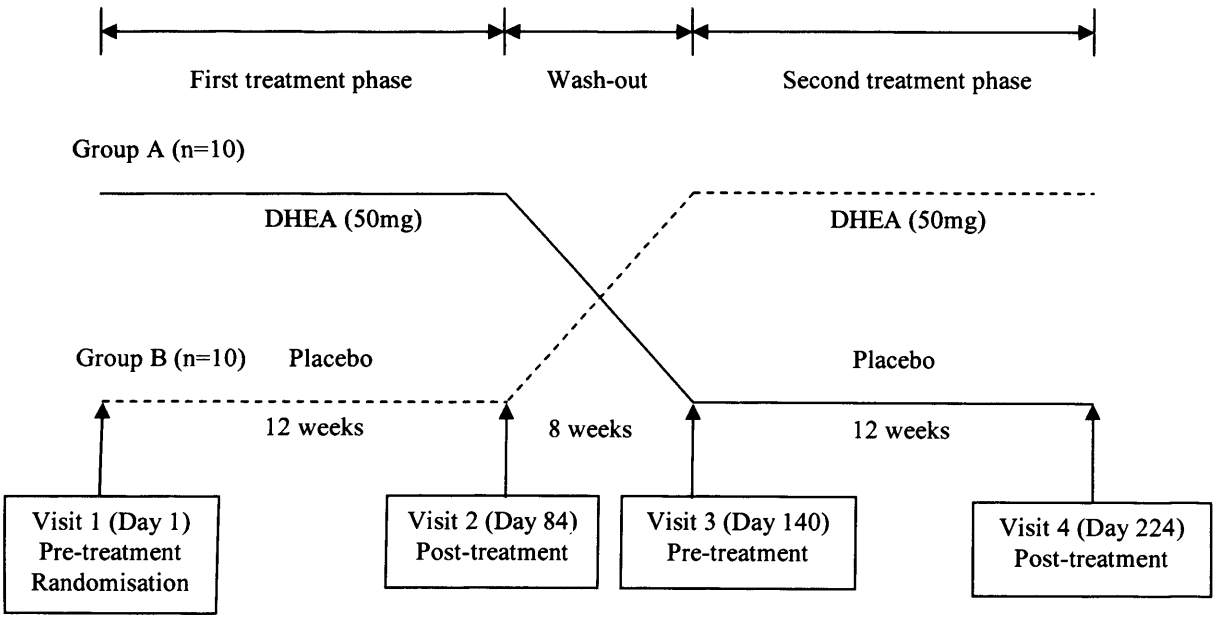



Fig 4.1 Clinical trial overview

50mg DHEA per day increases DHEAS levels to those of young adults and administration of this dose of DHEA over 1 year in an elderly population was shown to be safe with no harmful consequences [294]. Mild facial acne has been reported in some women [13] which is usually self-limiting, and subjects were monitored for these effects with withdrawal of therapy where appropriate. A minor reversible rise in serum aminotransferase concentration has also been reported, though other studies have not reported similar findings [13]. Serial hepatic enzyme measurements were performed at each study visit and study drug discontinued where necessary.

### 4.1.8 Investigational therapy

Patients were assessed at four points: baseline tests at visit 1 and at the end of the wash-out phase (visit 3) and after each treatment (DHEA or placebo) phase (visits 2 and 4; see fig 4.1). Following an overnight fast, subjects were admitted to the Clinical Research Facility at 08:00 hours. Patients were not assessed if they had evidence of recent or ongoing infection as this would skew measurement of C-reactive protein. Pre-menopausal women underwent a pregnancy test and were excluded from participation if this was positive.

Baseline anthropometric data, consisting of blood pressure, height, weight body mass index (BMI), waist/hip circumference and body composition measured by bioelectrical impedance were collected. Body height (without shoes) was measured to the nearest 0.5 cm using a stadiometer and body weight (in light clothing without shoes) to the nearest 0.5 kg on a balance beam scale. Waist circumference was measured at minimal respiration and reported to the nearest 0.5 cm by positioning a flexible anthropometric

tape parallel to the floor and immediately above the iliac crest. Hip circumference was measured at the maximum circumference over the buttocks and recorded to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Waist:hip ratio (WHR) was calculated as waist circumference divided by hip circumference. Standard bioimpedance was performed using a commercial bioimpedance meter (Tanita Body Fat analyser TBF-305, Tanita Corporation, Japan).

Blood samples were collected for analysis of DHEAS, testosterone, insulin-like growth factor-1 (IGF-1; SAI group only), SHBG, LH, FSH and androstenedione (the immediate downstream metabolite of DHEA). In addition, fasting blood samples were collected for measurement of total, HDL and LDL cholesterol, triglycerides, high sensitivity C-reactive protein (HSCRP), PAI-1, insulin, glucose, adiponectin and liver function tests. HSCRP measurements have emerged as powerful predictors of cardiovascular risk in a broad spectrum of patient populations [295] and the haemostatic factor PAI-1 is a circulating marker of endothelial function [180, 296]. Furthermore, DHEA therapy has been shown to reduce PAI-1 levels in middle-aged men with hypercholesterolaemia [180]. Adiponectin has emerged as a metabolically active adipoctykine the levels of which are inversely correlated with amounts of body fat [122]. Serum concentrations of adiponectin are lower in type II diabetes [120] and data suggest it may protect against the development of insulin resistance in normal individuals. Estimates of the degree of insulin resistance in the study population were performed by the homeostatic model assessment (HOMA) method. The HOMA method has been validated as a good index of insulin resistance in subjects with a broad range of insulin sensitivity [297] and

demonstrates good correlation with insulin-mediated glucose uptake calculated by the euglycaemic hyperinsulinaemic glucose clamp [297].
Serum samples collected at each visit were subsequently stored at -20°C until later analysis.

Measures of arterial stiffness and endothelial function in our cohort were performed by means of pulse wave analysis (PWA) and pulse wave velocity (PWV) using a non-invasive technique (SphygmoCor system, AtCor medical). Traditional methods for assessing endothelial function centre on measuring the response to an endothelium-dependent, NO-mediated stimulus such as acetylcholine or reactive hyperaemia, and a direct, endothelium-independent nitrovasodilator such as sodium nitroprusside or glyceryl trinitrate (GTN). These methods, however, are time-consuming and can prove difficult to reproduce accurately and are thus not ideally suitable for inclusion in large-scale trials. PWA combined with provocative administration of GTN and salbutamol has been shown to provide a simple, repeatable, non-invasive means of assessing endothelial function *in vivo* [298] . Aortic PWV, a classical index of aortic stiffness, can be easily measured in humans using non-invasive methods of high reproducibility [203, 299]. These techniques make use of the SphygmoCor system and the principle of applanation tonometry first described by O'Rouke and collegues in Australia [202]. This method involves placing a sensor (termed a tonometer) over the point of maximal palpable pulsation, typically at the radial pulse (also carotid or femoral). Minimum downward pressure is then applied to the sensor which compresses the vessel beneath against underlying structures (e.g. radius) thus increasing the lateral vessel diameter. This has the effect of equalising

circumferential pressure conducive to high-fidelity waveform capture by the tonometer (Fig 4.2).

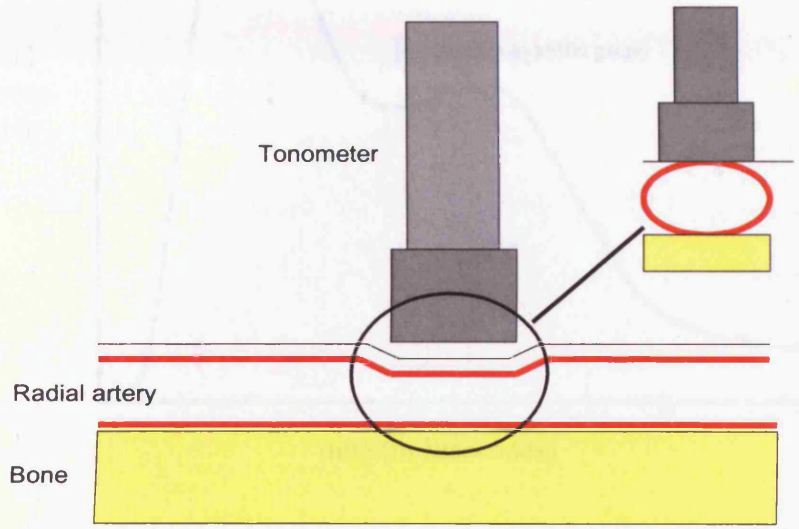



Fig 4.2 Applanation tonometry schematic

The waveforms detected by this method comprise paired systolic peaks, the first peak representing the out-going systolic pulse wave, the second representing the reflected wave from peripheral resistance vessels (Fig 4.3).

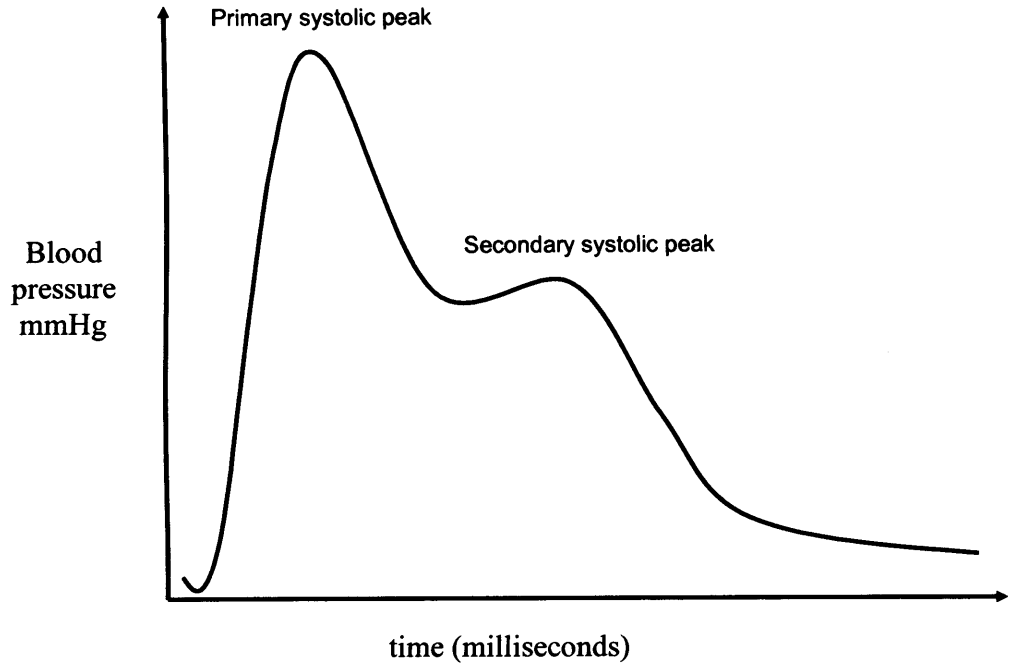
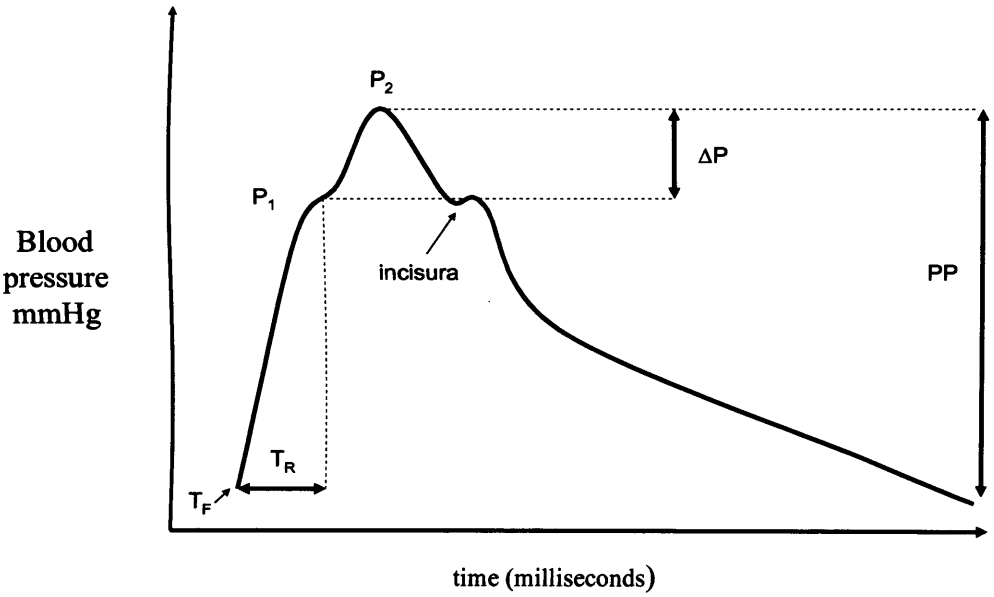



Fig 4.3 Radial pulse wave analysis recorded by applanation tonometry

Once the pressures are recorded at the radial artery a validated [300, 301] and generalised transfer factor is applied in order to generate the corresponding central wave form (Fig 4.4). The transfer function calculation is performed by the SphygmoCor software and is typically applied to the radial pulse waveform as the radial artery is less compliant relative to the aorta and tends to alter less with ageing and following the introduction of pharmacological agents. The transfer function used by the SphygmoCor system is based on a population with a mean age of 51 years [302] and can be defined as an arithmetical description of the difference in pulsatile phenomena experienced at the peripheral (radial) and proximal (ascending aorta) sites in terms of modulus and phase. The term generalised refers to the ability of the same transfer function to be used in different individuals under

different circumstances [300, 302]. Individualised [300], disease-specific [303] and gender-specific [304] transfer function calculations may be marginally more accurate in certain populations but this is not likely to interfere with a study of this design. Following application of the transfer function a central pressure waveform can then be determined and from that the augmentation index (Ai) (difference between the first and second systolic peaks (ΔP) expressed as a percentage of the pulse pressure (PP)), a measure of arterial stiffness can be derived. Central waveform pressures represent more accurately the left ventricular work load [305] than peripheral waveform pressures and thus potentially provide a better marker of cardiovascular risk.




$P_1$- First systolic peak
$P_2$- Second systolic peak PP- Pulse pressure
ΔP- difference between $P_1$ and $P_2$
$T_F$- Foot of Wave
$T_R$- time between $T_F$ and inflection point
Augmentation index (Ai) = ΔP/PP (%)

Fig 4.4 Central aortic waveform calculated from peripheral wave form using transfer factor showing $P_2$ augmentation of $P_1$

Overt cardiovascular complications are preceded by structural changes within the arterial wall that becomes less elastic or distensible. This in turn leads to a reduction in vessel capacity to accommodate volume changes occurring throughout the cardiac cycle. In systole the aorta will distend less well and so generate higher systolic pressures and during diastole, secondary to impaired elastic recoil, maintenance of pulse pressure will be impaired. The less compliant, diseased vessels generate peripheral and central arterial waveforms that are different in shape from waveforms generated form healthy vessels and this can be easily detected using the SphygmoCor system. 'Stiffer' vessels produce waveforms exhibiting exaggerated reflected wave augmentation as well as increased pulse wave velocities.

Arterial wave forms are a composite of a pulse pressure wave produced in systole by left ventricular contraction and a reflected wave produced as the forward wave hits smaller vessels with increased resistance and is reflected back on itself. Waveforms will differ depending on which artery is examined, as the distance from the left ventricle increases and the composition of the arterial wall becomes less elastic more distally. In stiffer arteries waveforms will travel faster and the reflected wave will be seen earlier in the formation of the forward wave. If the reflected wave appears early enough, before aortic valve closure, it will add to or augment the pressure produced by the initial wave, producing an increase in central systolic pressure [306]. The extent of this augmentation can be measured peripherally and expressed as a percentage. However central augmentation and pressures are of greater importance as these reflect more directly left ventricular workload. To determine central arterial values a validated transfer function

[299] can be applied to the peripheral arterial assessment and from this central aortic pressures can then be generated as discussed above.

Central ascending aorta pressures and waveforms are influenced by both height [307], and heart rate [308]. The higher Ai values observed in females are therefore likely to be a function of vessel length which is in turn a function of overall height. Alterations in the timing of the reflected wave and thus the extent to which it augments the forward wave explain the differences seen in association with pulse rate.

In the context of end stage renal disease, hypertension and glucose intolerance PWV has been shown to predict mortality [198-200, 309]. Increased arterial stiffness is associated with some of the classical cardiac risk factors such as age and male sex [310] and has also been shown to precede overt atheroma formation [311]. Arterial stiffness is also intimately involved with endothelial function as discussed in chapter 1. Ai has also been demonstrated to be an independent risk marker for coronary artery disease [186]; moreover the high central arterial pressures (that can be generated via the transfer function from peripheral pulse waveforms as discussed above) have been associated with increased arterial stiffness [312]. Theoretically central blood pressure better predicts cardiovascular status as it more accurately describes left ventricular pressure exposure and this is evidenced by the observation that central blood pressure, as opposed to peripheral blood pressure, has been shown to be an independent predictor of total and cardiovascular mortality [313].

### 4.1.9 Treatment assignment/randomisation methods/blinding

Subjects were randomised into two groups in a double-blind fashion: for each cohort (PAI and SAI), group A (n=10) received DHEA 50mg followed by placebo treatment and group B (n=10) received placebo followed by DHEA 50mg treatment for 12 weeks separated by an 8 week washout. The treatment assigned to each patient was determined according to a computer generated randomisation list, produced by St Mary's Pharmaceutical Unit using Microsoft XL random number generator. The patient packs/study drug were labelled with a unique patient identification number. When a patient was found to be eligible for the study and had completed all baseline procedures, he/she was allocated a unique patient identification number in sequential, chronological order. The patient was then treated with the medication labelled with the same number. Should a patient withdraw from the study, his or her identification number was not reallocated.

### 4.1.10 Emergency procedure for unblinding

The randomisation code for each patient was delivered to the Pharmacy department at the University Hospital of Wales in individually sealed envelopes for emergency use only. In the absence of any emergency, patient allocation details were kept coded and remained confidential until the trial was complete.

Only in the case of a serious adverse event was there a plan for the investigator to open the relevant sealed envelope containing the patient's treatment allocation and only if this would be of help in the treatment of the patient.

### 4.1.11 End of study unblinding

Following the last patient visit and completed biochemical analysis an electronic database was prepared containing all generated results. Once complete, a research governance officer from Cardiff University undertook a study closedown visit which included obtaining a CD copy of the dataset as evidence of the final, completed and verified data. St Mary's Pharmaceutical Unit was then informed by the Sponsor of study completion and permission to release the treatment allocation codes was given. The data were then analysed in conjunction with Professor Robert Newcombe, Department of Primary Care and Public Health, Centre for Health Sciences Research, Cardiff University.

### 4.1.12 Concomitant therapy

All medications taken during the four weeks prior to visit 1 and throughout the study were recorded. As indicated above, patients were expected to take their usual glucocorticoid or mineralocorticoid replacement therapy with dosage and timing being kept unaltered before and during the study, though brief increases in glucocorticoid therapy were permitted in cases of intercurrent illness. These were recorded. Similarly, patients with secondary adrenal insufficiency took their usual hormone replacement therapy with no alteration in dose or drug timing prior to or during the study. Investigators discouraged patients from taking any medication during the study that was not being taken at baseline, an exception being medication required to treat an adverse event.

### 4.1.13 Procedures and Instructions

Instructions on the reporting procedures for serious adverse events

Information about all non-serious and serious adverse events (SAEs), irrespective of causality, whether volunteered by the subject, discovered by investigator questioning, or detected through physical examination, laboratory test or other means, were collected and recorded. An adverse event was defined as any undesirable sign, symptom or medical condition occurring after starting the study treatment. Study treatment included the study drug under evaluation (DHEA) or placebo given during any phase of the trial. Adverse events were followed as appropriate.

Medical conditions/diseases present before starting the study were considered adverse events if they worsened after starting the study drug. Abnormal laboratory values or test results constituted adverse events only if they induced clinical signs or symptoms or required therapy, or were considered clinically significant for any reason, in which case they were recorded.

Where possible, each adverse event was also described by:

1. Its duration
2. The severity grade (mild, moderate, severe)
3. Its relationship to study drug (suspected, not suspected)
4. The action(s) taken

Serious adverse events

Information about all SAEs was collected and recorded. A SAE was defined as an undesirable sign, symptom or medical condition which:

1. was fatal or life-threatening
2. required or prolonged hospitalisation
3. was significantly or permanently disabling or incapacitating
4. constituted a congenital anomaly or a birth defect
5. was medically significant, jeopardised the subject or required medical or surgical intervention to prevent one of the outcomes listed above.

Any SAE, irrespective of causality, occurring in a patient after providing informed consent and until sixteen weeks after ending study participation was recorded. Suspected unexpected serious adverse reactions (SUSARS) were reported immediately to the trial sponsor (Cardiff University), the Research Ethics Committee and the Medicines and Healthcare products Regulatory Agency (MHRA) as per European Union requirements and ICH-GCP (International Conference of Harmonisation of Good Clinical Practice).

### 4.1.14 Endpoints

Based on previous studies in middle-aged men with hypercholesterolaemia, an improvement in endothelial function following DHEA administration was anticipated. Change in augmentation index alone and measured post-salbutamol versus post-GTN, central aortic blood pressure and pulse wave velocity from baseline were therefore designated the primary outcome measures. Secondary end-points included changes in

DHEAS, androstenedione, testosterone (in females), PAI-1, HSCRP, insulin resistance (HOMA-IR), IGF-1, lipid profile, adiponectin and measures of body composition.

### 4.1.15 Sample size and power calculations

Sample size advice was sought from Professor R. Newcombe, Department of Medical Statistics and Epidemiology, School of Medicine, Cardiff University. The design for these two studies was based largely on a precedent study by Kawano *et al* [180]. Important differences between the proposed study and the precedent study relate to the patient population studied, the method used to measure endothelial function, and the fact that Kawano *et al* used a parallel groups design, for reasons that are not made clear.

Based on Kawano *et al*'s figures, a difference of 8.4 – 4.4 = 4.0 FMD units (i.e. % of baseline) was postulated at 12 weeks i.e. when equilibrium is reached. Kawano *et al* gave a standard error of 0.7 units for the mean at 12 weeks in the DHEA group based on 12 subjects. The corresponding SD is $0.7 \times \sqrt{12} = 2.4$ units. Disregarding within-patient correlation, the standard error of the mean change from 0 to 12 weeks based on 20 subjects all receiving the same treatment was expected to be $2.4 \times \sqrt{(2/20)} = 0.77$ units. The standard error of the mean change from 0 to 12 weeks based on 20 subjects all receiving active minus the mean change from 0 to 12 weeks based on 20 subjects all receiving placebo was expected to be $0.77 \times \sqrt{2} = 1.08$ units. The resulting critical ratio, 4.0 / 1.08 = 3.7, corresponded to a 96% power to detect a 4.0 unit difference at the conventional 5% alpha level. Since the main rationale for using a crossover study is that a substantial within-subject correlation was anticipated, we therefore anticipated that the power should be well in excess of this figure. Any potential disadvantages of reduced

precision using augmentation index compared with FMD (Kawano *et al*'s study) to assess endothelial function should be outweighed by the within-subject correlation.

### 4.1.16 Statistical methods

The recommended method of analysis for a cross-over study design was described by Hills & Armitage [314]. The assessment of treatment efficacy compares the within-subjects period I minus period II differences between the treatment sequence groups AP (active-placebo) and PA (placebo-active) by unpaired t-test. In the event of serious departure from Gaussian distributional form (Kawano *et al* gave no evidence that this occurs), scale transformation or an equivalent non-parametric approach [315] was used. Point and interval estimates of the treatment difference (half the AP – PA difference) were given as well as p-values.

## 4.2 Project Management

The day-to-day conduct of the study, recruitment, consent, blood sampling and measurements of pulse wave velocity and augmentation index in the study participants were performed by myself. I was also responsible for data entry and storage. The Clinical Trials Section of the Pharmacy Directorate (managed by Kathryn Bethuene, Clinical Trials pharmacist) was responsible for DHEA/placebo dispensing and storage and destruction. The randomisation process was performed by St Mary's Pharmaceutical unit, Cardiff, using a Microsoft XL random number generator. The same organisation sourced, produced and packaged the investigational medicinal product (IMP) (DHEA 50mg) as well as the placebo capsules.

Dr Aled Rees (Principal Investigator) acted directly as project supervisor. Formal meetings took place on a weekly basis to discuss all aspects of the study and to plan the work for the forthcoming week. Formal training was undertaken on all aspects of pulse wave analysis including attendance at an arterial stiffness course (Addenbrooke's Hospital, Vascular Research Unit, Cambridge, March 2005). A run in study was performed to ensure optimal operator quality with minimal variability prior to study commencement. Informal discussions regarding any difficulties that arose with the project took place as required. In the event of an emergency I was contactable on a 24 hour basis and my contact details were provided on all IMP/placebo containers.

### 4.2.1 Administrative Procedures

Changes to the protocol: Changes or additions to the protocol required a written protocol amendment. Amendments significantly affecting the safety of subjects, the scope of the investigation or the scientific quality of the study were submitted for additional approval by the Local Research Ethics Committee, Cardiff and Vale NHS Trust Research and Development department and the Medicines and Healthcare products Regulatory Agency.

Auditing procedures: As part of Good Clinical Practice, Dr Rees ensured that the study protocol and documentation were closely monitored. All study documentation was available for inspection at any time by appropriate regulatory authorities including internal audits by the Cardiff and Vale NHS Trust and Cardiff University Research and Development audit officers.

Handling of study medication: All study medication was dispensed by the Pharmacy department at UHW. Drug supplies were stored in an appropriate, secure area and accurate records of the dispensing of study drug were kept in a drug accountability ledger. An accurate record of the date and amount of study drug dispensed to each subject was available for inspection at any time.

### 4.2.2 Consent

As outlined (see section on recruitment), potential participants were initially identified from the Endocrinology database at the University Hospital of Wales and eligible subjects were invited to participate through a letter from the clinician responsible for their care (Dr J. S. Davies, Professor M. F. Scanlon, Dr D. A. Rees). Potential participants were also identified as they passed through the Endocrine clinic systems at UHW (Professor M. F. Scanlon, Dr J. S. Davies, Dr D. A. Rees) and a patient information sheet was provided (appendix IV).

This explained the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it might entail. Each subject was informed that participation in the study was voluntary and he/she could withdraw from the study at any time and that withdrawal of consent would not affect his/her subsequent medical treatment or relationship with the treating physician. Patients were given adequate time to review the patient information sheet and ask questions about any aspects of the study. Consent was obtained in the ward area of the Clinical Research Facility, University Hospital of Wales.

This informed consent was given by means of a standard written statement, written in non-technical language. The subject read and considered the statement before signing and dating it, and was given a copy of the signed document. If the subject could not read or sign the documents, oral presentation was made or signature given by the subject's legally appointed representative, if witnessed by a person not involved in the study, mentioning that the patient could not read or sign the documents. No patient could enter the study before his/her informed consent had been obtained.

### 4.3 Investigator variability

In order to determine intra-operator variability 20 fasted subjects underwent tonometry for Ai on 2 occasions 30 minutes apart. These subjects consisted of healthy individuals from the Centre for Endocrine and Diabetes Sciences at the University Hospital of Wales. Measurements took place either in the Clinical Research Facility at the University Hospital of Wales and were performed in duplicate (table 4.1).

| Subject | Gender | Age (yrs) | Height (cm) | Weight (kg) | BP (mmHg) | Ai 1 (%) | Ai 2 (%) |
|---|---|---|---|---|---|---|---|
| 1 | M | 37 | 166 | 70 | 127/84 | 22.5 | 22.5 |
| 2 | M | 35 | 175 | 71 | 120/81 | 15 | 15.5 |
| 3 | M | 42 | 180 | 102 | 132/74 | 21.5 | 22 |
| 4 | M | 38 | 175 | 71 | 128/64 | 15.5 | 12.5 |
| 5 | F | 40 | 156 | 59 | 127/81 | 24.5 | 28 |
| 6 | F | 56 | 163 | 57 | 138/88 | 28 | 28 |
| 7 | F | 37 | 160 | 53 | 121/94 | 30 | 26.5 |
| 8 | F | 29 | 160 | 69 | 119/88 | 17.5 | 16 |
| 9 | M | 33 | 173 | 80 | 120/80 | 5 | 3.5 |
| 10 | F | 31 | 160 | 78 | 132/78 | 30 | 28 |
| 11 | M | 35 | 173 | 78 | 134/83 | 15 | 14.5 |
| 12 | F | 36 | 158 | 75 | 118/75 | 29.5 | 27.5 |
| 13 | F | 38 | 158 | 80 | 144/104 | 40.5 | 38 |
| 14 | F | 40 | 161 | 58 | 98/70 | 16.5 | 16 |
| 15 | F | 34 | 146 | 74 | 116/75 | 26 | 25.5 |
| 16 | F | 37 | 164 | 123 | 131/68 | 16.5 | 18 |
| 17 | F | 28 | 165 | 83 | 141/93 | -4.5 | -6 |
| 18 | F | 20 | 167 | 78 | 132/73 | 15 | 18.5 |
| 19 | F | 22 | 180 | 69 | 122/67 | -8 | -5 |
| 20 | F | 26 | 167 | 72 | 110/71 | 11.5 | 10.5 |
| **Overall intra-operator variability is <1%** | | | | | | | |

Table 4.1 Ai in control subjects 30 minutes apart demonstrating overall investigator variability of less than 1%

### 4.4 Procedure for measurement of arterial stiffness and endothelial function

Ai, PWV and endothelial function were all determined using the SphygmoCor equipment (SphygmoCor model MM3, AtCor Medical Ltd, Sydney, Australia) and software (SphygmoCor version 8.0 for Microsoft Windows). Required input data entered into the software prior to pulse wave measurements were gender, height, weight (Tanita Body Fat analyser TBF-305, Tanita corporation, Japan), and brachial blood pressure (British Hypertensive Society approved Omron 705IT, Omron Matsusaka Co Ltd, Japan).

Ai was calculated by the SphygmoCor software from tonometry performed over the radial pulse. Recordings were repeated until 2 readings were obtained within 5% of each other with operator quality index over 75%. The 2 measurements collected were averaged prior to data entry.

The SphygmoCor system was used to determine pulse wave velocity [203] by recording the time point at which the reflected wave occurred in the waveform of a proximal artery (carotid) compared to a distal artery (radial or femoral) in milliseconds. The vessel length (in millimeters) was determined (radial – distance from sternal notch to radial pulse in an out-stretched arm; femoral - distance from suprasternal notch to umbilicus and umbilicus to femoral pulse) and then subtracted from the distance from the suprasternal notch to the carotid pulsation by the SphygmoCor software. The subject was then attached to the basic electrocardiogram (ECG) component of SphygmoCor MM3 and Ai readings taken firstly from the distal vessel (radial or femoral) and then the proximal vessel (carotid). The use of the ECG allows the system to ascertain precisely the point of cardiac contraction (taken to be the R wave peak) and this point is taken as waveform time zero. The software thus generated a pulse wave velocity for both the carotid-radial (brachial pulse wave velocity - bPWV) and carotid-femoral (aortic pulse wave velocity - aPWV) sites. Measurements were repeated until 2 recordings were obtained within 0.5 meters per second of each other that were then averaged prior to data entry.

The precise endothelial function assessment protocol was obtained form I.B. Wilkinson's group in Cambridge (Vascular Research Clinic, Addenbrooke's Hospital, Cambridge, UK) [298] and is described in appendix IV. The pharmacological agents utilised in this protocol were 400μg salbutamol (four of a 100μg metered inhaler dose) (an endothelial

dependent nitric oxide-mediated vasodilator) [Salamol CFC-free inhaler, IVAX Pharmaceuticals, London, UK] and 500μg glyceryl trinitrate (GTN) (an endothelial-independent vasodilator) [Alpharma, Barnstable, UK]. An estimation of endothelial function was established through the of generation Ai values from peripheral waveforms following treatment with these agents, the vasodilating effects of which decrease Ai correspondingly. The vasodilating action of GTN generated data corresponding to optimum dilating ability (by NO endothelial saturation) whereas the vasodilating action of salbutamol produced data corresponding to the ability of the endothelium to dilate given current endothelial microenvironment.

The effect of firstly GTN and then salbutamol on Ai was determined due to the slower clearance of salbutamol. Endothelial function was defined as the maximum change in Ai following salbutamol treatment occurring within 20 minutes of drug administration. Each Ai recording was repeated until 2 were obtained within 5% of each other with an operator quality index of over 75%. Measurements were averaged prior to data entry.

**4.5 Assay characteristics of biochemical analyses**

At each visit blood was collected for a number of measurements. All biochemical analysis was performed at the Department of Medial Biochemistry at the University Hospital of Wales, apart from adiponectin which was measured in-house using the ELISA method outlined in chapter 3.

DHEAS was assayed by electrochemiluminescent immunoassay on an automated system (Roche Codas E170 Immunoassay analyser). Insulin was measured by a solid phase two-site immunoassay (Mercodia Iso-insulin ELISA, Mercodia AB, Uppsala, Sweden).

SHBG was determined via an electrochemiluminescence immunoassay on an automated immunoassay system (Roche Elecsys 170 immunoassay analyser). LH and FSH were analysed via a two-site sandwich immunoassay on an automated analyser (ADVIA Centaur LH and FSH assay and Siemens Healthcare Diagnostics immunoassay analyser); the same analyser was used to measure testosterone through competitive immunoassay (ADVIA Centaur Testosterone assay). IGF-1 was measured using a chemiluminescent immunometric assay (IMMULITE® 1000 chemiluminescent immunoassay system, Diagnostic Products Corporation, Llanberis, Wales, UK).

Androstenedione was manually assayed in duplicate using the coat-a-count system (Diagnostic Products Corporation, Llanberis, Wales, UK). Glucose, liver function tests and serum lipids (total cholesterol, triglyerides, HDL and LDL cholesterol) were all measured using the AEROSET general chemistry photometric system (Abbott Diagnsotics, Berkshire, UK). HSCRP was assayed via nephelometry (BN™ II system, Dade Behring). PAI-1 was measured using a tissue plasminogen activator coated ELISA plate that assays only active PAI-1 (ZYMUTEST, Hyphen Biomed, Nodia, Amsterdam). The performance characteristics of each of these assays are summarised in table 4.2.

| Test | Coefficient of variation | Lower limit Of detection | Reference range |
|---|---|---|---|
| Insulin | 4.1-4.9% | 1 mU/L | N/A |
| IGF-1 | 5.8-8.4% | 20 ng/mL | Age and sex specific |
| Androstenedione | 3.2-15.6% | 0.14 nm/L | 4.4-10.6 nmol/L |
| Glucose | 1.5-2.2% | 0.1 mmol/L | ≥ 5.6 mmol/L (IDF diagnostic criteria for the metabolic syndrome see chapter 3) |
| Triglyerides | 0.8-1.1% | 0.07 mmol/L | 0.6-2.0 mmol/L |
| HDL | 5.5-1.4% | 0.13 mmol/L | 0.7-1.7 mmol/L |
| Cholesterol | 0.8-1.6% | 0.16 mmol/L | Evaluate in conjunction with other cardiac risk factors (BP, FMH, DM, smoking) |
| DHEAS | 2.3-3.3% | 0.1 µmol/L | Age and sex specific |
| SHBG | 1.1-4.0% | 0.35 nmol/L | Male 11.4-52.3 nmol/L<br>Female premenopausal 19.8-122 nmol/L<br>Female post menopausal 14.1-68.9 nmol/L |
| HSCRP | 2.5-4% | 0 mg/L | 0-3.0 mg/L |
| LH | 2.7-3.8% | 0.07 U/L | 1.5-9.3 U/L |
| FSH | 2.2-3.9% | 0.3 U/L | 1.4-18.1 U/L |
| Testosterone | 2.7-7.6% | 0.35 nmol/L | 8.0-30.3 nmol/L (male) 3nmol/L (females) |
| PAI-1 | not available | 0.5 ng/mL | < 5.0 ng/mL |

Table 4.2 Biochemical test characteristics
(BP-blood pressure, FMH-family history, DM-diabetes Mellitus)

# Chapter 5

# A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function and body composition in patients with primary and secondary adrenal insufficiency – results

## 5.1 Overview

From the currently available evidence the use of DHEA therapy, in the clinical setting at least, can only be justified in females with primary adrenal insufficiency/Addison's disease, although its use is generally restricted to individuals with reduced quality of life and/or complaints of a reduced libido [12, 13].

There is however a sufficient body of work that suggests that DHEA/DHEAS deficiency and therefore its replacement may have further beneficial effects relating to potential actions on adipose tissue [139-141, 152, 316] and the vasculature [180, 208, 210, 317]. Many of these studies have however been performed in the *in vitro* setting or in animal models and the human studies performed have often been in cases of physiological rather than pathological deficiency. These various studies have been reviewed earlier in this thesis and are supported by some of the *in vitro* data presented in chapters 2 and 3.

Extrapolation of data from *in vitro* and animal models to human physiology is associated with a number of potential problems. As previously discussed the high and fluctuating concentration of DHEA/S are only observed in humans and higher primates; hence supplementation of DHEA into animal feeds or culture conditions often generates non-physiological environments [56]. Furthermore any data generated from studies performed *in vitro* by definition removes material of interest from its native environment and in doing so prevents the occurrence of any *in vivo* co-activating or co-suppressing interactions. For these reasons it becomes necessary to confirm (or refute) any *in vitro* observations *in vivo*.

Addison's disease provides a particularly good vehicle with which to examine the impact of DHEA replacement as DHEA deficiency may potentially be the condition's sole

uncorrected hormonal abnormality, assuming optimal glucocorticoid and mineralocorticoid replacement and the correct treatment of any other associated autoimmune disease. Addison's disease is a relatively rare condition; however utlisation of a cross-over study design and recruiting subjects from a large tertiary referral endocrine centre, enabled sufficient recruitment and maximal power. Addison's disease was therefore chosen as the first group in which to evaluate the effect of DHEA replacement on vascular function and adiposity.

Secondary adrenal insufficiency is a component of hypopituitarism which is known to be associated with impaired survival as well as cardiovascular risk [244-246]. Unlike primary adrenal insufficiency, however, the aetiology of hypopituitarism is more heterogeneous resulting from an array of pathological processes. Nevertheless DHEA deficiency is a feature of secondary adrenal insufficiency although replacement with DHEA is not commonplace as previously discussed. The accumulation of adipose tissue and excessive vascular risk in the context of hypopituitarism may be secondary to a number of factors such as sub-physiological (thyroxine, growth hormone) or supra-physiological (cortisol) hormone replacement, hypothalamic appetite centre damage secondary to either disease processes or therapy/treatment (radiotherapy, surgery) in addition to DHEA deficiency.

### 5.1.1 The flow of patients through each study phase

Figure 5.1 summarises the flow of patients through each phase of the study.

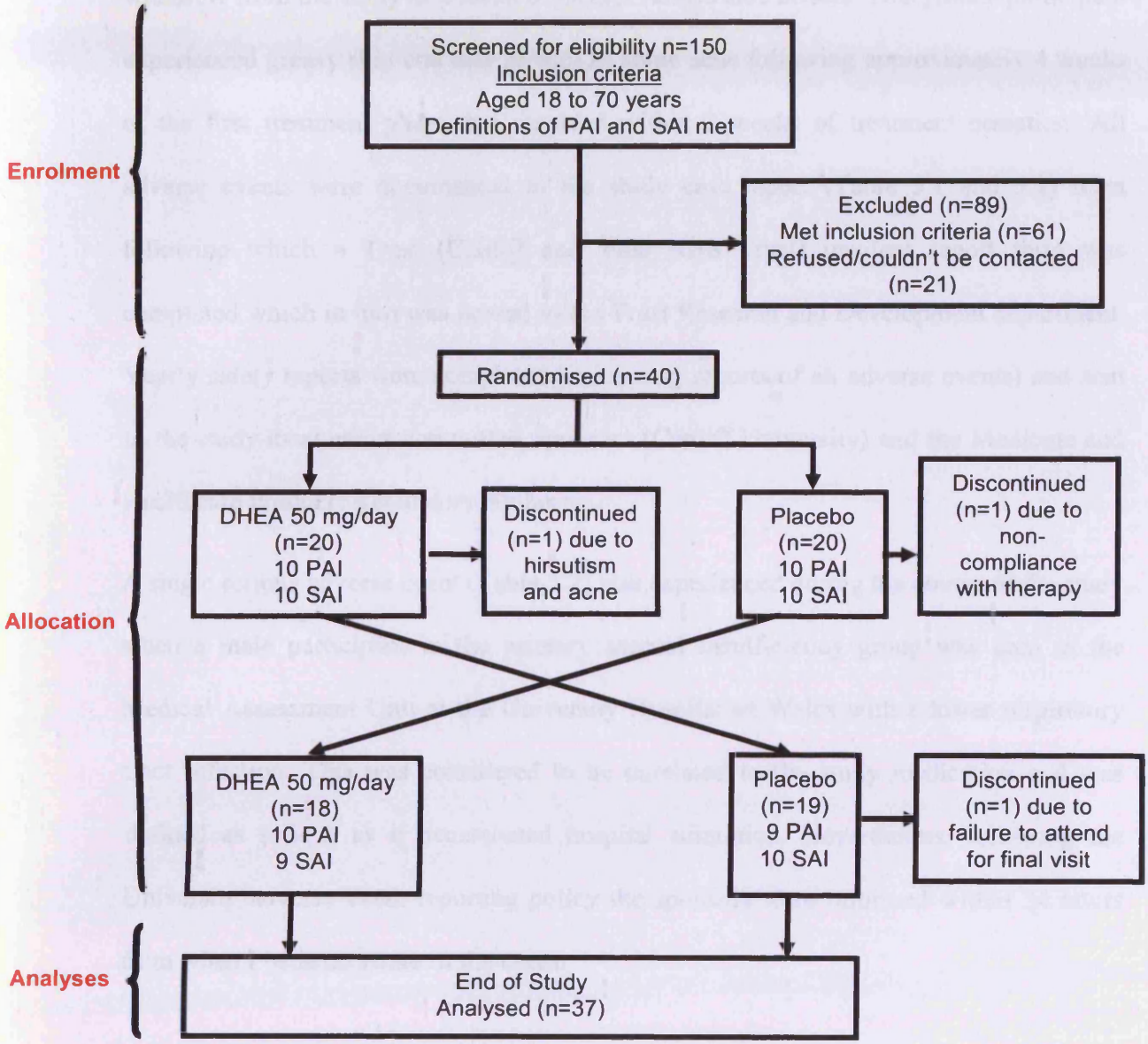



Fig 5.1 Flow of patients through each phase of the study

## 5.2 Safety, tolerability and compliance

Both the DHEA and placebo preparations were tolerated well and only one individual withdrew from the study as a result of DHEA related side effects. This female participant experienced greasy skin and hair as well as some acne following approximately 4 weeks of the first treatment phase that resolved within 2 weeks of treatment cessation. All adverse events were documented in the study case report (Table 5.1 and 5.2) form following which a Trust (Cardiff and Vale NHS Trust) incident report form was completed which in turn was passed to the Trust Research and Development department. Yearly safety reports were completed (containing reports of all adverse events) and sent to the study local ethics committee, sponsors (Cardiff University) and the Medicine and Healthcare Products Regulatory Authority.

A single serious adverse event (Table 5.2) was experienced during the course of the study when a male participant in the primary adrenal insufficiency group was seen in the Medical Assessment Unit at the University Hospital of Wales with a lower respiratory tract infection. This was considered to be unrelated to the study medication and was defined as serious as it necessitated hospital admission. Nevertheless, following the University adverse event reporting policy the sponsors were informed within 24 hours from when I became aware of the event.

A summary of the adverse events are outlined below:

| AE number | Patient ID | Description of event | Start Date | Finish Date | outcome | Intensity | Expectedness | Causality | Seriousness |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 101 | Acne Greasy skin | 24/9/06 | 5/10/06 | resolved | Mild | Expected | probably related | not serious |
| 2 | 102 | Rash | 10/12/06 | 1/04/07 | resolved | Mild | Unexpected | not related | not serious |
| 3 | 103 | Acne Greasy skin | 21/11/06 | 1/2/07 | resolved | Mild | Expected | probably related | not serious |
| 4 | 119 | Faulty pregnancy test kit | 30/5/07 | 19/6/07 | repeat test negative | Mild | Unexpected | not related | not serious |
| 5 | 115 | muscle ache | 26/7/07 | 6/8/07 | resolved | Mild | Unexpected | not related | not serious |
| 7 | 219 | abdominal cramps | 15/10/07 | 27/11/07 | resolved | Mild | Unexpected | not related | not serious |

Table 5.1 Summary of adverse events (n=6)

| AE number | Patient ID | Description of event | Start Date | Finish Date | outcome | Intensity | Expectedness | Causality | Seriousness |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 120 | Upper respiratory tract infection – seen in hospital and discharged | 31/10/7 | 31/10/7 | resolved | Moderate | Unexpected | not related | resulted in hospitalisation |

Table 5.2 Summary of serious adverse events (n=1)

Liver function tests were performed on study participants at each study visit and no increases were observed in these during the course of the study.

A further 2 individuals were withdrawn from the study due to protocol violations. One individual failed to attend for the final study visit due to work commitments and a second individual reported poor/variable compliance not only with the study medication but with other hormone replacements. Overall, however, compliance was good as DHEAS levels were found to be elevated in all but 2 subjects following the active treatment phase. One of these individuals was unable to have blood taken at the time of the visit and attended

10 days later, and the second individual had stopped taking the medication of her own accord 4 weeks prior to the study visit.

### 5.3.1 Primary adrenal insufficiency baseline demographic and anthropometric data

20 subjects with the diagnosis of Addison's disease were recruited into the study over a 9 month period. The table below describes the demographic and anthropometric baseline data of this group.

| Subject Number | Age (years) | Sex | Disease duration (years) | Height (cm) | Weight (Kg) | BMI ($Kg/m^2$) | % Fat | Waist (cm) | Hip (cm) |
|---|---|---|---|---|---|---|---|---|---|
| **101** | 52 | F | 20 | 157 | 81.0 | 33.0 | 44.5 | 99 | 116 |
| **102** | 47 | M | 15 | 179 | 83.9 | 26.2 | 23.0 | 96 | 109 |
| **103** | 59 | F | 25 | 159 | 72.0 | 28.5 | 39.5 | 94 | 104 |
| **104** | 67 | F | 18 | 160 | 71.0 | 27.7 | 38 | 90 | 104 |
| **105** | 57 | F | 7 | 151 | 65.0 | 28.5 | 35 | 85 | 103 |
| **106** | 68 | F | 22 | 153 | 78.0 | 33.3 | 45 | 100 | 116 |
| **107** | 30 | M | 4 | 177 | 83.0 | 26.5 | 25.5 | 99 | 98 |
| **108** | 50 | F | 2 | 161 | 74.0 | 28.6 | 40.5 | 97 | 106 |
| **109** | 39 | M | 23 | 168 | 104.0 | 36.9 | 45.5 | 126 | 118 |
| **110** | 40 | F | 12 | 167 | 95.0 | 34.1 | 48 | 89 | 125 |
| **111** | 44 | M | 12 | 175 | 77.0 | 25.1 | 18 | 85 | 95 |
| **112** | 62 | F | 37 | 153 | 51.0 | 21.8 | 28 | 79 | 90 |
| **113** | 50 | F | 22 | 165 | 68.1 | 25.0 | 36.5 | 81 | 102 |
| **114** | 52 | F | 16 | 156 | 80.8 | 33.2 | 44.5 | 100 | 113 |
| **115** | 39 | F | 2 | 173 | 60.3 | 20.2 | 24.5 | 83 | 93 |
| **116** | 38 | F | 7 | 155 | 87.0 | 36.2 | 47 | 105 | 113 |
| **117** | 61 | M | 22 | 178 | 79.3 | 25.0 | 22.5 | 96 | 97 |
| **118** | 19 | M | 1.5 | 170 | 52.1 | 18.0 | 6.5 | 73 | 83 |
| **119** | 24 | F | 14 | 168 | 102.7 | 36.4 | 48 | 99 | 125 |
| **120** | 67 | M | 51 | 183 | 107.6 | 32.1 | 29 | 114 | 123 |
| **MEAN** | **48.25** | **F 13 M 7** | **16.625** | **F 159.8 M 175.7** | **F 75.8 M 83.8** | **28.8** | **F 39.9 M 24.3** | **F 92.4 M 98.4** | **F 108.5 M 103.3** |

Table 5.3 Demographic and anthropometric data from subjects recruited into the study with PAI

The baseline characteristics indicate that the study cohort is representative of the general population with Addison's disease; the increased numbers of females is consistent with

the expected higher disease frequency in women. Five individuals were current smokers. Overall both males and females in the study population demonstrated a tendency for increased adiposity with the mean BMI within the overweight range. As expected, females were shorter, had a higher fat percentage, greater hip circumference and lower waist circumference than males.

### 5.3.2 Primary adrenal insufficiency baseline arterial stiffness and endothelial function data

| Subject Number | Sitting BP (mmHg) | Supine BP (mmHg) | Ai (%) | Central BP (mmHg) | bPWV (m/s) | aPWV (m/s) | Endothelial Function (% reduction in Ai post salbutamol) |
|---|---|---|---|---|---|---|---|
| **101** | 125/80 | 117/70 | 47.5 | 122/81 | 7.6 | 6.9 | -9 |
| **102** | 160/101 | 152/93 | 30 | 148/103 | 8.45 | 7.0 | -3 |
| **103** | 143/86 | 137/81 | 32 | 134/87 | 8.6 | 7.6 | -9 |
| **104** | 160/97 | 140/90 | 41 | 157/97 | 7.65 | 7.8 | -13.5 |
| **105** | 144/112 | 156/103 | 31.5 | 140/113 | 8.25 | 5.9 | -20.5 |
| **106** | 131/95 | 130/91 | 39.5 | 126/98 | 9.1 | 7.05 | -24 |
| **107** | 134/98 | 126/83 | 9 | 121/97 | 7.3 | 6.7 | -11 |
| **108** | 134/95 | 134/88 | 31.5 | 128/96 | 8.15 | 6.55 | -9 |
| **109** | 146/107 | 140/98 | 24.5 | 138/109 | 6.85 | 7.8 | -18 |
| **110** | 118/87 | 124/84 | 21.5 | 111/88 | 7.6 | 6.4 | -9.5 |
| **111** | 141/90 | 125/83 | 28 | 133/91 | 6.9 | 7.35 | -18.5 |
| **112** | 125/70 | 137/98 | 33 | 116/72 | 9.55 | 8.6 | -17.5 |
| **113** | 145/97 | 136/92 | 36.5 | 138/97 | 8.05 | 7.25 | -33.5 |
| **114** | 107/67 | 101/64 | 31.5 | 100/68 | 8.6 | 4.95 | -9.5 |
| **115** | 140/95 | 134/98 | 36.5 | 135/96 | 10.6 | 7.1 | -12.5 |
| **116** | 112/83 | 114/86 | 25.5 | 106/84 | 6.6 | 6.35 | -13 |
| **117** | 125/75 | 130/76 | 18.5 | 110/74 | 8.1 | 8.3 | -9 |
| **118** | 135/96 | 135/79 | 1 | 120/98 | 7.65 | 4.6 | -7 |
| **119** | 136/87 | 131/86 | 16.5 | 119/87 | 7.9 | 5.5 | -11 |
| **120** | 163/102 | 147/89 | 29.5 | 153/103 | 11.4 | 11.2 | -4 |
| **MEAN** | **136.3/91** | **132.3/86.6** | **28.2** | **127.8/92** | **8.2** | **7.0** | **-13.1** |

Table 5.4 Baseline arterial stiffness and endothelial function data in the PAI group

Half of the individuals had peripheral blood presures above or equal to that defined as hypertension in the IDF definition of the metabolic syndrome (≥138/85). The mean Ai of

28.2% was approximately 10% higher than that found in an as yet unpublished reference population (Cockcroft J, Cardiff, UK) of 405 individuals (239 males, 166 females) with the same mean age.

### 5.3.3 Primary adrenal insufficiency baseline biochemical data

Table 5.5 summarises the baseline biochemical, metabolic and other characteristics of the PAI cohort.

| Subject Number | DHEAS (µmol/l) | Testosterone (nmol/l) | HSCRP (mg/l) | Fasting glucose (mmol/l) | Fasting lipids | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | TC (mmol/l) | HDL (mmol/l) | LDL (mmol/l) | TAG (mmol/l) |
| **101** | 0.2 | 0.7 | 6.14 | 4.6 | 8.7 | nd | nd | 1.7 |
| **102** | 1.3 | 10.4 | 2.12 | 4.7 | 7.9 | nd | nd | 1.7 |
| **103** | 0.2 | 0.7 | 1.15 | 5.4 | 5.9 | nd | nd | 2.2 |
| **104** | 0.5 | 0.7 | 1.11 | 5.1 | 6.0 | nd | nd | 1.9 |
| **105** | 0.5 | 0.7 | 0.47 | 4.7 | 7.1 | nd | nd | 0.9 |
| **106** | 0.2 | 0.7 | 2.41 | 5.5 | 7.7 | nd | nd | 1.5 |
| **107** | 2.9 | 13.8 | 1.2 | 5.5 | 4.9 | 1.5 | 5.5 | 1.2 |
| **108** | 0.0 | 0.7 | 0.7 | 6.5 | 4.2 | 1.3 | 3.1 | 2.1 |
| **109** | 0.5 | 11.5 | 13.1 | 5.3 | 4.8 | 1 | 2.2 | 0.9 |
| **110** | 0.8 | 0.9 | 1.49 | 5.6 | 5.1 | 0.9 | 3.5 | 0.7 |
| **111** | 1.0 | 10.6 | 0.69 | 5.5 | 3.9 | 1.2 | 3.2 | 1.4 |
| **112** | 0.5 | 0.7 | 1.23 | 5.3 | 4.4 | 1.9 | 2.1 | 1.1 |
| **113** | 0.5 | 0.7 | 5.62 | 6.3 | 7.0 | 2 | 2.0 | 0.7 |
| **114** | 0.5 | 0.7 | 2.38 | 5.5 | 5.6 | 1.3 | 4.7 | 1.0 |
| **115** | 2.2 | 1.9 | 2.39 | 5.4 | 5.1 | 1.8 | 3.8 | 1.2 |
| **116** | 0.2 | 0.7 | 3.47 | 4.9 | 8.3 | 1.4 | 5.6 | 2.8 |
| **117** | 0.8 | 11.4 | 0.8 | 5.5 | 6.6 | 1.3 | 4.0 | 2.8 |
| **118** | 3.4 | 25.7 | 0.16 | 4.5 | 4.6 | 1.5 | 2.6 | 1.2 |
| **119** | 2.0 | 2.1 | 2.47 | 4.5 | 4.3 | 1.3 | 2.4 | 1.4 |
| **120** | 0.6 | 8.4 | 0.7 | 4.6 | 4.1 | 1.2 | 2.5 | 0.9 |
| **MEAN** | **0.96** | ♂ **13.1** ♀ **0.81** | **3.04** | **5.25** | **5.34** | **1.4** | **3.37** | **1.36** |

Table 5.5 Baseline biochemical data in study subjects with PAI (nd - not done)

40% of subjects within this study group met the diagnostic criteria for the metabolic syndrome; approximately double the estimated population prevalence (www.idf.org).

### 5.3.4 Primary adrenal insufficiency baseline data correlations

Although the number of cases is comparatively low (n=20) significant correlations (Pearson's) were confirmed between Ai and age (p=0.001) and Ai and height (p=0.05) (Figs 5.2).

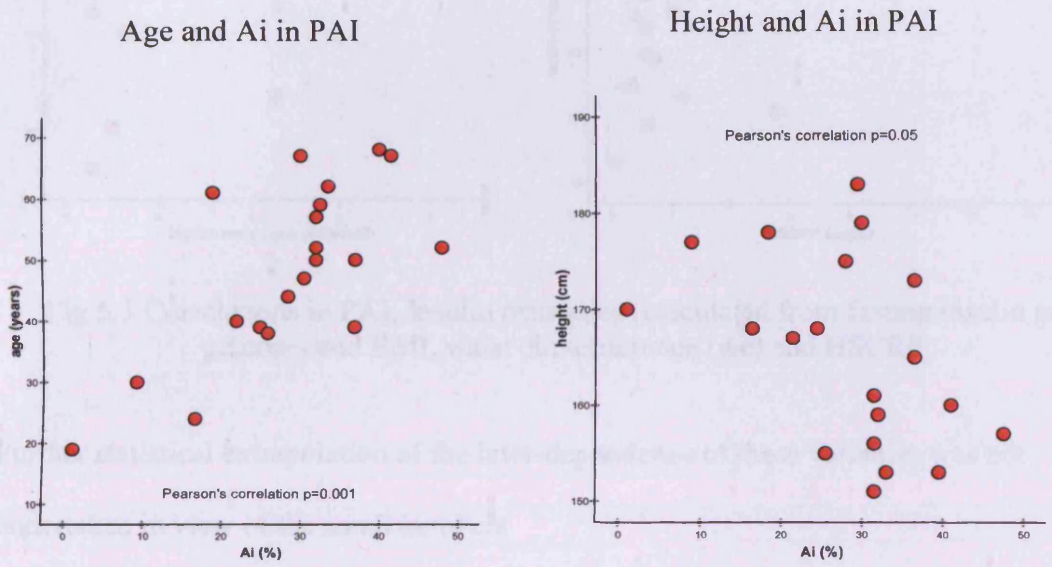
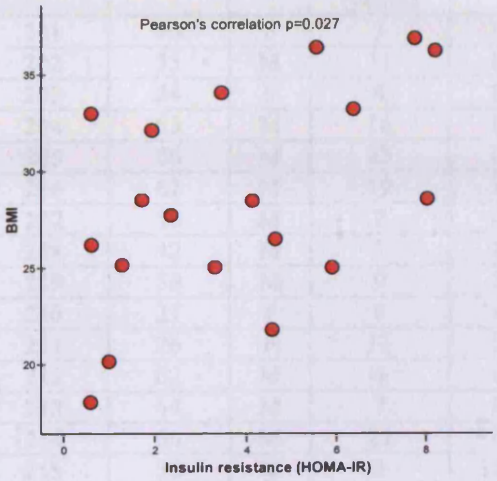



Fig 5.2 Patients with PAI Ai correlations with age and hight

Furthermore, as expected measures of body fat (BMI, percentage fat) correlated positively (but not strongly) with insulin resistance (HOMA-IR) (Correlation with BMI shown in Fig 5.3). A positive correlation between waist circumference and HSCRP was also observed (Fig 5.3).

BMI and insulin resistance in PAI




wc and HSCRP in PAI

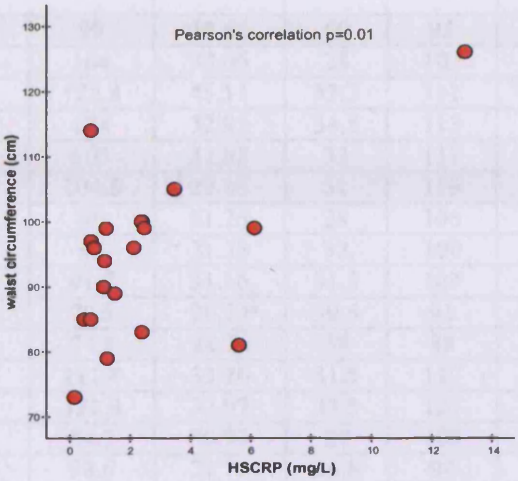



Fig 5.3 Correlations in PAI, Insulin resistance (calculated from fasting insulin and glucose) and BMI, waist circumference (wc) and HSCRP

Further statistical extrapolation of the inter-dependence of these variables was not undertaken in view of the small numbers

### 5.3.5 Secondary adrenal insufficiency baseline demographic and anthropometric data

20 subjects with the diagnosis of hypopituitarism secondary to either non-functioning pituitary tumors or craniopharyngiomas treated surgically with or without concomitant radiotherapy on full hormone replacement therapy including growth hormone were recruited into the study over a 9 month period. The table below shows the demographic and anthropometric baseline data of this group.

| Subject Number | Age (years) | Sex | Disease duration (years) | Height (cm) | Weight (Kg) | BMI ($Kg/m^2$) | % Fat | Waist (cm) | Hip (cm) |
|---|---|---|---|---|---|---|---|---|---|
| **201** | 40 | F | 3 | 158 | 99 | 39.66 | 50 | 95 | 139 |
| **202** | 35 | M | 11 | 186 | 104 | 30.06 | 28 | 102 | 110 |
| **203** | 24 | F | 4 | 165 | 122.8 | 45.11 | 53.3 | 131 | 148 |
| **204** | 63 | M | 16 | 194 | 124 | 32.95 | 34.5 | 113 | 114 |
| **205** | 66 | M | 33 | 177 | 103 | 32.88 | 33 | 111 | 109 |
| **206** | 62 | M | 19 | 188 | 104.8 | 29.65 | 31 | 119 | 120 |
| **207** | 50 | M | 7 | 178 | 99 | 31.25 | 28 | 106 | 107 |
| **208** | 42 | M | 7 | 174 | 95 | 31.38 | 32 | 100 | 108 |
| **209** | 58 | M | 9 | 171 | 91.7 | 31.36 | 31.5 | 103 | 107 |
| **210** | 21 | F | 8 | 158 | 70.5 | 28.24 | 39.5 | 91 | 100 |
| **211** | 26 | F | 11 | 161 | 74.5 | 28.74 | 38 | 88 | 98 |
| **212** | 62 | M | 4 | 183 | 111.4 | 33.26 | 31.5 | 112 | 108 |
| **213** | 64 | M | 7 | 193 | 122.8 | 32.97 | 33.5 | 125 | 127 |
| **214** | 60 | M | 21 | 165 | 84.2 | 30.93 | 27 | 109 | 106 |
| **215** | 38 | M | 4 | 188.5 | 98.6 | 27.75 | 26.8 | 94 | 108 |
| **216** | 70 | M | 15 | 186 | 97.2 | 28.01 | 25 | 106 | 102 |
| **217** | 18 | F | 12 | 162 | 53.6 | 20.42 | 35 | 89 | 90 |
| **218** | 63 | F | 12 | 156 | 92.8 | 38.13 | 50.5 | 117 | 124 |
| **219** | 37 | F | 29 | 158 | 98.6 | 39.44 | 45.5 | 126 | 138 |
| **220** | 48 | F | 13 | 170 | 74 | 25.6 | 39.5 | 93 | 107 |
| **MEAN** | **47.35** | **F 8 M 12** | **12.25** | **F 161 M 182** | **F 85.7 M 103** | **31.9** | **F 43.9 M 30.2** | **F 103.8 M 108.3** | **F 118 M 110.5** |

Table 5.6 Baseline demographic and anthropometric characteristics of subjects recruited with SAI

More males were recruited into the SAI group. As expected the males were taller, heavier and had greater waist circumference; hip circumference, fat percentage and BMI were again greater in females. There was a high prevalence of obesity (mean BMI of 31.9). Three individuals within this group were current smokers.

### 5.3.6 Secondary adrenal insufficiency baseline arterial stiffness and endothelial function data

| Subject Number | Sitting BP (mmHg) | Supine BP (mmHg) | Ai (%) | Central BP (mmHg) | bPWV (m/s) | aPWV (m/s) | Endothelial Function (% reduction in Ai post salbutamol) |
|---|---|---|---|---|---|---|---|
| **201** | 134/91 | 129/99 | 27.5 | 124/91 | 7.85 | 5.8 | -17 |
| **202** | 157/91 | 140/82 | 12 | 137/93 | 6.65 | 7 | -9 |
| **203** | 156/96 | 165/106 | 30.5 | 143/100 | 9.4 | 5.85 | -9 |
| **204** | 115/79 | 103/61 | 24.5 | 107/80 | 6.1 | 5.95 | -4 |
| **205** | 141/90 | 142/81 | 35 | 134/90 | 7.8 | 8.2 | -8 |
| **206** | 138/106 | 134/80 | 24 | 131/107 | 7.8 | 7.95 | -14.5 |
| **207** | 134/83 | 145/78 | -5.5 | 133/85 | 6.75 | 6 | 1.5 |
| **208** | 146/94 | 130/82 | 17.5 | 131/96 | 7.8 | 6.4 | -5 |
| **209** | 156/89 | 161/98 | 26 | 142/91 | 8.1 | 9.8 | -9.5 |
| **210** | 118/70 | 122/72 | -10.5 | 98/71 | 6.05 | 4.25 | -20 |
| **211** | 126/84 | 112/68 | 5.5 | 111/85 | 6.7 | 4.8 | -12 |
| **212** | 183/107 | 186/107 | 26.5 | 168/108 | 7.75 | 8.3 | -0.5 |
| **213** | 164/101 | 155/96 | 29.5 | 156/101 | 7.65 | 6.1 | -10 |
| **214** | 150/100 | 145/97 | 29 | 141/102 | 6.85 | 8 | -7 |
| **215** | 125/83 | 117/73 | 8 | 111/82 | 7.5 | 7.65 | 0 |
| **216** | 160/93 | 147/88 | 30 | 150/94 | 8 | 9.1 | -18 |
| **217** | 114/74 | 107/65 | 21.5 | 104/75 | 6.2 | 4.95 | -49.5 |
| **218** | 96/78 | 136/63 | 21 | 91/78 | 6.15 | Nd | -5.5 |
| **219** | 139/79 | 132/79 | 25 | 125/81 | 6.75 | Nd | -14.5 |
| **220** | 117/81 | 115/70 | 28 | 111/79 | 8.15 | 7.75 | -10.5 |
| **MEAN** | **138.5/88.5** | **136.2/82.3** | **20.25** | **127.4/89.4** | **7.3** | **6.88** | **-11.1** |

Table 5.7 Baseline arterial stiffness and endothelial function data in the SAI group (nd - not done)

40% of the recruited subjects demonstrated overall peripheral BPs above or equal to that defined as hypertension in the IDF definition of the metabolic syndrome (≥138/85). The mean Ai of 20.25 was higher than that found in an unpublished reference population but Ai, aPWV and bPWV were all marginally lower (slower) than those observed in the PAI group; this is likely a reflection of an increased number of males and thus overall height in the SAI group.

### 5.3.7 Secondary adrenal insufficiency baseline biochemical data

The table below summarises the baseline biochemical and other characteristics of the SAI cohort.

| Subject Number | DHEAS (mmol/l) | Testosterone (nmol/l) | HSCRP (mg/l) | Fasting glucose (mmol/l) | Fasting lipids | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | TC (mmol/l) | HDL (mmol/l) | LDL (mmol/l) | TAG (mmol/l) |
| **201** | 0.1 | 0.7 | 10.8 | 4.7 | 3.9 | 1.0 | 2.3 | 1.4 |
| **202** | 3.3 | 21 | 0.68 | 5.6 | 6.9 | 1.0 | 4.8 | 2.4 |
| **203** | 0.9 | 0.7 | 22.2 | 4.9 | 6.0 | 1.2 | 4.3 | 1.2 |
| **204** | 0.1 | 20 | 0.77 | 5.9 | 4.8 | 0.8 | 3.0 | 2.2 |
| **205** | 0.1 | 6.4 | 0.88 | 5.3 | 5.0 | 1.3 | 3.2 | 1.2 |
| **206** | 0.5 | 0.7 | 8.24 | 4.7 | 5.1 | 1 | 2.8 | 2.8 |
| **207** | 0.4 | 8.4 | 9.0 | 6.8 | 3.8 | 0.9 | 2.3 | 1.4 |
| **208** | 0.4 | 33.9 | 3.33 | 5.4 | 4.7 | 1.4 | 2.9 | 0.8 |
| **209** | 0.1 | 5.9 | 7.7 | 4.5 | 6.4 | 1 | 4.5 | 2 |
| **210** | nd | nd | Nd | nd | nd | nd | nd | nd |
| **211** | 0.1 | 0.7 | 7.44 | 4.3 | 5.6 | 1.4 | 3.8 | 0.9 |
| **212** | 0.6 | 7.4 | Nd | 11.3 | 5.1 | 0.7 | 3.1 | 3 |
| **213** | 0.3 | 27.3 | 0.5 | 4.7 | 4.5 | 0.7 | 3.2 | 1.3 |
| **214** | 0.2 | 10.4 | 0.67 | 5.1 | 4.6 | 1.3 | 2.6 | 1.6 |
| **215** | 0.4 | 25.7 | 1.0 | 4.6 | 5.6 | 1.1 | 4.2 | 0.6 |
| **216** | 0.4 | 32 | 0.24 | 5.4 | 4.5 | 1.0 | 3.0 | 1.2 |
| **217** | 0.9 | 0.7 | 2.23 | 4.8 | 7.3 | 1.7 | 5.2 | 0.8 |
| **218** | 0.2 | 0.7 | 1.3 | 4.3 | 3.6 | 0.9 | 1.5 | 2.7 |
| **219** | 0.2 | 0.7 | 3.91 | 7.6 | 4.8 | 1.2 | 2.1 | 3.4 |
| **220** | 0.2 | 0.7 | 0.93 | 5.0 | 7.4 | 1.5 | 5.2 | 1.5 |
| **MEAN** | **0.49** | ♂ **16.5**<br>♀ **0.70** | **4.55** | **5.52** | **5.24** | **1.11** | **3.37** | **1.71** |

Table 5.8 Baseline biochemical data collected in study subjects with SAI (nd – not done)

Based on the IDF 2005 criteria 55% of this study population met the diagnostic criteria for the metabolic syndrome (see chapter 3); this is over double what would be expected in the background population (www.idf.org) and is slightly greater than that demonstrated in the PAI group.

### 5.3.8 Secondary adrenal insufficiency baseline data correlations

As in the analysis of the PAI baseline data the number of cases is small (n=20) and must be considered when interpreting the correlation data. However a number of parameters demonstrated significant correlations (Pearson's correlation).

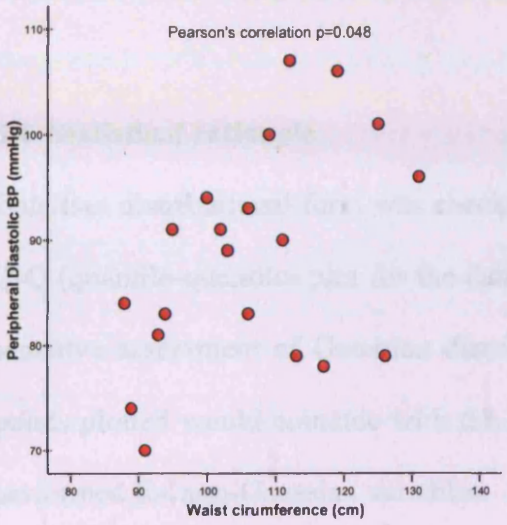
Significant correlations (Pearson's) in metabolic syndrome parameters in study subjects with SAI at baseline

| | Age | Sex | BMI | %fat | wc | dia BP | Cdia BP | Insulin | HSCRP | HOMA | HDL | LDL | aPWV | Ai |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age** | | - | - | - | 0.03 | - | - | - | - | - | - | - | 0.001 | 0.024 |
| **Sex** | - | | - | - | - | - | - | - | - | - | - | - | 0.006 | - |
| **BMI** | - | - | | - | - | - | - | - | 0.013 | - | - | - | - | - |
| **%fat** | - | - | - | | - | - | - | - | 0.024 | - | - | - | 0.034 | - |
| **wc** | 0.03 | - | - | - | | 0.048 | 0.032 | 0.013 | - | 0.008 | 0.032 | 0.021 | - | - |
| **diaBP** | - | - | - | - | 0.048 | | - | - | - | - | - | - | - | - |
| **CdiaBP** | - | - | - | - | 0.032 | - | | - | - | - | - | - | - | 0.035 |
| **Insulin** | - | - | - | - | 0.013 | - | - | | - | - | - | - | - | - |
| **HSCRP** | - | - | 0.013 | 0.024 | - | - | - | - | | - | - | - | - | - |
| **HOMA** | - | - | - | - | 0.008 | - | - | - | - | | - | - | - | - |
| **HDL** | - | - | - | - | 0.032 | - | - | - | - | - | | - | - | - |
| **LDL** | - | - | - | - | 0.021 | - | - | - | - | - | - | | - | - |
| **aPWV** | 0.001 | 0.006 | - | 0.034 | - | - | - | - | - | - | - | - | | - |
| **Ai** | 0.024 | - | - | - | - | - | 0.035 | - | - | - | - | - | - | |

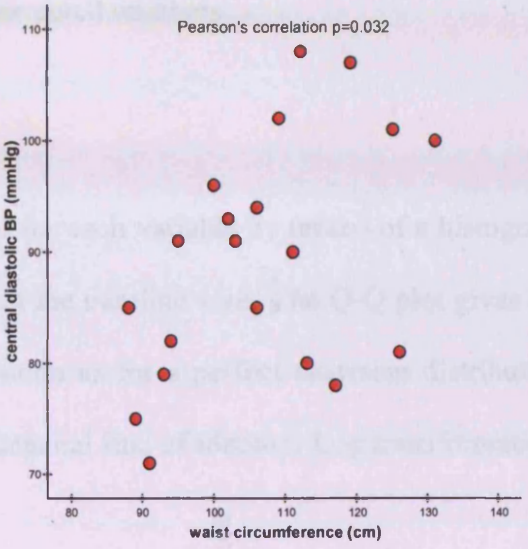
Table 5.9 Significant baseline data correlations (Pearson's correlation) expressed as p-values in study subjects with SAI

Waist circumference (wc), as a measure of central (visceral) adiposity correlates positively with age, peripheral and central diastolic blood pressure, insulin and insulin resistance and negatively with HDL (Fig 5.4 shows graphical examples).

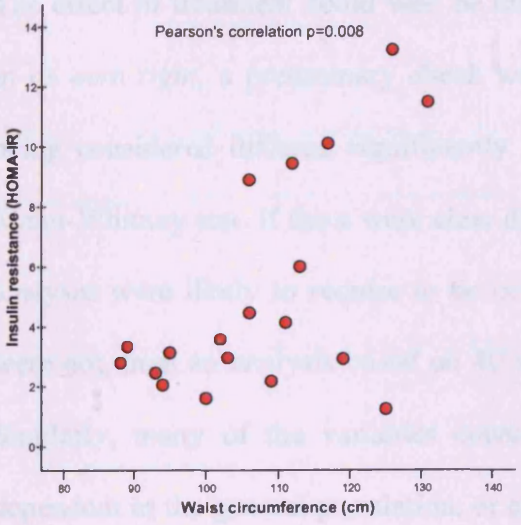
wc and peripheral diastolic BP




wc and central diastolic BP




wc and insulin resistance




wc and HDL

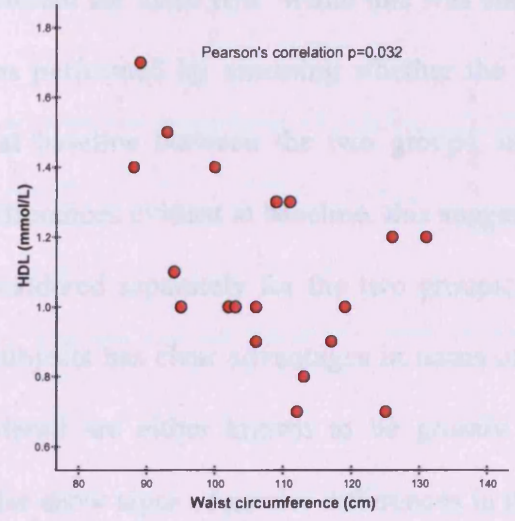



Fig 5.4 Correlations (Pearson's) with waist circumference at baseline in study subjects with SAI (wc – waist circumference, HDL – high density lipoprotein)

Fat percentage also correlated with HSCRP and as anticipated, measures of arterial stiffness (aPWV) correlated positively with age and sex though negatively correlation

with fat percentage. Again, further statistical exploration of the inter-dependence of these variables was not undertaken in view of the small numbers.

### 5.4 Statistical rationale

Gaussian distributional form was checked for each variable by means of a histogram and Q-Q (quantile-quantile) plot for the data at the baseline visit. The Q-Q plot gives a more sensitive assessment of Gaussian distribution as for a perfect Gaussian distribution the points plotted would coincide with the diagonal line of identity. Log transformation was performed for non-Gaussian variables.

The total study population comprised 40 individuals, half of whom had PAI and half SAI. The effect of treatment could well be different for these two. While this was considered in its own right, a preliminary check was performed by assessing whether the variable being considered differed significantly at baseline between the two groups, using the Mann-Whitney test. If there were clear differences evident at baseline, this suggested that analyses were likely to require to be considered separately for the two groups; if there were not, then an analysis based on 40 subjects has clear advantages in terms of power. Similarly, many of the variables considered are either known to be grossly gender-dependent in the general population, or else show signs of gender differences in the study population. This would suggest splitting by gender also but analyses split by group and gender simultaneously would have grossly inadequate power. To decide for each variable whether to split by group and/or gender, we took into account both the evidence from the study data and our prior knowledge concerning the variable in question.

### 5.5 Effects on primary end points - vascular parameters

A summary of the vascular outcomes of the entire cohort is given in Table 5.10. From these results it is clear that DHEA therapy did not improve any measure of arterial stiffness or endothelial function, although results for both brachial pulse wave velocity and Tr (time of travel of the reflected wave) almost reached significance with p-values of 0.07 and 95% confidence intervals which just include the null hypothesis value of zero. If confirmed in larger trials, an increase in Tr and reduced bPWV would both be consistent with reduced regional arterial stiffness in the brachial tree. However, the absence of any change in Ai suggests that wave reflection is not significantly affected. Furthermore, carotid-femoral pulse wave velocity (aPWV), which has the greatest amount of epidemiological evidence for its predictive value for cardiovascular events, did not change following DHEA treatment. There were no changes in peripheral or central systolic or diastolic blood pressure. Furthermore, the magnitude of reduction in Ai post-GTN, a direct endothelium-independent smooth muscle vasodilator, was unaffected by DHEA compared with placebo. Similarly, there were no changes following salbutamol in this parameter, suggesting that endothelial function was not affected by DHEA treatment.

Vascular outcomes of the entire study cohort

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Tr (milliseconds) | +3.7 | -0.25 to +7.61 | 0.066 |
| bPWV(metres/second) | -0.37 | -0.72 to +0.01 | 0.068 |
| aPWV (metres/second) | +0.48 | -0.33 to -0.24 | 0.753 |
| Ai (%) | +0.16 | -2.55 to +2.86 | 0.907 |
| Peripheral systolic BP (mmHg) | +0.22 | -3.64 to +4.08 | 0.909 |
| Peripheral diastolic BP (mmHg) | +0.43 | -2.13 to +3.0 | 0.734 |
| Central systolic BP (mmHg) | +0.52 | -4.6 to +2.68 | 0.594 |
| Central diastolic BP (mmHg) | -0.19 | -2.91 to +2.52 | 0.886 |
| Ai post-GTN (%) | +0.83 | -1.6 to +3.26 | 0.493 |
| Ai post-salbutamol (%) | -1.8 | -4.86 to + 1.25 | 0.239 |

Table 5.10. Entire cohort vascular data. Tr, time of travel of the reflected wave; bPWV, brachial pulse wave velocity; aPWV, aortic pulse wave velocity; Ai, augmentation index. Data shown are for mean difference, 95% confidence interval for the difference (CI) and p-value

Stratification of the data by study group did not show any differences in baseline values between subjects with PAI and SAI for Tr ($p = 0.33$), aPWV ($p = 0.83$), systolic peripheral blood pressure ($p = 0.76$), systolic diastolic blood pressure ($p = 0.34$), central systolic blood pressure ($p = 0.99$), central diastolic blood pressure ($p = 0.38$), Ai post-GTN ($p = 0.07$) or Ai post salbutamol ($p = 0.2$). However there was evidence for a difference between groups for bPWV ($p = 0.009$) and for Ai ($p = 0.02$). Nevertheless, analyses of these and other vascular end points stratified by study group (Table 5.11) did not show any changes in vascular outcome with DHEA therapy apart from minor changes in peripheral and central systolic blood pressure (Fig 5.5). However, the reduction seen in peripheral systolic blood pressure and central systolic blood pressure with DHEA in the SAI group and increase in peripheral systolic blood pressure in subjects with Addison's disease should be interpreted with caution given the wide confidence intervals evident.

Furthermore, because peripheral systolic blood pressure is a major input variable in calculation of central systolic blood pressure, it is clear that change in peripheral blood pressure would appear to be the principal driver of the reduced central systolic BP given that Ai did not change.

Change in vascular outcomes stratified by group

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Tr (milliseconds) | | | |
| PAI | +2.75 | -3.35 to +8.85 | 0.352 |
| SAI | +4.42 | -0.83 to 9.67 | 0.093 |
| bPWV(metres/second) | | | |
| PAI | -0.42 | -1.08. to +0.24 | 0.199 |
| SAI | -0.31 | -0.74 to +0.12 | 0.139 |
| aPWV (metres/second) | | | |
| PAI | +0.02 | -0.4 to +0.45 | 0.906 |
| SAI | -0.07 | -0.47 to +0.32 | 0.689 |
| Ai (%) | | | |
| PAI | +1.75 | -2.19 to +5.68 | 0.357 |
| SAI | -1.39 | -5.38 to 2.59 | 0.467 |
| Peripheral systolic BP (mmHg) | | | |
| PAI | +6.34 | +0.84 to +11.58 | 0.027 |
| SAI | -6.08 | -10.63 to -1.52 | 0.012 |
| Peripheral diastolic BP (mmHg) | | | |
| PAI | +3.27 | -0.3 to 6.85 | 0.07 |
| SAI | -2.23 | -5.7 to 1.23 | 0.184 |
| Central systolic BP (mmHg) | | | |
| PAI | +4.65 | -0.85 to +10.15 | 0.089 |
| SAI | -6.66 | -10.74 to -2.59 | 0.003 |
| Central diastolic BP (mmHg) | | | |
| PAI | +2.27 | -1.8 to +6.35 | 0.256 |
| SAI | -2.66 | -6.42 to +1.09 | 0.151 |
| Ai post-GTN (%) | | | |
| PAI | +1.6 | -2.43 to +5.63 | 0.402 |
| SAI | -0.08 | -3.99 to 3.84 | 0.967 |
| Ai post-salbutamol (%) | | | |
| PAI | -2.28 | -7.98 to +3.41 | 0.407 |
| SAI | -1.47 | -4.91 to +1.98 | 0.369 |

Table 5.11 Vascular data stratified by study group. Tr, time of travel of the reflected wave; bPWV, brachial pulse wave velocity; aPWV, aortic pulse wave velocity; Ai, augmentation index. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Stratification of the data by gender did not show any differences in baseline values between male and female subjects for bPWV ($p = 0.35$), or Ai post-GTN ($p = 0.89$). However there was evidence for a difference between genders for Tr ($p = 0.02$) aPWV ($p = 0.02$) Ai ($p = 0.04$), peripheral systolic blood pressure ($p = 0.004$) peripheral diastolic blood pressure ($p = 0.02$), central systolic blood pressure ($p = 0.02$) central diastolic blood pressure ($p = 0.02$), and Ai post salbutamol ($p = 0.003$). Nevertheless, analyses of these and the other vascular end points stratified by gender (Table 5.12) did not show any changes in vascular outcome with DHEA therapy.

Change in vascular parameters stratified by gender

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Tr (milliseconds) | | | |
| Male | +5.69 | -1.28 to +12.66 | 0.096 |
| Female | +2.62 | -4.66 to 9.9 | 0.442 |
| bPWV(metres/second) | | | |
| Male | -0.14 | -0.64 to +0.36 | 0.538 |
| Female | -0.64 | -1.32 to +0.03 | 0.061 |
| aPWV (metres/second) | | | |
| Male | +0.24 | -0.72 to +0.24 | 0.301 |
| Female | +0.19 | -0.19 to +0.57 | 0.28 |
| Ai (%) | | | |
| Male | -0.6 | -7.23 to +6.02 | 0.835 |
| Female | +0.3 | -2.83 to +3.43 | 0.842 |
| Peripheral Systolic BP (mmHg) | | | |
| Male | -1.17 | -7.34 to +5.01 | 0.687 |
| Female | +2.39 | -4.88 to +9.67 | 0.472 |
| Peripheral Diastolic BP (mmHg) | | | |
| Male | +0.79 | -4.4 to +5.98 | 0.733 |
| Female | +0.26 | -3.69 to +4.2 | 0.891 |
| Systolic central BP (mmHg) | | | |
| Male | -2.33 | -8.24 to +3.58 | 0.411 |
| Female | +0.55 | -6.31 to +7.42 | 0.855 |
| Diastolic central BP (mmHg) | | | |
| Male | +0.88 | -4.46 to +6.21 | 0.713 |
| Female | -1.52 | -6.1 to +3.05 | 0.48 |
| Ai post-GTN (%) | | | |
| Male | -0.56 | -2.93 to +1.8 | 0.621 |
| Female | +2.39 | -2.39 to +7.17 | 0.294 |
| Ai post-salbutamol (%) | | | |
| Male | -1.9 | -7.2 to +3.6 | 0.345 |
| Female | -1.8 | -7.9 to +4.2 | 0.522 |

Table 5.12 Vascular data stratified by gender. Tr, time of travel of the reflected wave; bPWV, brachial pulse wave velocity; aPWV, aortic pulse wave velocity; Ai, augmentation index. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Effects of supplementing DHEA on central systolic blood pressure in PAI and SAI

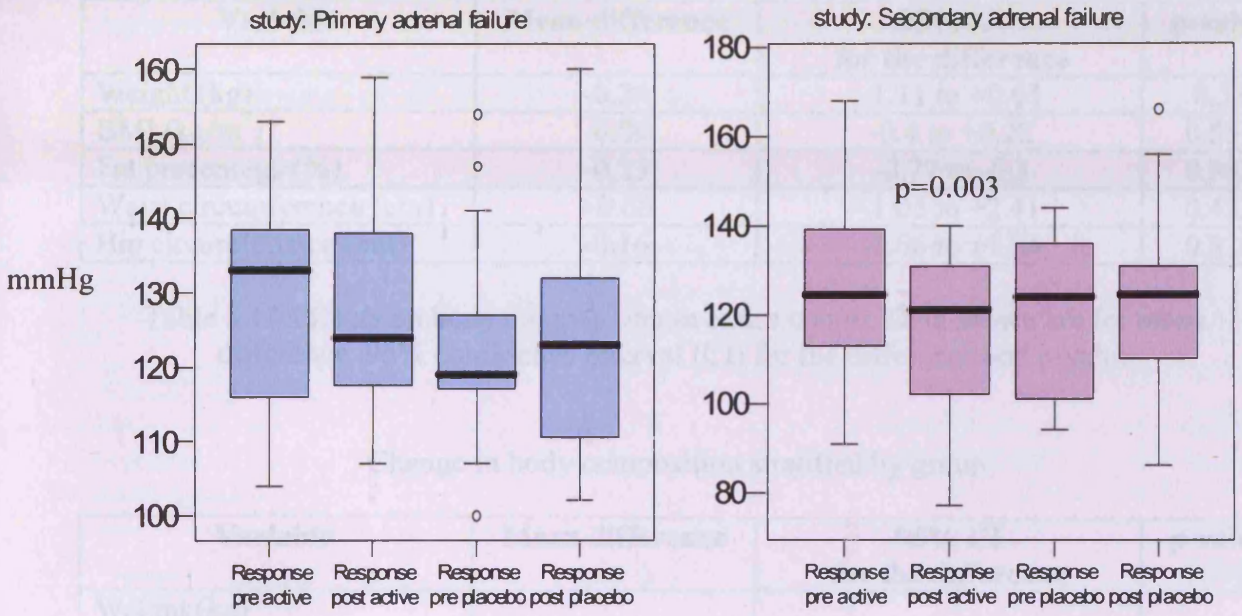



Fig 5.5 Change in central systolic blood pressure following 12 weeks of DHEA/placebo treatment in PAI and SAI.

## 5.6 Effects on secondary end points

### 5.6.1 Body composition

A summary of the effects on body composition for the entire cohort is given in Table 5.13. From these results it is clear that DHEA therapy did not affect weight, body mass index, fat percentage, waist circumference or hip circumference. Stratification of the data by study group did not show any differences in baseline values between subjects with PAI and SAI for body mass index ($p= 0.16$) or hip circumference ($p = 0.19$). However there was evidence for a difference between groups for weight ($p = 0.003$) fat percentage ($p = 0.003$) and waist circumference ($p = 0.006$). Nevertheless, analyses of these and all other body composition end points stratified by study group (Table 5.14) did not show any effects of DHEA therapy on any of these variables.

Change in body composition for the entire cohort

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Weight (kg) | -0.24 | -1.11 to +0.63 | 0.58 |
| BMI (kg/m$^2$) | -0.09 | -0.4 to +0.22 | 0.551 |
| Fat percentage (%) | -0.23 | -0.77 to -0.3 | 0.383 |
| Waist circumference (cm) | +0.68 | -1.05 to +2.41 | 0.423 |
| Hip circumference (cm) | -0.16 | -1.66 to +1.34 | 0.828 |

Table 5.13 Effects on body composition in entire cohort. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Change in body composition stratified by group

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Weight (kg) | | | |
| PAI | +0.32 | -0.67 to +1.13 | 0.498 |
| SAI | -0.91 | -2.66 to +0.84 | 0.267 |
| BMI (kg/m$^2$) | | | |
| PAI | +0.12 | -0.24 to +0.47 | 0.498 |
| SAI | -0.34 | -0.95 to +0.27 | 0.233 |
| Fat percentage (%) | | | |
| PAI | -0.1 | -0.9 to +0.7 | 0.786 |
| SAI | -0.38 | -1.19 to +0.44 | 0.337 |
| Waist circumference (cm) | | | |
| PAI | +1.82 | -0.85 to +4.49 | 0.161 |
| SAI | -0.58 | -3.04 to +1.89 | 0.61 |
| Hip circumference (cm) | | | |
| PAI | +0.83 | -1.23 to +2.9 | 0.407 |
| SAI | -1.08 | -3.58 to +1.43 | 0.362 |

Table 5.14 Changes in body composition stratified by group. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Stratification of the data by gender did not show any differences in baseline values between male and female subjects for BMI ($p = 0.44$) or hip circumference ($p = 0.75$). However, there was evidence for a difference between genders at baseline for weight ($p =$

0.002), fat percentage (p = 0.002) and waist circumference (p = 0.02). Nevertheless, analyses of all body composition end points stratified by gender (Table 5.15) did not show any changes following DHEA treatment.

Change in body composition stratified by gender

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Weight (kg) | | | |
| Male | +0.13 | -1 to +1.26 | 0.807 |
| Female | +0.54 | -1.31 to +0.99 | 0.77 |
| BMI ($kg/m^2$) | | | |
| Male | +0.02 | -0.31 to +0.36 | 0.883 |
| Female | -0.05 | -0.48 to +0.37 | 0.798 |
| Fat percentage (%) | | | |
| Male | +0.5 | -0.28 to +1.28 | 0.18 |
| Female | -0.59 | -1.23 to +0.04 | 0.065 |
| Waist circumference (cm) | | | |
| Male | +2.58 | -1.12 to +6.29 | 0.136 |
| Female | +0.02 | -2.13 to +2.16 | 0.986 |
| Hip circumference (cm) | | | |
| Male | +1.58 | -0.38 to +3.54 | 0.105 |
| Female | -0.86 | -2.48 to +0.77 | 0.28 |

Table 5.15 Changes in body composition stratified by gender. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

### 5.6.2 Serum Androgens

Table 5.16 summarises the change in DHEAS, androstenedione and testosterone levels for the total cohort. As expected supplementation of DHEA significantly increased serum DHEAS levels (Fig 5.6). Androstenedione concentrations were also increased significantly with mean concentrations marginally above the reference range (Fig 5.8). Unsurprisingly testosterone concentration was not altered in the total cohort.

Stratification by study group (table 5.17) did not show any differences in baseline values between subjects with PAI and SAI for DHEAS ($p = 0.73$) androstenedione ($p = 0.41$) or testosterone ($p = 0.34$). Analysis of the data stratified by study group showed that the increases in DHEAS and androstenedione levels following DHEA treatment were maintained in both groups although again testosterone levels did not change. Stratification of the data by gender showed clear differences at baseline between male and female subjects for both androstenedione ($p = 0.002$) and testosterone ($p<0.001$). DHEAS levels were not different between males and females at baseline ($p = 0.41$). DHEAS levels increased following DHEA supplementation in both males and females as did androstenedione levels (Table 5.18). Table 5.18 also shows that testosterone increased in women but was unaffected in men (Fig 5.7), which is not surprising as testicular Leydig cells are the major source of testosterone in men and all males with hypopituitarism were optimally replaced with testosterone therapy.

Change in DHEAS, androstenedione and testosterone levels for the total cohort

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| DHEAS (μmol/l) | +12.94 | +10.68 to +15.18 | <0.001 |
| Androstenedione (nmol/l) | +13.86 | +11.24 to +16.47 | <0.001 |
| Testosterone (nmol/l) | +1 | -1.83 to +3.38 | 0.447 |

Table 5.16 Change in DHEAS, androstenedione and testosterone levels for the total cohort. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Change in DHEAS, androstenedione and testosterone levels stratified by group

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| DHEAS (μmol/l) | | | |
| PAI | +14.42 | +10.99 to +17.85 | <0.001 |
| SAI | +11.16 | +7.52 to 14.81 | <0.0001 |
| Androstenedione (nmol/l) | | | |
| PAI | +14.46 | +9.86 to +19.06 | <0.001 |
| SAI | +12.99 | +9.29 to +16.69 | <0.001 |
| Testosterone (nmol/l) | | | |
| PAI | +0.5 | -0.45 to +1.44 | 0.267 |
| SAI | +1.3 | -5.04 to 7.63 | 0.667 |

Table 5.17 Change in DHEAS, androstenedione and testosterone levels stratified by group. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Change in DHEAS, androstenedione and testosterone levels stratified by gender

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| DHEAS (μmol/l) | | | |
| Male | +12.29 | +10.12 to +14.47 | <0.001 |
| Female | +14.31 | +10.03 to 18.59 | <0.0001 |
| Androstenedione (nmol/l) | | | |
| Male | +12.3 | +8.1 to +16.5 | <0.001 |
| Female | +15.41 | +9.82 to +21 | <0.001 |
| Testosterone (nmol/l) | | | |
| Male | +0.74 | -7.59 to +9.1 | 0.84 |
| Female | +0.45 | +0.24 to 0.69 | 0.002 |

Table 5.18 Change in DHEAS, androstenedione and testosterone levels stratified by gender. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value

Effect of DHEA replacement on serum DHEAS concentrations in the entire cohort

Effect of DHEA replacement on serum androstenedione concentrations in the entire cohort

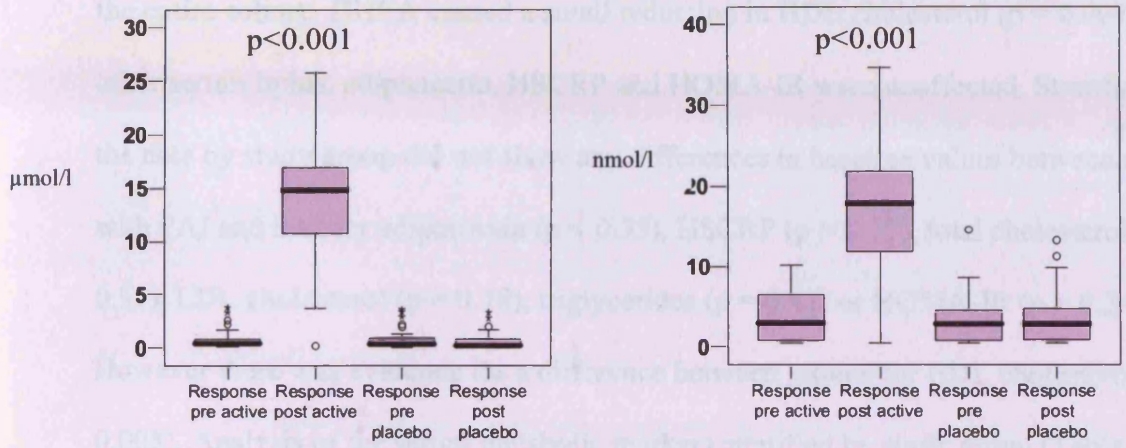



Fig 5.6 Serum DHEAS and androstenedione concentrations following 12 weeks of treatment with DHEA/placebo in PAI and SAI.

Effect of DHEA replacement on testosterones concentrations according to gender

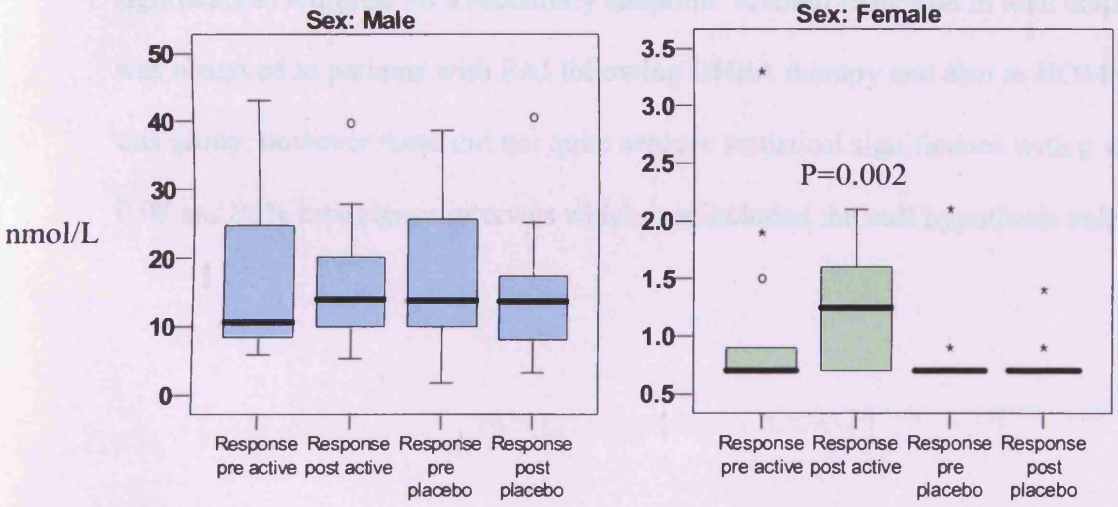



Fig 5.7 Serum testosterone concentrations following 12 weeks of treatment with DHEA/placebo in males and females

### 5.6.3 Metabolic Biochemistry

Table 5.19 provides a summary of the effects of DHEA on serum metabolic markers for the entire cohort. DHEA caused a small reduction in HDL cholesterol ($p = 0.007$) but other serum lipids, adiponectin, HSCRP and HOMA-IR were unaffected. Stratification of the data by study group did not show any differences in baseline values between subjects with PAI and SAI for adiponectin ($p = 0.55$), HSCRP ($p = 0.57$), total cholesterol ($p = 0.31$), LDL cholesterol ($p = 0.78$), triglycerides ($p = 0.43$) or HOMA-IR ($p = 0.34$). However there was evidence for a difference between groups for HDL cholesterol ($p = 0.008$). Analysis of the serum metabolic markers stratified by study group (Table 5.20) did not show any changes with DHEA therapy apart from a small increase in HSCRP in patients with SAI and a small reduction in HDL cholesterol in subjects with PAI. However, neither of these quite met the level of statistical stringency (1% level of significance) required for a secondary endpoint. A small reduction in total cholesterol was observed in patients with PAI following DHEA therapy and also in HOMA-IR in this group; however these did not quite achieve statistical significance with p values of 0.08 and 95% confidence intervals which just included the null hypothesis value of zero.

Summary of the effects of DHEA on serum metabolic markers for the entire cohort

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Adiponectin (pg/ml) | +7.3 | -46.65 to +61.25 | 0.785 |
| HSCRP (mg/l) | +0.23 | -0.06 to + 0.52 | 0.117 |
| Insulin (mU/l) | -1.71 | -5.07 to +1.65 | 0.308 |
| Glucose (mmol/l) | -0.05 | -0.56 to +0.45 | 0.824 |
| HOMA-IR | -0.58 | -1.42 to +0.26 | 0.166 |
| HDL (mmol/l) | -0.08 | -0.13 to -0.02 | 0.007 |
| LDL (mmol/l) | -0.07 | -0.25 to +0.1 | 0.4 |
| TAG (mmol/l) | +0.05 | -0.21 to +0.31 | 0.709 |
| TC (mmol/l) | -0.13 | -0.34 to 0.09 | 0.232 |
| IGF-1 (ng/ml) (SAI only) | -0.75 | -3.53 to +2.03 | 0.555 |

Table 5.19 Effects of DHEA on serum metabolic markers for the entire cohort. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value (HSCRP – high sensitivity c-reactive protein, HOMA-IR – homeostatic model of insulin resistance, HDL – high density lipoprotein cholesterol, LDL – low density lipoprotein cholesterol, TAG – triacylglycerides, TC – total cholesterol, IGF-1 – Insulin-like growth factor-1)

Change in serum metabolic markers stratified by group

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Adiponectin (pg/ml) | | | |
| PAI | +11.27 | -93.34 to +115.87 | 0.823 |
| SAI | +6.51 | -33.84 to +46.86 | 0.735 |
| HSCRP (mg/l) | | | |
| PAI | +0.12 | -0.33 to +0.58 | 0.575 |
| SAI | +0.38 | +0.01 to +0.75 | 0.043 |
| Insulin (mU/l) | | | |
| PAI | -2.62 | -7.05 to +1.81 | 0.215 |
| SAI | -1.16 | -7.6 to +5.27 | 0.7 |
| Glucose (mmol/l) | | | |
| PAI | +0.17 | -0.05 to +0.39 | 0.119 |
| SAI | -0.33 | -1.57 to +0.92 | 0.559 |
| HOMA-IR | | | |
| PAI | -0.7 | -0.15 to +0.1 | 0.08 |
| SAI | -0.56 | -2.41 to +1.28 | 0.506 |
| HDL (mmol/l) | | | |
| PAI | -0.13 | -0.23 to -0.03 | 0.011 |
| SAI | -0.02 | -0.07 to +0.03 | 0.345 |
| LDL (mmol/l) | | | |
| PAI | -0.1 | -0.3 to +0.11 | 0.339 |
| SAI | -0.04 | -0.39 to 0.31 | 0.81 |
| TAG (mmol/l) | | | |
| PAI | -0.02 | -0.43 to +0.39 | 0.919 |
| SAI | +0.1 | -0.25 to 0.45 | 0.54 |
| TC (mmol/l) | | | |
| PAI | -0.25 | -0.52 to 0.03 | 0.076 |
| SAI | -0.001 | -0.37 to +0.37 | 0.994 |

Table 5.20 Effects of DHEA on serum metabolic markers stratified by group. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value (HSCRP – high sensitivity c-reactive protein, HOMA-IR – homeostatic model of insulin resistance, HDL – high density lipoprotein cholesterol, LDL – low density lipoprotein cholesterol, TAG – triacylglycerides, TC – total cholesterol)

Stratification of the data by gender (Table 5.21) did not show any differences in baseline values between male and female subjects for adiponectin ($p = 0.39$), total cholesterol ($p = 0.11$), LDL cholesterol ($p = 0.7$), triacylglycerides ($p = 0.73$) or HOMA-IR ($p=0.18$).

However, HDL cholesterol did show a gender difference at baseline ($p = 0.004$) and HSCRP also showed a gender difference at baseline of borderline statistical significance ($p = 0.05$). Analysis of metabolic outcomes stratified by gender showed no effects of DHEA therapy on any of the variables examined apart from confirmation of HDL cholesterol lowering in females, with mild reduction in HDL cholesterol and increase in HSCRP in males almost reaching significance with p-values of 0.06 and 0.08 respectively.

Effects of DHEA on serum metabolic markers stratified by gender

| Variable | Mean difference | 95% CI for the difference | p-value |
|---|---|---|---|
| Adiponectin (pg/ml) | | | |
| Male | +15.84 | -65.43 to +97.12 | 0.675 |
| Female | -11.62 | -107.14 to +83.91 | 0.795 |
| HSCRP (mg/l) | | | |
| Male | +0.34 | -0.05 to +0.73 | 0.082 |
| Female | +0.19 | -0.18 to +0.55 | 0.297 |
| Insulin (mU/l) | | | |
| Male | -0.76 | -3.92 to +2.4 | 0.615 |
| Female | -2.77 | -9.9 to +4.4 | 0.41 |
| Glucose (mmol/l) | | | |
| Male | +0.12 | -0.22 to +0.46 | 0.437 |
| Female | -0.1 | -0.88 to +0.69 | 0.795 |
| HOMA-IR | | | |
| Male | -0.25 | -1 to +0.51 | 0.493 |
| Female | -0.97 | -2.43 to +0.5 | 0.18 |
| HDL (mmol/l) | | | |
| Male | -0.05 | -0.11 to +0.002 | 0.056 |
| Female | -0.12 | -0.23 to -0.01 | 0.032 |
| LDL (mmol/l) | | | |
| Male | +0.03 | -0.19 to +0.25 | 0.742 |
| Female | -0.13 | -0.41 to 0.15 | 0.325 |
| TAG (mmol/l) | | | |
| Male | +0.08 | -0.33 to +0.49 | 0.662 |
| Female | +0.03 | -0.31 to 0.38 | 0.836 |
| TC (mmol/l) | | | |
| Male | +0.01 | -0.24 to 0.26 | 0.915 |
| Female | -0.23 | -0.55 to +0.09 | 0.147 |

Table 5.20 Effects of DHEA on serum metabolic markers stratified by gender. Data shown are for mean difference, 95% confidence interval (CI) for the difference and p-value (HSCRP – high sensitivity c-reactive protein, HOMA-IR – homeostatic model of insulin resistance, HDL – high density lipoprotein cholesterol, LDL – low density lipoprotein cholesterol, TAG – triacylglycerides, TC – total cholesterol)

## 5.7 Discussion

To my knowledge, this is only the second clinical trial to report on the effects of DHEA supplementation on vascular function in subjects with Addison's disease and the first of its kind in subjects with optimally treated panhypopituitarism. The study failed to demonstrate any clear benefits, or risks, of short-term physiological DHEA supplementation on endothelial function or arterial stiffness in these patient groups, although results for both brachial pulse wave velocity and Tr almost reached significance with p-values of 0.07 and 95% confidence intervals which just included the null hypothesis value of zero. If confirmed, an increase in Tr and reduced brachial PWV would both be consistent with reduced regional arterial stiffness in the brachial tree, and would corroborate Fukui and colleagues' observation of an independent, inverse correlation of serum DHEAS levels with brachial-ankle PWV in men with type 2 diabetes [318]. However, the absence of any change in Ai suggests that wave reflection is not significantly influenced by DHEA. Furthermore, aortic pulse wave velocity, which has the greatest amount of epidemiological evidence for its predictive value for cardiovascular mortality [198-200], did not change.

A cross-over design was employed for this study because a substantial within-subject correlation is anticipated, such that smaller sample sizes are required to detect significant differences. Care was taken to minimise the potential for a carry-over effect, which was not evident in the study, by using a washout period of 8 weeks. The sample size calculations indicated that the study had more than adequate power to detect any clinically relevant changes in endothelial function if present, especially when both study

populations were combined. Furthermore, any disadvantages of reduced precision using augmentation index compared with FMD to assess endothelial function [319] should have been outweighed by the within-subject correlation. It was felt important to recruit both males and females to redress an imbalance in the literature which has predominantly focused on females with Addison's disease. Moreover, interventional studies of DHEA supplementation in other conditions have shown benefits on vascular risk in both sexes [180, 187].

Many previous studies in this area support the contention that DHEA has important actions on the vasculature. The epidemiological data, which have demonstrated either an inverse [8, 320] or no [321] relationship between cardiovascular mortality and circulating DHEAS levels in men, are controversial but animal studies [210, 219, 322, 323] and *in vitro* experiments [187, 211, 212, 324-329] have both shown the potential for anti-atherosclerotic actions of DHEA. Indeed, the *in vitro* data support both a direct and non-genomic action of DHEA on the endothelium, conclusions based on the rapidity of action on endothelial nitric oxide synthase and failure to block endothelial cell activation with selective oestrogen or testosterone receptor antagonists [187, 211, 325]. Recent studies have extended these observations to demonstrate roles for DHEA in vascular endothelial cell survival [327], proliferation/angiogenesis [328], and activation, including transcriptional regulation of endothelin-1 [329]. Studies in rodents [322] and other mammals [210, 219, 323] have also demonstrated many vascular benefits of DHEA, although the validity of a number of these findings must be questioned in view of the

often supraphysiological DHEA doses used and the low circulating DHEAS levels present in lower order mammals.

Despite the compelling evidence for vasculoprotective actions of DHEA from *in vitro* and animal studies, human clinical trials of DHEA replacement on metabolic and vascular function have shown conflicting results, partly because interpretation of these trials is difficult due to significant heterogeneity in study design, including major differences in DHEA dose (ranging from 25 to 1600mg), treatment duration (ranging from 4 to 52 weeks) and underlying condition (healthy volunteers, hypercholesterolaemic men, postmenopausal women, obesity and adrenal insufficiency) [147]. Nevertheless, Kawano and colleagues were able to show improved endothelial function with low doses of DHEA (25mg daily) in middle-aged hypercholesterolaemic men [180], an observation later substantiated in post-menopausal women [187], albeit using high DHEA doses (100mg/day). However, our findings are largely in keeping with the only other clinical trial of DHEA action on vascular function in adrenal insufficiency [241]. In this report, Christiansen *et al* examined magnetic resonance cardiac output, endothelial function (FMD), echocardiography, 24 hour ambulatory blood pressure and maximal oxygen consumption in 10 women with Addison's disease or isolated ACTH deficiency. They did not find any effects of DHEA therapy on these parameters but the comparatively small subject number may have left their study underpowered to detect any minor changes.

Recognising that the effects of DHEA replacement might well be different for subjects with primary adrenal failure and hypopituitarism we repeated our analyses with the data

stratified by study group. These analyses demonstrated discordant effects of DHEA on systolic blood pressure, with an increase in peripheral systolic blood pressure in subjects with Addison's disease and a reduction in peripheral and central systolic blood pressure in subjects with hypopituitarism, the latter driven largely by reduced peripheral systolic pressure as Ai did not change. These discrepancies are not easily resolved but the confidence intervals are wide, and the changes may simply represent type I errors. A previous study has shown a beneficial action of growth hormone replacement on central systolic blood pressure in subjects with hypopituitarism [330]. Given that DHEA has been shown to reduce growth hormone dose requirements in hypopituitarism [83] we hypothesised that the reduction in central systolic blood pressure might be attributable to improved GH sensitivity; however, IGF-I levels did not change with treatment.

Further analyses were undertaken to determine whether any of the effects of DHEA in the study populations were dependent on gender. Other than the anticipated increase in serum testosterone in women but not men, DHEA did not seem to affect any of the vascular or metabolic endpoints in a gender-dependent manner, although it is conceded that the study had very limited power to support either these analyses or the logically preferable interaction analyses.

DHEA did not affect any of the anthropometric variables studied (weight, body mass index, fat percentage, waist circumference or hip circumference). This is perhaps not surprising given the physiological replacement dose chosen and the comparatively short timeframe of treatment. Nevertheless, direct, non-genomic, inhibitory actions of DHEA

on preadipocyte cell proliferation and adipogenesis have been previously reported *in vitro* [139, 289] and some interventional studies in humans [152, 331] in which adipose mass is reduced with DHEA therapy. However, most other studies, including a recent long-term clinical trial of physiological DHEA replacement in adrenal insufficiency [240], do not support a significant effect of DHEA on fat mass. Given that measures of fat mass did not change with DHEA treatment, it is perhaps not surprising that adiponectin concentrations were also unaffected, although increases in adiponectin mRNA expression have previously been reported in white adipose tissue of DHEA-treated rats [332].

In agreement with some studies a small reduction in HDL cholesterol was observed, notably in women, an observation that others have attributed to androgenic conversion of DHEA [12, 152, 177], with consequent increased hepatic lipase activity [230] and/or increased HDL uptake by scavenger receptor B1 and so augmenting reverse cholesterol transport [229]. However, the magnitude of fall is modest and of the same order as that observed with low dose testosterone replacement. Although these changes might be considered proatherogenic, it is important to note that the antiatherogenic effect of HDL cholesterol is largely attributable to changes in HDL cholesterol metabolism rather than concentration *per se* [333]; HDL subclass analysis was not undertaken in this study.

I utilised fasting insulin and glucose measurements to determine insulin resistance (HOMA-IR) and noted that changes in this parameter almost reached statistical significance in the PAI group ($p=0.08$). A previous study by Dhataryia *et al* [10] has shown improvements in insulin sensitivity in this patient group using a more robust

method (hyperinsulinaemic euglycaemic clamp). My study was limited in only using a single fasting value of glucose and insulin to calculate HOMA-IR, in contrast to recommendations which employ the triple fasting insulin measurement (5 minutes apart) [297]. Hence, a more robust measurement protocol incorporating the mean of 3 measures might have compensated more adequately for variation in insulin pulsatility, and might have resulted in statistically significant changes in HOMA-IR.

I acknowledge the heterogeneous nature of my study population whereby males and females were recruited from a wide age range, in addition to subjects with both primary and secondary adrenal failure (of varying disease duration), and oestrogen replete and deplete females. This is a limitation of my study and a more focused study group may well have generated different results to those observed. Indeed, Kawano's study, which demonstrated improved endothelial function following DHEA treatment, confined recruitment to middle aged men with hypercholesterolaemia [180], in line with similar benefits in postmenopausal women [187]. I had originally planned to determine whether any improvements in vascular function might be confined to either gender but acknowledge that the study had limited power to address this question.

In conclusion, these data do not support a major action of short-term DHEA on vascular risk reduction in subjects with Addison's disease or hypopituitarism, and suggest that pathological DHEA/DHEAS deficiency does not provide a unifying explanation for the increased cardiovascular mortality reported in these conditions [239, 244, 245, 247]. Based on these findings, routine DHEA replacement in Addison's disease or hypopituitarism for vascular protection cannot presently be recommended and its use in

the clinic should continue to be restricted to individuals with significantly impaired quality of life and/or reduced libido.

# Chapter 6

# GENERAL DISCUSSION

## 6.1 Overview

The *in vitro* studies generated in this thesis have yielded some results that are novel and others that are in support of previous findings. Previous investigators have demonstrated inhibition of preadipocyte proliferation by DHEA [139] as well as possible pro-BAT [334] effects and an inability of adipose tissue to sulphate DHEA [87]. The major novel findings are:

1. The differential effect of DHEA on adipogenesis of primary human omental (inhibited) and subcutaneous (neutral effect) preadipocytes, and
2. The cell cycle blockade observed following DHEA treatment in cell line preadipocytes.

These novel data therefore represent potential advancements in this area of biochemical endocrinology. More recent studies continue to demonstrate beneficial anthropometric and metabolic effects following DHEA treatment. A recent study has shown improvements in insulin sensitivity in ageing rats [335] following DHEA treatment and also reductions in measures of body fat and adiponectin levels in aged rats [336]. A further study has linked low DHEAS levels with the more severe types of non-alcoholic fatty liver disease, a condition associated with insulin resistance and the metabolic syndrome [337]. Furthermore a paper by Hernandez-Morante and colleagues showed gender-specific effects on lipolysis of adipose tissue *ex vivo*, with DHEAS stimulating lipolysis in subcutaneous adipose tissue in females but in visceral adipose tissue in males [316].

The clinical trial reported in this thesis was conducted to a high standard with comprehensive ethical, statistical and regulatory body review both at pre-initiation and study completion. It therefore represents the first comprehensive trial of DHEA supplementation on vascular risk in hypopituitarism and Addison's disease. However, I was unable to confirm my hypothesis that DHEA, through its action on adipose tissue, has a beneficial effect on vascular health in pathological states of DHEA deficiency. Nevertheless, the study has generated some interesting results within sub-groups which might warrant further analysis in the future. A number of more recent studies analysing the potential influence that DHEA may have on vascular health *in vivo* have been performed during the course of this body of work, and these continue to be inconclusive. Once more these various studies have tended to focus on physiological DHEA deficiency or specific disease states rather than pathological DHEA deficiency. In a study by Fukui and colleagues [318], using similar methods of evaluating arterial stiffness to those used in this study, a significant inverse correlation was noted between serum DHEAS and brachial pulse wave velocity in men with Type II diabetes, one of the core features of the metabolic syndrome. However, a similar independent correlation was also seen with serum testosterone. A placebo controlled therapeutic trial of DHEA supplementation in the elderly (75 mg in men and 50 mg in women) for two years (mean age in the treatment arms 68.4 years for both men and women) failed to demonstrate any improvements in insulin sensitivity [338]. A further observational study failed to identify any association between serum DHEAS and the presence or severity of coronary angiography – identified cardiovascular disease in 502 men [339]. Serum DHEAS was, however, found to be inversely associated with atherosclerosis in diabetic post-menopausal women [317] and

endothelial function in post-menopausal women with coronary risk factors [340]. A further myocardial infarction study in women (predominantly post menopausal), however, showed a positive, albeit modest, association between DHEA and DHEAS and risk of myocardial infarction [341], although another study by Kawano *et al* demonstrated significantly lower DHEAS levels in cases of congestive cardiac failure [342]. It is therefore clear that most reports in this field are focused on observational studies, which do not necessarily imply causation nor tell us about the directionality of effects. Moreover, as previously discussed, positive inverse associations of serum DHEAS with any of the above outcome measures may merely represent an epiphenomenon, perhaps reflecting a stress-induced decrease in DHEAS to cortisol ratio. The question as to whether DHEA should be routinely supplemented in pathological DHEA deficiency remains unanswered, but I believe the various studies undertaken within this body of work will positively influence this area of uncertainty.

### 6.2 *In vitro* and *ex vivo* studies

It appears clear that DHEA can negatively influence both cell line and primary preadipocyte biology in agreement with some previous studies [139, 258, 289]. Proliferation is inhibited in cell line and primary culture preadipocytes and I have been able to demonstrate that this is due, at least in part, to cell cycle blockade. The inhibition of proliferation in isolation could be either inhibitory or stimulatory to adipogenesis. Since termination of proliferation is a prerequisite for preadipocyte differentiation, the induced cell cycle arrest could promote differentiation. However, the reduced number of precursors could result in a smaller adipose tissue depot. The differential effect on

adipogenesis in omental- and subcutaneous-derived preadipocytes is a novel finding and would tend to support the initial hypothesis. The extent to which this effect may influence fat depots *in vivo* is, however, unclear, since the extent to which latent precursor fat cell maturation influences human adult adiposity is not known. A recent study using the novel carbon-14 dating method suggested that adipocyte number does not increase in association with the development of obesity after adolescence, such that an increase in fat cell size causes the enlargement of adipose depots and fat cell number is therefore determined before the onset of adulthood [343]. This conclusion is drawn from observations that fat cell turnover remains constant irrespective of BMI, and adipocyte age is no different in thin and obese individuals despite the presence of more adipocytes in the obese individuals [343]. Data from patients with phaeochromocytomas would tend to counter this argument and suggest that under certain circumstances precursor cell adipogenesis can, and does affect adipocyte depot mass. In this situation there is a demonstrable increase in BAT secondary to exaggerated adrenergic stimulus [89], even though there is likely to be only very little BAT in human adults despite a previous study having identified functioning BAT in adult humans using positron emission tomography [344]. The development of obesity is therefore likely to be secondary to a combination of these factors, although the relative contribution of adipogenesis from latent precursors is likely to be significantly less than the simple, widespread increase in adipocyte accumulation of triglyceride and overall cell diameter. What is potentially more interesting still is that DHEA may promote adipogenesis in BAT. The caveats to these data are discussed in Chapter 3 relating to the high Ct values observed following QPCR. This observation may explain why DHEA is so avidly generated by the foetal adrenal

gland and also why this falls away soon after birth. Furthermore, these data are supported by a previous study that demonstrated an increase in UCP-1 in genetically obese rats following DHEA treatment [334]. Studies concerning modulation of BAT are particularly emotive as any agent promoting BAT over WAT is effectively causing a change in function of adipose tissue shifting the focus from energy storage to one of energy dissipation. Such agents could have enormous implications considering the world wide health burden of obesity. Intra-adipose tissue DHEA concentration is a further area of uncertainty. The study by Féher [85] suggests that DHEA in adipose tissue is present in much higher concentrations than that found in the circulation and also that this increases further with obesity. These issues make the decision of which concentrations to base one's estimation of physiologically relevant effects difficult. It is clear that mouse adipocytes would not be exposed to the concentrations that are utilised in the study, but the human primary culture experiments are likely to reflect conditions that are close to physiology, particularly in the obese.

The primary culture experiments were, however, affected by difficulties that often beset such studies. The cells were slow growing, could be prone to infection and the omental samples contained significant numbers of mesothelial cells that demonstrated a greater proliferation potential than the preadipocytes. Furthermore, RNA yield from these experiments tended to be low, despite various measures to augment this; RNA quality was, however, most often preserved. These various factors lead to smaller numbers of samples suitable for final analysis and certainly more extensive *in vitro* studies would be required to confirm these findings.

A further area of discussion is that this study has demonstrated, as suggested by others [87], that adipocytes do not have the capacity to sulphate DHEA and thus generate DHEAS; hence there is a tendency to generate down-stream metabolites as opposed to adding to the serum DHEAS pool. The implications that this may have are not immediately obvious although obesity in males is known to be associated with central hypogonadism and adipose tissue primed for the conversion of androgenic precursors could represent part of the compensatory mechanism. This would, however, be against the gender-dependent tendency to produce oestrogen in males and testosterone in females [51-53], as confirmed by this work.

### 6.3 *In vivo* studies

The clinical trial did not demonstrate any significant improvement in body composition following active treatment although it is conceded that it was not powered to do so. This is corroborated by other studies [240], although most studies in this area have evaluated effects in primary and not secondary adrenal insufficiency. The lack of effect may be secondary to the relatively short treatment phase duration or to a reduced potency on adipose tissue when exposed *in vivo*, and at physiological concentration, to counter regulatory substances. Furthermore, even if DHEA does impact upon adipogenesis, the effect on mature adipocytes may be minimal if at all. It is clear that adipocytes have the ability to metabolise DHEA and it is conceivable that mature cells have a greater metabolic capacity than the precursors such that the metabolite conversation occurs before any anti-growth effects can be triggered. The preparation of DHEA and the doses used in the clinical trial generated circulating concentrations that tended to be marginally

above established reference ranges and this counters data from some previous studies, although a more recent study reported similar findings [240].

I hypothesised that a link between adipose tissue and the vasculature might be mediated by changes in the adipocytokine profile. Obesity causes alterations in the adipocytokine profile (as discussed in Chapter 1) with resultant effects on insulin sensitivity, inflammation and satiety. Many of these factors have been implicated in the development of the derangements apparent in the metabolic syndrome. This study was not able to confirm this hypothesis although I chose to focus solely on adiponectin and it is conceivable that DHEA might alter other adipokines. It was perhaps not surprising that adiponectin levels did not change given that there was no reduction in measures of adiposity. However, the ELISA kits used to assay the serum were designed for the analysis of conditioned culture medium from *in vitro* cultures in which relatively low concentrations of adiponectin may be detectable when compared to patient serum. It was therefore necessary to dilute the serum significantly to allow for ELISA detection. Serial 1 in 10 dilutions were performed in order to reduce the possibility of any pipetting error; however the results generated revealed wide variations. Although the major contributing factor to the variability observed here is likely to be a consequence of the great degree of heterogeneity in the differentiating potential of the preadipocytes cultured, minor experimental errors remain a possibility.

Although the characteristics of the subjects in both groups were largely representative of patients in the general population with Addison's disease and hypopituitarism, the

patients recruited into this clinical trial, as indeed in many clinical trials, tended to have certain characteristics that may have influenced some of the data generated. Overall they tended to be educated, professional (or retired professional) and health aware. I observed a small non-significant reduction in body weight, especially in subjects with hypopituitarism, even with placebo therapy which is likely a reflection of increased motivation as a result of participation in a clinical trial. This may have diluted any differences compared with active treatment. Many individuals who enrolled in the study were also retired. This likely relates to the fact that individuals at this time are more likely to have fewer commitments and are more available to participate in such endeavours. The numbers studied were too small to allow comparisons based on age, but an interesting area for future research might be to examine the effects of DHEA stratified by age.

The trial was adequately powered for the vascular primary end points, yet failed to demonstrate a benefit on arterial compliance or endothelial function. Nevertheless, the data for the entire cohort suggest that DHEA might improve arterial stiffness in the brachial tree but this fell just short of statistical significance. Even so, the magnitude of effect was not marked, and an increased Tr of 3.7 ms and reduced bPWV of 0.35 m/s, if confirmed in other studies, would only be of marginal clinical relevance. This, taken also in the context of no changes in Ai or aPWV, suggests that DHEA does not markedly affect vascular risk *in vivo*, at least in the short-term and in physiological replacement doses.

The methods used to determine arterial stiffness in this study appear to be relatively robust with little in the way of intra/inter-subject or investigator variability. However, I felt that the method of assessing endothelial function using the SphygmoCor system demonstrated excessive variability, which is in agreement with more recent comparisons of methods for measuring endothelial function [319]. The cause for this was unclear as I performed all but two of the endothelial function assessments, the medication used (GTN and salbutamol) was within use-by dates and identical equipment was used to both capture and analyse the data. Subjects were exposed to the same dose of medication although the salbutamol inhalation technique could conceivably have differed. Furthermore, the study subjects attended at the same or near same time at each visit and were asked to fast overnight; smokers were asked to refrain from smoking on the morning of the study visit. These numerous factors will have generated, as far as possible, standardised study visit conditions. The variations observed, however, may well have prevented the detection of any potential differences in endothelial function following DHEA therapy and, were the study to be repeated, a more robust method of determining endothelial function such as FMD should, I believe, be sought.

### 6.4 DHEA Therapy, Safety and Tolerability

Overall the study population tolerated the DHEA supplementation well. There were a few cases of the anticipated androgenic side effects experienced in females, although this only culminated in one withdrawal from the study. No male participants experienced drug-related side effects, although the only adverse event defined as serious occurred in a male subject, who coincidentally developed a viral upper respiratory tract infection. No issues

concerning the safety of DHEA were raised in this study and no alteration in liver function tests were observed during the course of the study. There are, however, no long-term safety data available on DHEA replacement, with the longest trials reporting follow-up periods of only two years. Nevertheless, it is possible to conclude that DHEA replacement, in the short term at least, is safe to use in this patient group. The potential for exacerbating hormone-dependent tumours remains and so its use cannot be advocated in cases of confirmed or suspected breast or prostate carcinoma. Furthermore, it might be prudent to undertake annual blood tests for prostate specific antigen (males over fifty years), liver function tests and full blood count on any one initiated on therapy.

### 6.5 Mortality and adrenal insufficiency – possible causes and further implications

The increased cardiovascular mortality observed in both primary and secondary adrenal insufficiency is, as far as my study can ascertain, not attributable to DHEA deficiency, although the blood pressure effects in the hypopituitarism group may warrant further investigation. Possible causes for the adverse mortality rates are discussed in Chapter 2 and include supraphysiological glucocorticoid replacement and associated illness. Further analysis of this area of vascular risk and adrenal insufficiency becomes more important when one considers the potential ramifications for the 1-3% of the wider general population with adrenal insufficiency secondary to chronic glucocorticoid therapy. It may therefore be the case that DHEA/DHEAS deficiency is also important in patients taking long term steroids, although dissecting DHEA-related from glucocorticoid- or condition-related health effects would clearly be difficult.

### 6.6 Future *in vitro* studies

Further studies, particularly assessing the impact on primary culture preadipoctyes would, I feel be important though cell line work using more human cell lines would also be useful (e.g. CHUB S7 see chapter 2). Preadipocyte extraction optimization could lead to greater experimental yield by reducing the possibility of mesothelial cell contamination and identify those cells with high differentiating potential. The isolation of PREF-1 positive preadipocytes would effectively generate an enriched cell population with higher and potentially more predicable levels of adipogenic potential though this would require larger fat samples.

Assessing the effect of DHEA on mature adipose tissue *ex vivo* is another area of important work that is more readily transferable to the human model and should ideally be included in any future planned *in vivo/ex vivo* studies.

As discussed in chapter 3 many individuals consenting for adipose tissue retrieval had the diagnosis of cancer and although these individuals were all having potentially curative procedures with no sign of distant metastasis it is possible that this diagnosis in itself had an adipose tissue effect. Cachexia is a well recognised feature of advanced cancer but there is the possibility of lesser, clinically unapparent effects with small tumour loads. Therefore further studies should ideally use adipose tissue samples retrieved from individuals undergoing surgical procedures for non-malignant reasons. Due to increased use of endoscopic abdominal surgery it is likely that in order for this to be achieved fat retrieval via this method will need to be investigated. Fat removal methods for cosmetic purposes (liposuction) is another possible source of adipose tissue for experimentation but would only provide subcutaneous samples. Bariatric surgery would provide a further

source of potential primary adipose tissue without the potential confounding factor of malignancy.

The possibility that DHEA may be involved in the modulation of BAT is also something I feel warrants further evaluation for reasons mentioned previously and positron emission tomography could provide an ideal, if expensive tool, with which to do this.

### 6.7 Future *in vivo* studies

Most of the data in relation to DHEA in vascular function have been acquired from observational, *in vitro*, or animal model sources. Given the limitations of each of these approaches, definitive conclusions on the role of DHEA in vascular protection and adrenal failure must rely on well conducted clinical trials. In this context the literature is currently sparse and my clinical trial has hopefully helped address this deficiency. The trial was conducted to 'gold standard' methodology and seemingly had more than adequate power to show an effect on arterial stiffness and endothelial function if present. Therefore, the failure to demonstrate any positive, or negative, effects of DHEA on vascular function is in itself important, and improves the evidence base considerably.

Any future *in vivo* studies might wish to focus specifically either on subjects with hypopituitarism, where we observed some reduction in central systolic blood pressure, or females where the reduction in bPWV almost reached significance. However, comparatively large numbers of patients would need to included, even with a cross-over design and its obvious advantages in terms of statistical power. Given the relative rarity of these conditions this might well require multi-centre involvement.

Finally, refinements in methodology to improve precision should be considered, especially in relation to measurement of endothelial function e.g. flow mediated dilatation, body composition, visceral fat area by CT or MRI and other cardiovascular variables (e.g. echocardiography and carotid intima-media thickness).

### 6.8 Personal Conclusion

It is my opinion that DHEA replacement in Addison's disease and hypopituitarism can have beneficial anthropometric and vascular effects but these actions are likely to be subtle, at least in the short-term. It is conceivable that the individuals that may benefit the most are those with the worst metabolic and anthropometric profile at baseline but my studies did not have the power to formally test this. Nevertheless, at the current time I do not anticipate that my clinical practice will change significantly as a result of this body of work; however, this is an area of clinical and biochemical endocrinology that undoubtedly requires further inspection.

# Appendices

## Appendix chapter II

**FRTL-5 cell culture medium**

DMEM/Hams F12 (ratio 1:1) (86+86 mLs)

5% calf serum (10mLs)

1.5% sodium bicarbonate (3mLs)

Pyruvate 2mLs

100u/mL Penicillin and 100µg/mL Streptomycin (4mLs)

TSH 2mLs/Transferrin 2mLs/Insulin 2mLs/ascorbic acid 2mLs/hydrocortisone 2uLs

**HACAT cell culture medium**

DMEM/Hams F12 (ratio 1:1)

10% foetal calf serum

**Loading buffer**

10% SDS 2mL

Glycerol 1mL

0.5M Tris (pH 6.8) 1mL

Distilled water 0.8mL

0.2% Pyronin Y 0.1mL

β-mercaptoethanol 0.1mL

**Stacking gel (4%)**

30% acrylamide 1.3mL

Distilled water 6.1mL

0.5M Tris (pH6.8) 2.5mL

10% SDS 100uL

10% APS 100uL

TEMED 10uL

**10% acrylamide (running) gel**

30% acrylamide 3.3mL

Distilled water 2.92mL

1M Tris (pH 8.8) 3.75mL

10% SDS 100uL

10% APS 100uL

TEMED 5uL

**Running buffer**

Tris 15g

Glycine 72g

SDS 5g

Make up to 1 litre with distilled water

**Blotting buffer**

0.025M Tris 3.025g

0.182M Glycine 13.66g

Methanol 200mL

Distilled water 800mL

Cool at -20 for 1 hour prior to use

**TBS (10x)**

Tris 24.2g

Sodium Chloride 80g

(adjust to pH 7.6 with hydrochloric acid)

Make up to 1 litre with distilled water

**TBS-T**

TBS (10x) 100mL

Distilled water 900mL

Tween 20 1mL

**Blocking buffer**

TBS-T 100mL

Marvel (powdered milk) 5g

**Stripping buffer**

0.5M Tris (pH 6.8) 6.25mL

10% SDS 10mL

Mercaptoethanol 0.35mL

Distilled water 33.5mL

**Appendix chapter III**

**Adipose tissue study Patient information sheet**

## PATIENT INFORMATION SHEET

Version 2
May 2006

**1. Title of study**
Physiological and pharmacological modulation of adipose/connective tissue remodelling.

**2. Introduction**
You are being invited to take part in a research study. Before you decide whether you wish to become involved it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

**3. What is the purpose of this study?**
A number of hormones can change the amount of fat that is stored in the body. Our research group is interested in studying the effects of some of these hormones, including thyroid hormones, growth hormone and a hormone called DHEA which is made in the adrenal gland, on the function of fat cells in the laboratory. Not all fat reacts in the same way. In some parts of the body hormones may cause fat to build up while in others it may break down.
In order for us to fully understand how these hormones affect fat cells, we are asking people to donate a small sample of their fat to us for our research studies. This sample will be obtained during your planned operation. We will measure how the cells grow and work in response to treatment with hormones.
We are also interested in studying whether changes in genes controlling growth hormone levels and function can have an effect on fat cells. In order for us to test this we will also ask you to donate a blood sample for us to analyse your growth hormone genes.

**4. Why have I been chosen?**
You have been chosen for this study as your surgeon has identified you as someone who will be undergoing an operation soon. A total of 100 patients will be studied.

**5. Do I have to take part?**
No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you withdraw your permission after we have obtained your samples, any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

**6. What will happen to me if I take part?**
A sample, about the size of one sugar cube, will be taken from the fat under your skin during your planned surgery. If you are having abdominal surgery we would also plan to remove a further sample of fat of the same size from the fat tissue that sits around your bowel (called omental or visceral fat). This procedure will not affect your operation scar. The fat samples would be used in laboratory experiments for further study. During these we would break up the fat and separate it into different cell types. We will measure how the cells grow and work in response to treatment with hormones.
The tests to examine your growth hormone genes (called GH1 and GHR) would involve us taking an extra sample of blood (equivalent to roughly two tablespoons). We would plan to take this sample while you are under anaesthetic during your operation so that you do not feel any discomfort.

**7. What do I have to do?**
There are no lifestyle or dietary restrictions and you can continue your daily activities normally. There will be no changes to the way in which you need to prepare for your operation and your surgical team will fully instruct you on this as normal. With your permission we will check with you and in your records on your previous medical health in case you have a medical condition or are taking any medication which may affect our ability to analyse your fat cells.

**8. What are the possible disdvantages of taking part?**
Occasionally patients may experience discomfort (temporary pain, swelling, bruising and rarely infection) caused by the collection of blood. Removing a small sample of fat from under the skin or from around the bowel is unlikely to cause any additional complications to those which could be expected from your type of operation.

The genetic blood sampling will test for variation in your growth hormone genes. These changes occur extremely commonly in the general population and can be seen as a variation of normal. The samples will be fully anonymised and the results of these genetic tests will have no implications for you in terms of inherited risk, reproductive decisions or insurance status.

**9. What are the possible benefits of taking part?**
The study may provide us with important information in identifying how hormones affect fat cell function. While there are unlikely to be any direct benefits to you, we hope that the results of these studies may help in the future treatment of patients with weight-related conditions such as obesity and diabetes.

**10. What if there is a problem?**
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Aled Rees 02920 745002). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

You are unlikely to be harmed as a result of taking part in this research project, but if you are you should be aware that there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

**11. Will my taking part in this study be kept confidential?**
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.
With your permission your GP will be informed of your participation in this study.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by the research team, by representatives of regulatory authorities and by authorised people from the Cardiff and Vale NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site. Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

**12. What will happen to any samples I give?**
After the sample of fat has been obtained at surgery the research doctors will immediately freeze this sample and store it for later analysis, or break it up and grow the fat cells in the laboratory. The blood samples will be used to test your growth hormone genes. Both the fat and blood samples will be stored securely in the Section of Endocrinology at the University Hospital of Wales (Room 168, 2$^{nd}$ Floor). The research investigators, Dr Aled Rees and Dr Marian Ludgate, will act as the day-to-day custodians for these samples. Only Dr Rees and Dr Ludgate, and immediate members of their research team will have access to the use of these samples. In addition, all the samples will be coded and anonymised such that your personal details will not be identifiable. We propose to store the fat and genetic samples for up to 15 years. After this time the samples will be destroyed by incineration.

It is possible that we may wish to use your fat sample for future studies looking at how other hormone systems could affect fat cell function. If this is the case then a further application will be made to the Research Ethics Committee for consideration. Similarly, we may wish to conduct genetic studies on genes other than the growth hormone system in the future. If this is the case then this will also be submitted to an Ethics Committee for consideration. The results of these tests done for research purposes are unlikely to have any direct clinical implications for you. It is possible that these studies may be carried out by researchers other than Dr Rees or Dr Ludgate, including researchers working for commercial companies.

There is little prospect that the current studies will have any commercial significance but if future information from our research using your tissue samples shows any likelihood of commercial benefit, such as the development of a new treatment or medical test, then you would not benefit financially.

**13. What will happen to the results of the research study?**
The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have been published in the relevant journal(s). Patients will not be identified in any report/publication.

**14. Who is organising and funding the research?**
The study is being organised by Dr Aled Rees and Dr Marian Ludgate in the Section of Endocrinology. Funding for the study is provided from the Section of Endocrinology and the doctors conducting the research are not being paid for including and looking after patients in the study, nor will they be able to sell for profit any samples that are collected.

**15. Who has reviewed the study?**
The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South East Wales Local Research Ethics Committee.

**16. Contact for further information**
Should you have any further questions or queries regarding this research study, then please do not hesitate to contact me on **029 20745002**. I can also be contacted via email (reesda@cf.ac.uk).

Thank you for considering taking part and taking time to read this sheet.

Aled Rees
Principal Investigator/Senior Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep

**Adipose tissue study consent from**

# CONSENT FORM

**Version 2, May 2006.**

**Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.**

Patient Identification Number for this study:

**Title of project:** Physiological and pharmacological modulation of adipose/connective tissue remodelling

**Name of researchers:** Dr Aled Rees and Dr Marian Ludgate

**Contact details for research team:** Centre for Endocrine and Diabetes Sciences, University Hospital of Wales, Heath Park, Cardiff CF14 4XN. Telephone: 02920 742182.

| | **Please initial boxes** |
|---|---|
| 1. I have read the attached information sheet on this project, dated May 2006 (version 2), and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved. | ☐ |
| 2. I agree to give a sample of fat for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. | ☐ |
| 3. I give permission for someone from the research team to look at my medical records to get information on my previous medical health. I understand that the information will be kept confidential. | ☐ |
| 4. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. | ☐ |
| 5. I know how to contact the research team if I need to, and how to get information about the results of the research. | ☐ |
| 6. I consent to my GP being informed of my participation in the study. | ☐ |

**7. Consent for storage and use in possible future research projects**
I agree that the sample I have given and the information gathered about me can be stored by Dr Aled Rees/Dr Marian Ludgate at the Centre for Endocrine and Diabetes Sciences, School of Medicine, Cardiff University for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Dr Aled Rees/Dr Marian Ludgate who ran the first project, including researchers working for commercial companies. ☐

**8. Consent for genetic research**
**A.** I give permission for growth hormone 1 (GH1) and growth hormone receptor (GHR) genotyping to be carried out on the samples I give, as part of this project ☐

**B.** I understand that the project/future research using the samples I give may include genetic research aimed at understanding the genetic influences on fat cell regulation but that the results of these investigations are unlikely to have any implications for me personally. ☐

| Name of patient (BLOCK CAPITALS) | Date | Signature |
|---|---|---|
| Name of person taking consent (if different from researcher) | Date | Signature |
| Name of Researcher | Date | Signature |

Would you like to be sent information about the progress of the project?

☐ Yes ☐ No

**Thank you for agreeing to participate in this research**
s
1 copy for patient; 1 for researcher; 1 to be kept with hospital note

**Appendix chapter IV**

**DHEA study Patient information sheet**

# Adrenal insufficiency and vascular dysfunction.

## PATIENT INFORMATION SHEET
**Version 4, September 2005.**

### 1. Title of study
A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function in patients with primary and secondary adrenal insufficiency.

### 2. Introduction
You are being invited to take part in a clinical research study. Before you decide whether you wish to become involved it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please do not hesitate to ask us if there is anything that is not clear or if you would like more information. Take time to consider whether or not you would wish to take part.
Thank you for reading this.

### 3. What is the purpose of this study?
Adrenal insufficiency or failure is a condition resulting in low or absent steroid hormone production by the adrenal glands. This can arise as a result of a problem in the adrenal glands themselves where it is termed primary adrenal insufficiency (often called Addison's disease), or from a failure of the pituitary glands where it is called secondary adrenal insufficiency. Treatment of these conditions requires replacement of steroid hormones, such as hydrocortisone or prednisolone, and, on occasions, a class of hormones called mineralocorticoids, such as fludrocortisone. However, recent studies have shown that replacing a third hormone called dehydroepiandrosterone (or DHEA in short) can have positive effects on mood and well-being. There have been no studies though on the effects of replacing DHEA on the function of blood vessels in adrenal failure. This may be an important area to study as research in elderly and middle-aged people without adrenal disease has shown that low levels of DHEA may increase the risk of heart disease. Another study recently has shown that treating healthy middle-aged men with DHEA improves the function of their blood vessels.

We wish to study the effects of replacing DHEA in adrenal insufficiency on blood vessel function. This study will involve four visits to the Endocrinology department at the Heath hospital and will last for a total of 32 weeks.

**4. Why have I been chosen?**
You have been chosen for this study as your doctor has identified you as having either Addison's disease (primary adrenal insufficiency) or pituitary disease causing secondary adrenal insufficiency, and you have been on stable hormone replacement treatment for some time. A total of 40 patients will be studied, 20 of whom will have primary adrenal insufficiency and 20 will have secondary adrenal insufficiency.

**5. Do I have to take part?**
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**6. What will happen to me if I take part?**
The study will last for a total of 32 weeks and you will be asked to attend the Endocrinology Investigation unit on 4 separate occasions. Each of these visits will last approximately 2 hours.

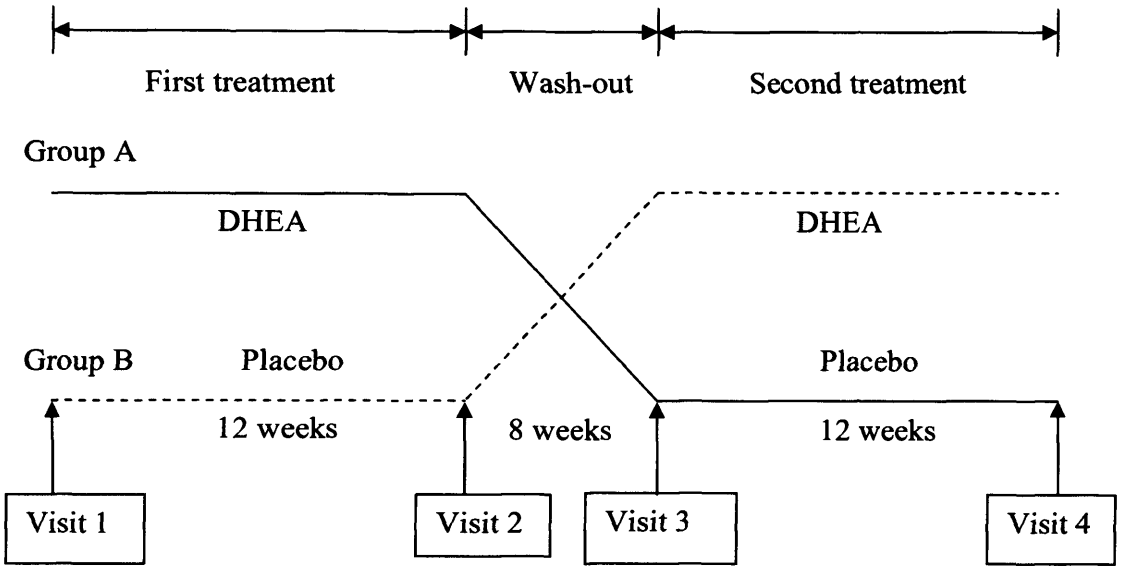
In order to know whether DHEA changes blood vessel function we need to make comparisons. DHEA will be compared in this study with a placebo tablet. A placebo is a dummy treatment such as a pill which looks like the real thing but is not. It contains no active ingredient.

People will be put into two groups and then compared. The groups are selected by a computer which has no information about the individual i.e. by chance (or randomised). Half the patients will be put into group A and will receive DHEA treatment for 12 weeks. The other half will be put into group B and will receive placebo treatment. These groups will be compared.

Neither you nor your doctor will know in which treatment group you are (although your doctor is able to find out if he/she needs to do so). This is to make sure that the results cannot be influenced in any way.

At the end of 12 weeks of treatment you will have a break of 8 weeks before taking the other treatment for a further 12 weeks (placebo treatment if you received DHEA for the first part of the study and DHEA if you received placebo initially). The break of 8 weeks between treatments will ensure that the first drug is cleared from your body before you start the new treatment.

A summary of what will happen and when the study visits will take place is shown in the following flowchart:




On each visit the measurements performed are designed to measure the stiffness of your blood vessels. This is known as Pulse Wave Analysis. **The techniques that we use are completely painless and 'non-invasive'**. The main equipment we use is called a "Sphygmocor" machine. This consists of a small pencil-like probe placed over the wrist, which measures the pulse's waveform. During the measurements you will also be asked to take a puff of a salbutamol inhaler (like that used in asthma) and a GTN tablet under your tongue (like that used in angina). Both these drugs act to change the shape of the pulse waveform that we measure. Both drugs are safe and are unlikely to produce any side effects although you may experience a slight headache after the GTN. We would then use the "Sphygmocor" machine to measure something called Pulse Wave Velocity. This looks at how fast your pulse travels along blood vessels. It is done in the same way by putting a pencil-like probe on the skin. This time it is put on the skin over three different arteries, at the wrist (the radial artery), in the neck (the carotid artery) and at the top of the leg (the femoral artery). Whilst this is being done you are put an ECG machine so that we can time the pulse with your heart beat.

During each visit we will also take blood samples (approximately three tablespoons) to check your hormones, cholesterol, insulin, clotting factors and CRP (a non-specific marker of inflammation in the body). Your height, weight and blood pressure will also be measured at each visit as well as something called Bioimpedance. This is measured using a specialised weighing scale and measures levels of body fat and body muscle.

**7. What do I have to do?**
It is important that you take your regular medication in the normal way without altering the dose or timing of these during the course of the study, though you will be permitted to increase the dose of your steroid tablets for a short while if you developed an illness coincidentally. You should inform us of any dose adjustments.
You will be asked to take the study medication regularly for the duration of the study. The tablets will be taken once a day.
There is no lifestyle or dietary restrictions and you can continue your daily activities normally. We request that you report any illnesses to us as they may influence the timing of the test visits.
For women, you should inform us immediately if you become pregnant.
For each of the study visits, we ask that you attend the Endocrine Investigation unit at 9 o'clock in the morning having fasted from midnight the previous night. You can drink water freely.

**8. What is the drug that is being tested?**
DHEA is a naturally occurring hormone that is produced by the adrenal glands and many patients with adrenal insufficiency are now taking this treatment on a regular basis. A dose of 50 mg is being used in this study as this is the dose that restores the levels of DHEA in the blood to normal. The drug comes in tablet form and we will provide you with a card to carry explaining the details of this trial. You should carry this with you at all times.

**9. What are the side effects of DHEA?**
DHEA is a naturally occurring hormone and is a safe, established treatment in adrenal insufficiency. No major side effects have been reported with this treatment. However, a small number of women have experienced minor acne in some studies and we will ask you to report any side effects to us at each study visit. There have been very rare cases of minor abnormal liver tests also reported and we will be monitoring these. You will be told if any new side effect is found as a result of this study or any other studies using DHEA.
If you become in any way concerned you should contact Dr Sam Rice on telephone number 02920 746357 or 07866 741 543.

**10. What are the possible disadvantages and risks of taking part?**
DHEA can cause some minor acne in women and abnormal liver tests have been recorded rarely in patients taking DHEA. Other than possible discomfort (temporary pain, swelling, bruising and rarely infection) caused by the collection of blood, no other side effects are anticipated from the study procedures.

The risks to an unborn human foetus or a breastfed child from DHEA are not known. Women who are pregnant or breastfeeding a child may therefore not participate in this trial, neither should women who plan to become pregnant during the study. Women who are at risk of pregnancy will have a pregnancy test before taking part in the study.

Women who could become pregnant must use an effective contraceptive during the course of this study. Any woman who finds that she has become pregnant while taking part in the study should immediately tell her research doctor.

**11. What are the potential benefits of taking part?**
Some patients may experience improved mood and well-being. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with adrenal insufficiency better.

**12. What if new information becomes available?**
Sometimes during the course of a research project, new information becomes available about the drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

**13. What happens when the research study stops?**
If you feel you would wish to continue DHEA therapy at the end of the study you will be able to discuss this with your Endocrinologist.

**14. What if something goes wrong?**
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

**15. Will my taking part in this study be kept confidential?**
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.
With your permission your GP will be informed of your participation in this study.

**16. What will happen to the results of the research study?**
The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have been published in the relevant journal(s). Patients will not be identified in any report/publication.
Patients will be able to discover which arm of the study they were in if they so wish, but only after the study has been completed in its entirety.

**17. Who is organising and funding the research?**
The study is being organised by Dr Aled Rees in the Section of Endocrinology (the Principal Investigator) and Dr Sam Rice (Clinical Research Fellow). Funding for the study is provided from the Section of Endocrinology and the doctors conducting the research are not being paid for including and looking after patients in the study.

**18. Who has reviewed the study?**
The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South East Wales Local Research Ethics Committee.

**19. Contact for further information**
Should you have any further questions or queries regarding this research study, then please do not hesitate to contact Dr Aled Rees on **029 20745002** or Dr Sam Rice on **029 20746357** . If an emergency should arise during the study Dr Rice can be contacted on **07866 741 543**.

Thank you for taking part in this study.

Dr Aled Rees
Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

**DHEA study consent form**

**Adrenal insufficiency and vascular dysfunction.**

Patient Identification Number for this trial:

# CONSENT FORM

**Version 4, September 2005.**

**Title of project:** A randomised, double blind, placebo-controlled, cross-over study of the effects of dehydroepiandrosterone replacement on vascular function in patients with primary and secondary adrenal insufficiency

Name of Researchers: Dr Aled Rees, Dr Sam Rice

| | **Please initial box** |
|---|---|
| 1. I confirm that I have read and understood the information sheet dated September 2005 (version 4) for the above study and have had the opportunity to ask questions. | ☐ |
| 2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected | ☐ |
| 3. I understand that sections of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. | ☐ |
| 4. Do you consent to your GP being informed of your participation in the Study. | ☐ |
| 5. If I am at risk of pregnancy I consent to a pregnancy test. | ☐ |
| 6. I agree to take part in the above study. | ☐ |

| | | |
|---|---|---|
| Name of patient | Date | Signature |
| Name of person taking consent (if different from researcher) | Date | Signature |
| Researcher | Date | Signature |

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

**Endothelial function assessment protocol**

**Run in**

| Time | | Augmentation index a | Augmentation index b |
|---|---|---|---|
| + 5 minutes | Blood pressure | | |
| | Heart rate | | |
| + 7 minutes | Augmentation Index | | |
| + 15 minutes | Blood pressure | | |
| | Heart Rate | | |
| + 17 minutes | Augmentation index | | |

**PART B (GTN – endothelial independent vasodilation)**

**A 500mcg GTN tablet is placed under the tongue and the stopwatch is started**

| Time | | Augmentation index a | Augmentation index b |
|---|---|---|---|
| +1 minute | Blood pressure | | |
| | Heart rate | | |
| +3 minutes REMOVE GTN | Augmentation Index | | |
| +5 minutes | Augmentation Index | | |
| +7 minutes | Blood pressure | | |
| | Heart rate | | |
| +10 minutes | Augmentation Index | | |
| +12 minutes | Blood pressure | | |
| | Heart rate | | |
| +15 minutes | Augmentation Index | | |
| +17 minutes | Blood pressure | | |
| | Heart rate | | |
| +20 minutes | Augmentation Index | | |
| +25 minutes | Blood pressure | | |
| | Heart rate | | |
| +28 minutes | Augmentation Index | | |

**PART C (SALBUTAMOL –endothelial independent vasodilation)**

**Patient practices breathing in and holding breath using spacer device. 2 puffs of salbutamol are delivered into device, patient takes inhales maximally and holds breath for 10 seconds then exhales, a further 2 puffs of salbutmol are delivered and the breathing manoeuvres are repeated a second time. The stopwatch is started following the final exhalation.**

| Time | | Augmentation index a | Augmentation index b |
|---|---|---|---|
| +2 minutes | Blood pressure | | |
| | Heart rate | | |
| +5 minutes | Augmentation Index | | |
| +7 minutes | Blood pressure | | |
| | Heart rate | | |
| +10 minutes | Augmentation Index | | |
| +12 minutes | Blood pressure | | |
| | Heart rate | | |
| +15 minutes | Augmentation Index | | |
| +17 minutes | Blood pressure | | |
| | Heart rate | | |
| +20 minutes | Augmentation Index | | |

# REFERENCES

1. Migeon, C., Plager JE., *Identification and isolation of dehydroisoandrosterone from peripheral human plasma.* The Journal of Biological Chemistry, 1954. **209**: p. 767-772.
2. Parker, L., *Adrenal Androgens in Clinical Medicine.* 1989, San Diego: Academic Press, Inc.
3. Orentreich, N., Brind, JL., Rizer, RL., Vogelman, JH., *Age changes and sex differences in serum dehydroepiandrosterone sulphate concentrations throughout adulthood.* Journal of Clinical Endocrinology and Metabolism, 1984. **59**(3): p. 551-555.
4. Bonny, R., Scanlon, MJ., Jones, DL., Beranek, PA., Reed, MJ., and James, VHT., *The interrelationship between plasma 5-ene adrenal androgens in normal women.* Journal of Steroid Biochemistry, 1984. **20**(6A): p. 1353-1355.
5. Tchernof, A., Despres, J, Bélanger, A., Dupont, A., Prud'homme, D., Moorjani, S. Lupien, PJ., Labrie, F., *Reduced testosterone and adrenal $C_{19}$ steroid levels in obese men* Metabolism, 1995. **44**(4): p. 513-519.
6. Couillard, C., Gargnon, J., Bergeron, J., Leon, AS., Roa, DC., Skinner, JS., Wilmore, JH., Després, J., Bouchard, C. , *Contribution of body fatness and adipose tissue disribution to the age variation in plasma steroid hormone concentrations in men: the HERITAGE family study.* Journal of Clinical Endocrinology and Metabolism, 2000. **85**(3): p. 1026-1031.
7. Haffner, S., Valdez, RA., Mykkänen, L., Stern, MP., Katz, MS., *Decreased testosterone and dehyroepiandrosterone sulfate concentrations are associated with increased insulin and glucose concentrations in nondiabetic men.* Metabolism, 1994. **43**(5): p. 599-603.
8. Trivedi, D., Khaw, KT., *Dehydroepiandrosterone sulphate and mortality in elderly men and women.* Journal of Clinical Endocrinology and Metabolism, 2001. **86**(9): p. 4171-4177.
9. Berr, C., Lafont, S., Deburie, B., Dartigues, J., Baulieu, E., *Relationship of dehydroepiandrosterone sulfate in the elderly with functional, psychological and mental status and short term mortality: A French community-based study.* Proceedings of the National Academy for Science U.S.A, 1996. **93**: p. 13410-13415.
10. Dhatariya, K., Bigelow, ML., Nair, SK., *Effect of dehydroepiandrosterone replacement on insulin sensitivity and lipids in hypoadrenal women.* Diabetes, 2005. **54**: p. 765-769.
11. Lasco, A., Frisina, N., Morabito, N., Gaudio, A., Morini, E., Trifiletti, A., Basile, G., Nicita-Mauro, V., Cucinotta, D., *Metabolic effects of dehyroepiandrosterone replacement therapy in postmenopausal women.* European Journal of Endocrinology, 2001. **145**: p. 457-461.
12. Arlt, W., Callies, F., Van Vlijmen, JC., Koehler, I., Reincke, M., Bidlingmaier, M., Heubler, D., Oettel, M., Ernst, M., Schult, HM., Allolio, B., *Dehyroepiandrosterone replacement in women with adrenal insufficiency* New England Journal of Medicine, 1999. **341**(14): p. 1013-1020.
13. Hunt, P., Gurnell, EM,. Huppert, FA,. Richards, C,. Prevost, AT,. Wass, JAH,. Herbert, J,. Chatterjee, VKK., *Improvement in mood and fatigue after*

*dehydroepiandrosterone replacement in Addison's disease in a randomized, double blind trial.* . Journal of Clinical Endocrinology and Metabolism, 2000. **85**: p. 4650-4656.

14. Mavoungou, D., Poaty-Mavoungou., Akoume, M., Ongali, B., Mavoungou, E., *Inhibition of human immunodeficiency virus type-1 (HIV-1) glycoprotein - mediated cell-cell fusion by Immunor (IM28).* Virology Journal, 2005. **2**(9).
15. Hernandez-Pando, R., Aguilar-Leon, D,. Orozco, H., Serrano, A., Ahelm, C.,Trauger, R,. Schramm, B., Reading, C., Frinke, J., Rook, GA., *16 alpha-Bromoepiandrosterone restores T-helper cell type 1 activity and accelerates chemotherapy -induced bacterial clearance in a model of progressive pulmonary tuberculosis* Journal of Infectious disease, 2005. **191**: p. 299-306.
16. dos Santos, C., Toldo, MPA., Junior, JCdP., *Trypanosoma cruzi: the effects of dehyroepiandrosterone (DHEA) treatment during experimental infection.* Acta. Trop, 2005. **95**: p. 109-115.
17. Liu, S., Ishikawi, H., Li, F., Ma, Z., Otsuyama, K., Asaoka, H,. Abroun, S., Zheng, X., Tsuyama, N., Obata, M., Kawano, MM., *Dehydroepiandrosterone can inhibit the proliferation of myeloma cells and the interleukin-6 production of bone marrow mononuclear cells from patients with myeloma.* Cancer Research, 2005. **65**(6): p. 2269-2276.
18. Osawa, E., Nakajima, A., Yoshida, S., Omura, M., Nagase, H., Ueno, N., Wada, K., Matsuhashi, N., Ochiai, M., Nakagama, H., Sekihara, H. , *Chemoprotection of precursors to colon cancer by dehydroepiandrosterone (DHEA).* Life Sciences, 2002. **70**: p. 2623-2630.
19. Muscarella, P., Boros, LG., Fisher, WE., Rink, C., Melvin, WS., *Oral dehydroepiandrosterone inhibits the growth of human pancreatic cancer in nude nice.* Journal of Surgical Research, 1998. **79**: p. 154-157.
20. McCormick, D., Johnson, WD., Kozub, NM., Roa, KVN., Lubet, RA., Steele, VE., Bosland, MC., *Chemoprevention of rat prostate carcinogenesis by dietary 16alpha-fluoro-5-androsten-17-one (fluasterone), a minimally androgenic analog of dehydroepiandrosterone.* Carcinogenesis, 2007. **28**(2): p. 398-403.
21. Oberbeck, R., Dahlweid, M., Koch, R., van Griensven, M., Emmendorfer, A., Tscherne, H., Pape, H., *Dehydroepiandrosterone decreases mortality rate and improves cellular immune function during polymicrobial sepsis.* Critical Care Medicine, 2001. **29**(2): p. 380-384.
22. Solerte, S., Ferrari, E., Cuzzoni, G., Locatelli, E., Guistina, A., Zamboni, M., Schifino, N, Rondanelli, M., Gazzaruso, C., Firoavanti, M. , *Decreased release of angiogenic peptide vascular endothelial growth factor in Alzheimer's disease: recovering effect with insulin and DHEA sulphate.* Dementia and Geriatric Cognitive Disorders, 2005. **19**(1): p. 1-10.
23. Payne, A., Hales, DB., *Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.* Endocrine Reviews, 2004. **25**(6): p. 947-970.
24. Mellon-Nussbaum, S., Welch, M., Bandy, L., and Lieberman, S., *The lipoidal derivatives of steroids as biosynthetic intermediates.* Journal of Biological Chemistry, 1980. **255**(6): p. 2487-2492.

25. Lieberman, S., Greenfield, N., and Wolfson, A., *A heuristic proposal for understanding steroidogenic processes.* Endocrine Reviews, 1984. **5**(1): p. 128-148.
26. Drucker, W., *Biological activity and metabolism of androgenic hormones: the role of adrenal androgens.* Bull. N. Y. Acad. Med., 1977. **53**(4): p. 347-358.
27. Adams, J., Mcdonald, D., *Enzymic synthesis of steroid sulphates.* Biochimica et Biophysica Acta, 1981. **664**: p. 460-468.
28. Boström, H., and Wengle, B., *Studies on ester sulphates: Distribution of phenol and steroid sulphokinases in adult human tissue.* Acta Endocrinologica 1967. **56**.
29. Payne, A., Singer, S., ed. *The role of steroid sulfatase and sulfotransferase enzymes in the metabolism of C21 and C19 steroids.* ed. R. Hobkirk. 1979, CRC press: Boca Raton, Florida.
30. Clair, P., Patricot, MC., Mathian, B., Revol, A., *Androgen metabolism in vitro by human leukocytes. Variations with sex and age.* Journal of Steroid Biochemistry, 1984. **20**(1): p. 377-381.
31. Milewich, L., Garcia, RL., Johnson, AR., *Steroid sulfatase activity in human lung tissue and in endothelial pulmonary cells in culture.* Journal of Clinical Endocrinology and Metabolism, 1983. **57**(1): p. 8-14.
32. Allolio, B., in *British Endocrine Society.* 2005: Harrogate.
33. Kumar, A., Woods, KS, Bartolucci, AA, Azziz, R., *Prevalence of adrenal androgen excess in patients with ploycystic ovary syndrome (PCOS).* Clinical Endocrinology, 2005. **62**: p. 644-649.
34. Plagger, J., *The binding of Androsterone Sulphate, Etiocholanolone Sulphte, and Dehydroisoandrosterone Sulphate by Human Plasma Protein* Journal of Clinical Investigation, 1965. **44**(7): p. 1234-1239.
35. Dunn, J., Nisula, BC., Rodbard, D. , *Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteriod-binding globulin in human plasma.* . Journal of Clinical Endocrinology and Metabolism, 1981. **53**(1): p. 58-68.
36. Luu The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, JL., Strickler, RC., Labrie, F., *Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase* Molecular Endocrinology, 1989. **3**(8): p. 1310-1312.
37. Pelletier, G., Dupont, E., Simard, J., Luu-The, V., Bélanger, A., Labrie, F., *Ontogeny and subcellular localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the human and rat adrenal, ovary and testis.* Journal of Steroid Biochemistry and Molecular Biology, 1992. **43**(5): p. 451-467.
38. Milewich, L., Shaw, CE., Mason, JI., Carr, BR., Blomquist, CH., Thomas, JL., *3β-hydroxysteroid dehydrogenase activity in tissues of the human fetus determined with 5α-androstane-3β,17β-diol and dehydroepiandrosterone as substrates.* Journal of Steroid Biochemistry and Molecular Biology, 1993. **45**(6): p. 525-533.
39. Martel, C., Melner, MH., Gagné, D., Simard, J., Fabrie, F., *Widespread tissue distribution of steroid sulphatase, 3β-hydroxysteroid dehydrogenase/Δ5 -Δ4*

*isomerase (3β-HSD), 17β-HSD 5α-reducase and aromatase activites in the rhesus monkey* Molecular and Cellular Endocrinology, 1994. **104**: p. 103-111.
40. Labrie, F., Simard, J., Luu-The, V., Bélanger, A., Pelletier, G., *Structure, function and tissue-specific gene expression of 3β-hydroxysteriod dehydrogenase/5-ene-4-ene isomerase enzymes in classical and peripheral intracrine steroidogenic tissues.* Journal of Steroid Biochemistry and Molecular Biology, 1992. **43**(8): p. 805-826.
41. Puddefoot, J., Barker, S., Vinson, GP. , *Trilostane in advanced breast cancer.* Expert Opinion on Pharmacotherapy, 2006. **7**(17): p. 2413-2419.
42. Puddefoot, J., Barker, S., Glover, HR., Malouitre, SD., Vinson, GP., *Non-competative steroid inhibition of oestrogen receptor funtions.* International Journal of Cancer, 2002. **101**: p. 17-22.
43. Luu-The, V., Zhang, Y., Poirier, D., Labrie, F., *Characteristics of human types 1, 2 and 3 17β-hydroxysteroid dehydrogenase activites: oxidatio-reduction and inhibition.* Journal of Steroid Biochemistry and Molecular Biology, 1995. **55**(5/6): p. 581-587.
44. Luu-The, V., Dufort, I., Pelletier, G., Labrie, F., *Type 5 17β-hydroxysteroid dehydrogenase: its role in the formation of androgens in women.* Molecular and Cellular Endocrinology 2001. **171**: p. 77-82.
45. Dufort, I., Rheault, P., Huang, XF., Soucy, P., Luu-The, V., *Characteristics of a highly labile human type 5 beta-hydroxysteroid dehydrogenase.* Endocrinology, 1999. **140**(2): p. 568-574.
46. Haung, X.-F., Luu-The, V., *Molecular characterization of a first human 3 (alpha-beta)-hydroxysteroid epimerase* Journal of Biological Chemistry, 2001. **275**(38): p. 29452-29457.
47. Luu-The, V., Sugimoto, Y., Puy, L., Labrie, Y., Solache, IL., Singh, M., Labrie, F., *Characterization, expression and immunohistochemical localisation of 5α-reductase in human skin.* Journal of Investigative Dermatology, 1994. **102**: p. 221-226.
48. Andersson, S., Berman, DM., Jenkins, EP., Russel, DW., *Deletion of steriod 5α-reductase 2 gene in male pseudohermaphroditism.* Nature, 1991. **354**: p. 159-161.
49. Labrie, F., *Intracrinology.* Molecular and Cellular Endocrinology, 1991. **78**(3): p. C113-C118.
50. Martel, C., Rhéaume, E., Takahashi, M., Trudel, C., Couët, J., Luu-The, V., Simard, J., Labrie, F., *Distribution of 17β-hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues.* Journal of Steroid Biochemistry and Molecular Biology 1992. **41**(3-8): p. 597-603.
51. Arlt, W., Justl, HG., Callies, F., Reincke, M., Hubler, D., Oettel, M., Ernst, M., Schulte, HM., Allolio, B. , *Oral dehydroepiandrosterone for adrenal androgen replacement: pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression.* Journal of Clinical Endocrinology and Metabolism, 1998. **83**(6): p. 1928-1934.
52. Arlt, W., Haas, J., Callies, F., Reincke, M., Hubler, D., Oettel, M., Ernst, M., Schulte, HM., Allolio, B. , *Biotransformation of oral dehydroepiandrosterone in*

*elderly men: significant increase in circulating estrogens.* Journal of Clinical Endocrinology and Metabolism, 1999. **84**(6): p. 2170-2176.
53. Young, J., Couzinet, B., Nahoul, K., Brailly, S., Chason, P., Baulieu, EE., Schaison, G., *Panhypopituitarism as a model to study the metabolism of dehydroepiandrosterone (DHEA) in humans.* Journal of Clinical Endocrinology and Metabolism, 1997. **82**(8): p. 2578-2585.
54. Bird, C., Murphy, J., Boroomand, K., Finnis, W., Dressel, D., Clark, AF., *Dehydroepiandrosterone: Kinetics of metabolism in normal men and women.* Journal of Clinical Endocrinology and Metabolism, 1978. **47**(4): p. 818-822.
55. Rosenfeld, R., Rosenberg, BJ., Fukushima, DK., Hellman, L., *24 Hours secertory pattern of dehydroepiandrosterone and dehydroepiandrosterone sulfate.* Journal of Clinical Endocrinology and Metabolism, 1975. **40**(5): p. 850-855.
56. Bélanger, B., Bélanger, A., Labrie, F., Dupont, A., Cusan, L., Monfette, G., *Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men.* Journal of Steroid Biochemistry, 1989. **32**(5): p. 695-698.
57. Rosenfeld, R., Hellman, L., Gallagher, T., *Metabolism and interconversion of dehydroisoandrosterone and dehydroisoandrosterone sulphate.* Journal of Clinical Endocrinology and Metabolism, 1972. **35**(2): p. 187-193.
58. Romanoff, L., Baxter, MN., *The secretion rates of deoxycorticosterone and corticosterone in young and elderly men.* Journal of Clinical Endocrinology and Metabolism, 1975. **41**(3): p. 630-633.
59. Flood, C., Gherondache, C., Pincus, G., Tait, JF., Tait, SAS., Willoughby, S., *The metabolism and secretion of aldosterone in elderly subjects.* Journal of Clinical Investigation, 1967. **46**(6): p. 960-965.
60. Hopper, B., Yen, SSC., *Circulating concentrations of dehydroepiandrosterone and dehydroepiandrosterone sulfate during puberty* Journal of Clinical Endocrinology and Metabolism, 1975. **40**(3): p. 458-461.
61. Rotter, J., Wong., L, Lifrak, ET., Parker, LN., *A genetic component to the variation of dehydroepiandrosterone sulfate.* Metabolism, 1985. **34**(8): p. 731-736.
62. de la Torre, B., Sjoberg, B,. Hedman, M., Bartfai, G., Diczalusy, E., *A study of the short-term variation and interrelationship of plasma hormone levels reflecting pituitary, adrenocortical and testicular function in fertile men.* Int. J. Androl., 1981. **4**(5): p. 532-545.
63. Deslypere, J., Bishop, G., Vermeulen, A., *Seasonal variation of plasma dehydroepiandrosterone sulphate and urinary androgen excretion in post-menopausal women.* Clinical Endocrinology, 1983. **18**: p. 25-30.
64. Easterling, W., Simmer, HH., Digram, WJ., Frankland, MV., Naftolin, F., *Neutral C-19 steroids and steroid sulfates in human pregnancy.* Steroids, 1966. **8**: p. 157-178.
65. Nahoul, K., Daffos, F., Forestier, F., Scholler, R., *Cortisol, cortisone and dehydroepiandrosterone sulfate levels in umbilical cord and maternal plasma between 21 and 30 weeks of pregnancy.* Journal of Steroid Biochemistry, 1985. **23**(4): p. 445-450.

66. Compagnon, N., Mellon, SH., *Dehydroepiandrosterone: a potential signaling molecule for neocortical organization during development.* Proceedings of the National Academy for Science U.S.A, 1998. **95**: p. 4678-4683.
67. Mesiano, S., Katz, SL., Lee, JY., Jaffe, RB., *Insulin-like growth factors augment steriod production and expression of steroidogenic enzymes in human fetal adrenal cortical cells: Implications for adrenal androgen regulation.* Journal of Clinical Endocrinology and Metabolism, 1997. **82**(5): p. 1390-1396.
68. de Peretti, E., Forest, M. , *Pattern of plasma DHEAS levels in humans from birth to adulthood: Evidence for testicular production.* Journal of Clinical Endocrinology and Metabolism, 1978. **47**: p. 572.
69. Rieter, E., Fuldauer, VG., Root, AW., *Secretion of the adrenal androgen, dehydroepiandrosterone sulphate, during normal infancy, childhood, and adolescence, in sick infants, and in children with endocrinologic abnormalities.* Journal of Paediatrics, 1977. **90**(5): p. 766-770.
70. Vermulen, A., Deslypere, J., Schelfhout, W., Verdonck, L., Rubens, R., *Adrenocortical function in old age: Response the acute ACTH stimulation.* Journal of Clinical Endocrinology and Metabolism, 1982. **54**(1): p. 187-191.
71. Parker, L., Eugene, J., Farber, D., Lifrak, E., Lai, M., and Juler, G., *Dissociation of adrenal androgen and cortisol levels in acute stress.* Hormone and Metabolism Research, 1985. **17**: p. 209.
72. Zumoff, B., Walsh, B., Katz, J., Levin, J., Rosenfeld, R., Kream, J., Weiner, H., *Subnormal plasma DHA to cortisol ratio in anorexia nervosa: A second hormonal parameter of ontogenic regression.* Journal of Clinical Endocrinology and Metabolism, 1983. **56**(4): p. 668-672.
73. Parker, L., Lifrak, ET., Odwell, WD., *A 60,000 molecular weight human pituitaty glycopeptide stimualtes adrenal androgen secretion.* Endocrinology, 1983. **133**: p. 2092-2096.
74. Anderson, D., *The adrenal androgen-stimulating hormone does not exist.* Lancet, 1980. **2**: p. 454-456.
75. Labrie, F., Luu-The, V., Bélanger, A., Lui, S-X., Simard, J., Pelletier, G., Labrie, C., *Is dehydroepiandrosterone a hormone?* Journal of Endocrinology, 2005. **187**: p. 169-196.
76. Majewska, M., Demirgören, S., Spivak, CE., London, ED. , *The neurosteroid dehydroepiandrosterone sulphate is an allosteric antagonist of the $GABA_A$ receptor.* Brain Research, 1990. **526**: p. 143-146.
77. Demirgören, S., Majeweska, MD., Spivak, CE., London, ED., *Receptor binding and electrophysiological effects of dehydroepiandrosterone sulphate, an antagonist of the $GABA_A$ receptor.* Neuroscience, 1991. **45**(1): p. 127-135.
78. Corpéchot, C., Robel, P., Axelson, M., Sjövall, M., Baulieu, E., *Characterization and measurement of dehydroepiandrosterone sulphate in rat brain* Proceedings of the National Academy for Science U.S.A, 1981. **78**(8): p. 4704-4707.
79. Okabe, T., Haji, M., Takayanagi, R., Adachi, M., Imasaki, K., Kurimoto, F., Watanabe, T., Nawata, H., *Up-regulation of high-affinity dehydroepiandrosterone binding activity in activted human T lymphocytes.* Journal of Clinical Endocrinology and Metabolism, 1995. **80**(10): p. 2993-2996.

80. Lui, D., Dillon, JS., *Dehydroepiandrosterone activates endothelial cell nitric-oxide synthase by a specific plasma membrane receptor coupled to* $G\alpha_{i2,3}$. Journal of Biological Chemistry, 2002. **277**(24): p. 21379-21388.
81. Liu, D., Dillon, JS., *Dehydroepiandrosterone stimulates nitric oxide release in vascular endothelial cells: evidence for a cell surface receptor.* Steroids, 2004. **69**: p. 279-289.
82. Chen, F., Knecht, K., Birzin, E., Fisher, J., Wilkinson, H., Mojena, M., Morena, CT., Shmidt, A., Harada, S., Freedman, LP., Reszka AA. , *Direct agonist/antagonist functions of dehydroepiandrosterone.* Endocrinology, 2005. **146**(11): p. 4568-4576.
83. Brooke, A., Kalingag, LA., Miraki-Moud, F., Camacho-Hübner, C., Maher, KT., Walker, DM., Hinson, JP., Monson, JP., *Dehydroepiandrosterone (DHEA) replacement reduces growth hormone (GH) dose requirement in female hypopituitary patients on GH replacement.* Clinical Endocrinology, 2006. **65**: p. 673-680.
84. Morales, A., Nolan, JJ., Nelson, JC., Yen, SSC., *Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age.* Journal of Clinical Endocrinology and Metabolism, 1994. **78**: p. 1360-1367.
85. Fehér, T., Bodrogi, L., *A comparative study of steroid concentrations in human adipose tissue and the peripheral circulation.* Clinica Chimica Acta, 1982. **126**: p. 135-141.
86. Bleau, G., Roberts, KD., Chapdelaine, A. , *The in vitro and in vivo uptake and metabolism of steroids in human adipose tissue.* Journal of Clinical Endocrinology and Metabolism, 1974. **39**(2): p. 236-246.
87. Dalla Valle, L., Toffolo, V., Nardi, A., Fiore, C., Bernante, P., Di Liddo, R., Parnigotto, PP., Colombo, L., *Tissue-specific transcriptional initiation and activity of steroid sulfatase complementing dehyroepiandrosterone sulfate uptake and intracriine steroid activations in human adipose tissue.* Journal of Endocrinology, 2006. **190**: p. 129-139.
88. Garruti, G., Ricquier, D., *Analysis of uncoupling protein and its mRNA in adipose tissue of adult humans.* International Journal of Obesity and Related Metababolic Disorders, 1992. **16**(5): p. 383-390.
89. Lean, M., James, WP., Jennings, G., Trayhurn, P., *Brown adipose tissue in patients with phaeochromocytoma.* International Journal of Obesity, 1986. **10**(3): p. 219-227.
90. Rehnmark, S., Nedergaard, J., *DNA synthesis in mouse brown adipose tissue is under beta-adrenergic control.* Experiamtal and Cellular Research, 1989. **180**(2): p. 574-579.
91. Lowell, B., Flier, JS., *Brown adipose tissue, beta 3-adrenergic receptors, and obesity.* Annual Reviews in Medicine, 1997. **48**: p. 307-316.
92. Puigserver, P., Wu, Z., Park, CW., Graves, R., Wright, M., Speigelman, BM. , *A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis.* Cell, 1998. **92**: p. 829-839.
93. Reaven, G., *Banting lecture 1988. Role of insulin resistance in human disease.* Diabetes, 1988. **37**(12): p. 1595-1607.

94. Rosen, E., Macdougald, OA., *Adipocyte differentiaiton from the inside out.* Molecular cell biology, 2006. **7**: p. 885-896.
95. Tontonoz, P., Hu, E., Spiegelman, BM. , *Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activted transcription factor* Cell, 1994. **79**: p. 1147-1156.
96. Zhang, J., Fu, M., Cui, T., Xiong, C., Xu, K., Zhong, W., Xiao, Y., Floyd, D., Liang, J., Li, E., Song, Q., Chen, YE. , *Selective disruption of PPARγ2 impairs the development of adipose tissue and insulin sensitivity.* Proceedings of the National Academy for Science U.S.A, 2004. **101**(29): p. 10703-10708.
97. Tamoro, Y., Masugi, J., Nishino, N., Kasuga, M., *Role of peroxisome proliferator-activated receptor-γ in maintenance of the characteristics of mature 3T3-L1 adipocytes.* Diabetes, 2002. **51**: p. 2045-2055.
98. Savage, D., Tan, GD., Acerini, CL., Jebb, SA., Agostini, M., Gurnell, M., Williams, RI., Umpleby, M., Thomas, EL., Bell, JD., Dixon, AK., Dunne, F., Boiani, R., Cinti, S., Vidal-Puig, A., Karpe, F., Chaterjee, VKK., O'Rahilly, S., *Human metabolic syndrome resulting from dominant-negative mutations in the nuclear receptor peroxisome proliferator-activated receptor-γ.* Diabetes, 2003. **52**(4): p. 910-917.
99. Tong, Q., Dalgin, G., Xu, H., Ting, C., Leiden, JM., Hotamisligil, GS., *Function of GATA transcription factors in preadipocyte-adipocyte transition* Science, 2000. **290**: p. 134-138.
100. Fajas, L., Fruchart, J., Auwerx, J., *PPARγ3 mRNA: a distinct PPARγ mRNA subtype transcribed from an independent promotor* FABS letters, 1998. **438**(1-2): p. 55-60.
101. Mukherjee, R., Jow, L., Croston, GE., Paterniti., JR, Jr., *Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARγ2 versus PPARγ1 and activation with retinoid X receptor agonists and antagonists* Journal of Biological Chemistry, 1997. **272**(12): p. 8071-8067.
102. MacDougald, O., Cornelius, P., Lin, F., Chen, SS., Lane, MD. , *Glucocorticoids reciprocally regulate expression of the CCAAT/Enhancer-binding protein α and δ genes in 3T3-L1 adipocytes and adipose tissue.* Journal of Biological Chemistry, 1994. **269**(29): p. 19041-19047.
103. Linhart , H., Ishimura-Oka, K., DeMayo, F., Kibe, T., Repka, D., Poindexter, B., Bick, RJ., Darlington, GJ., *C/EBPα is required for differentiation of white but not brown adipose tissue.* Proceedings of the National Academy for Science U.S.A, 2001. **98**(22): p. 12532-12537.
104. Mori, T., Sakaue, H., Iguchi, H., Gomi, H., Okade, Y., Takashima, Y., Nakamura, K., Nakamura, T., Yamauchi, T., Kubota, N., Kadowaki, T., Matsuki, Y., Ogawa, W., Hiramatsu, R., Kasuga, M. , *Role of krupple-like factor 15 (KLF15) in transcriptional regulation of adipogenesis.* Journal of Biological Chemistry, 2005. **280**(13): p. 12867-12875.
105. Gray, S., Feinberg, MW., Hull, S., Kuo, CT., Watanabe, M., Banerjee, SS., Depina, A., Haspel, R., Jain, MK., *The krupple-like factor KLF15 regulates the*

*insulin-sensitive glucose transporter GLUT4.* Journal of Biological Chemistry, 2002. **277**(37): p. 34322-34328.

106. Banjeree, S., Feinberg, MW., Watanebe, M., Grey, S., Haspel, RL., Denkinger, DJ., Kawahara, R., Hauner, H., Jain, MK., *The krupple-like factor KLF2 inhibits peroxisome proliferator-activated receptor-γ expression and adipogenesis.* Journal of Biological Chemistry, 2003. **278**(4): p. 2581-2584.
107. Chen, Z., Torrens, JI., Anand, A., Spiegelman, BM. Friedman, JM., *Krox20 stimulates adipogenesis via C/EBPβ -dependent and -independent mechanisms.* Cell Metabolism, 2005. **1**: p. 93-106.
108. Kim, J., Wright, HM., Wright, M., Spiegelman, BM., *ADD1/SREBP1 activates PPARγ through the production of endogenoues ligand* Proceedings of the National Academy for Science U.S.A, 1998. **95**: p. 4333-4337.
109. Frühbeck, G., Gomez-Ambrosi, J., Muruzabal, FJ., Burrell, MA., *The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation* American Journal of Physiology Endocinololgy and Metabolism, 2001. **280**: p. E827-E847.
110. Kisseleva, T., Bhattacharya, S., Braunstein, J., Schindler, CW. , *Signaling through the JAK/STAT pathway, recent advances and future challenges.* Gene, 2002. **285**: p. 1-24.
111. Kolch, W., *Meaningful relationship: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions.* Biochemistry Journal, 2000. **351**: p. 289-305.
112. Bost, F., Aouadi, M., Caron, L., Binetruy, B., *The role of MAPKs in adipocyte differentiaiton and obesity.* Biochimie, 2005. **87**: p. 51-56.
113. Aouadi, M., Binetury, B., Caron, L., Le Marchand-Brustel, Y., Bost, F. , *Role of MAPKs in development and differentiation: lesons from knockout mice.* Biochimie, 2006. **88**: p. 1091-1098.
114. Garofalo, R., Orena, SJ., Rafidi, K., Torchia, AJ., Stock, JL., Hilderbrandt ,AL., Coskran, T., Black, SC., Brees, DJ., Wicks, JR., McNeish, JD., Coleman, KG. , *Severe diabetes, age-dependant loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKBβ* Journal of Clinical Investigation, 2003. **112**(2): p. 197-208.
115. Chung, J., Park, YC., Ye, H., Wu, H., *All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF- mediated signal transduction.* Journal of Cell Science, 2002. **115**: p. 679-688.
116. Ross, S., Erickson, RL., Gerin, I., DeRose, PM., Bajnok, L., Longo, KA., Misek, DE., Kuick, R., Hanash, SM., Atkins, KB., Anderson, SM., Nedd, HI., Madsen, L., Kristiansen, K., MacDougald, OA., *Microarray analyses during adipogenesis: understanding the effects of Wnt signaling on adipogenesis and the roles of liver X receptor α in adipocyte metabolism* Molecular and Cellular Biology, 2002. **22**(16): p. 5989-5999.
117. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G. Negrel, R., Pfieffer, EF., *Promoting effect of glucocorticoids on differentiation of human adipoctye precursor cells cultured in a clinically defined medium.* Journal of Clinical Investigation, 1989. **84**: p. 1663-1670.

118. Özcan, U., Cao, Q., Yilmaz, E., Lee, A., Iwakoshi, N., Özdelen, E., Tuncamn, G., Görgün, C., Glimcher, LH., Hotamisligil, GS. , *Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes.* Science, 2004. **306**: p. 457-461.
119. Fruebis, J., Tsao, TS., Javorschi, S., Ebbets-Reed, D., Erickson, MR., Yen, TF., Bihian, BE., Lodish, HF. , *Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice.* Proceedings of the National Academy for Science USA, 2001. **98**(4): p. 2005-2010.
120. Kadowaki, T., Yamauchi, T., *Adiponectin and adiponectin receptors.* Endocrine Reviews, 2005. **26**(3): p. 439-451.
121. Arita, Y., Kilhara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shiromomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita,S., Okubo, K., Matsubara,K., Muraguchi, M., Ohmoto,Y., Funahashi, T., Matsuzawa, Y., *Paradoxical decrease of an adipose-specific protien, adiponectin, in obesity.* Biochemical and Biophysical Research Communications, 1999. **257**: p. 79-83.
122. Hu, E., Liang, P., Spiegelman, BM., *AdipoQ is a novel adipose-specific gene dysregulated in obesity.* . Journal of Biological Chemistry, 1996. **271**(18): p. 10697-10703.
123. Hotamisligil, G., Spiegelman, BM., *Tumor necrosis factor-α : a key component of the obesity-diabetes link.* Diabetes, 1994. **43**(11): p. 1271-1278.
124. Hotamisligil, G., Peraldi, P., Budavari, A., Ellis, R., White, MF., Spiegelman, BM., *IRS-1 mediated inhibition of insulin receptor tyrosine kinase activity in TNFα- and obesity-induced insulin resistance* Science, 1996. **271**: p. 665-668.
125. Frühbeck, G., Jebb, SA., Prentice, AM., *Leptin: physiology and pathophysiology* Clinical Physiology, 1998. **18**(5): p. 399-419.
126. Hennige, A., Stefan, N., Kapp, K., Lehman, R., Weigert, C., Beck, A., Moeschel, K., Mushack, J., Schleicher, E., Haring, H-U., *Leptin down-regulates insulin action through phosphorylation of serine-318 in insulin receptor substrate 1.* FASEB Journal, 2006. **20**: p. 1206-1208.
127. Pelleymounter, M., Cullen, MJ., Baker, MB., Hecht, R., Winters, D., Boone, T., Collins, F., *Effects of the obese gene product on body weight regulation in ob/ob mice.* Science, 1995. **269**: p. 540-543.
128. Kumohara, S., Burcelin, R., Halaas, JL., Friedman, JM., Charron, M. , *Acute stimulation of glucose metabolism in mice by leptin treatment* Nature, 1997. **389**: p. 374-377.
129. Juhan-vague, I., Alessi, MC., Mavri, A., Morange, PE. , *PAI-1, obesity, insulin resistance and risk of cardiovascular events.* Journal of Thrombosis and Haemostasis, 1997. **1**(7): p. 1575-1579.
130. Steppan, C., Baily, ST., Bhat, S., Brown, EJ., Banjeree, RR., Wright, CM., Patel, HR., Ahima, RS., Lazar, MA., *The hormone resistin links obesity to diabetes.* Nature, 2001. **409**: p. 307-312.
131. Axelsson, J., Bergsten, A., Qureshi, AR., Heimburger, O., Barany, P., Lonnqvist, F., Lindholm, B., Nordfors, L., Alvestrand, A., Stenvinkel, P. , *Elevate resistin levels in chronic kidney disease are associated with decreased glomerular*

*filtration rate and inflammation, but not with insulin resistance.* Kidney International, 2006. **69**: p. 596-604.
132. Harris, T., Ferrucci, L., Tracy, RP., Corti, MC., Wacholder, S., Ettinger, WH., Hemiovitz, H., Cohen, HJ., Wallace, R., *Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly.* American journal of Medicine, 1999. **106**: p. 506-512.
133. Yudkin, J., Kumari, M., Humphries, SE., Mohammed-Ali, V., *Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?* Atherosclerosis, 2000. **148**: p. 209-214.
134. Samad, F., Pandey, M., Loskatoff, DJ., *Tissue factor gene expression in the adipose tissues of obese mice.* Proceedings of the National Academy for Science U.S.A, 1998. **95**: p. 7591-7596.
135. Sakaue, S., Nishihira, J., Hirokawa, J., Yoshimura, H., Honda, T., Aoki, K., Tagami, S., Kawakami, Y., *Regulation of macrophage migration inhibitory factor (MIF) expression by glucose and insulin in adipocytes in vitro.* Molecular Medicine, 1999. **5**: p. 361-371.
136. Van Dielen, F., Buurman, WA., Hadfoune, M., Nijhuis, J., Greve, JW., *Macrophage inhibitory factor, plasminogen activator inhibitor-1, other acute phase proteins, and inflammatory mediators normalize as a result of weight loss in mobidly obese subjects treated with gastric restrictive surgery.* Journal of Clinical Endocrinology and Metabolism, 2003. **89**(8): p. 4062-4068.
137. Tan, B., Chen, J., Lenhert, H., Kennedy, R., Randeva, HS., *Raised serum, adipocyte, and adipose tissue retinol-binding protein 4 in overwieight women with polycystic ovary syndrome: Effects of gonadal and adrenal steroids* Journal Clinical Endocrinology and Metabolism, 2007. **92**(7): p. 2764-2772.
138. Gordon, G., Newitt, JA., Shantz, LM., Weng, DE., Talalay, P., *Inhibition of the conversion of 3T3 fibroblast clones to adipocytes by dehydroepiandrosterone and related anticarcinogenic steroids.* Cancer Research, 1986. **46**: p. 3389-3395.
139. Lea-Currie, Y., Wen, P., Mclntosh, MK., *Dehydroepiandrosterone reduces proliferation and differentiation of 3T3-L1 preadipocytes.* Biochemical and Biophysical Research Communications, 1998. **248**: p. 497-504.
140. Lea-Currie, Y., Wen, P., Mclntosh, MK., *Dehydroepiandrosterone-sulphate (DHEAS) reduces adipocyte hyperplasia associated with feeding rats a high-fat diet.* Internatrional Journal of Obesity and Related Metabolic Disorders, 1997. **21**: p. 1058-1064.
141. Hansen, P., Han, DH., Nolte, LA., Chen, M., Holloszy, JO., *DHEA protects againts viseral obesity and muscle insulin resistance in rats fed a high-fat diet.* American Journal of Physiology - Regulatory, integrative and comparative physiology, 1997. **273**: p. 1704-1708.
142. Ishizawa, M., Ishizuka, T., Kajita, K., Miura, A., Kanoh, Y., Kimura, M., Yasuda, K., *Dehydroepiandrosterone (DHEA) stimulates glucose uptake in rat adipocytes: activation of phospholipase D.* Comparative Biochemistry and Physiology, 2001. **130**: p. 359-364.
143. Ishizuka, T., Kajita, K., Miura, A., Ishizawa, M., Kanoh, Y., Itaya, S., Kimura, M., Muto, N., Mune, T., Morita, H., Yasuda, K., *DHEA improves glucose uptake*

*via actvations of protein kinase C and phosphatidylinositol 3-kinase.* American Journal of Physiology - Endocrinology and Metabolism, 1999. **276**: p. 196-204.
144. Kajita, K., Ishizuka, T., Mune, T., Miura, A., Ishizawa, M., Kanoh, Y., Kawai, Y., Natsume, Y., Yasuda, K., *Dehydroepiandrosterone down-regulates the expression of peroxisome proliferator-activated receptor γ in adipocytes.* Endocrinology 2003. **144**(1): p. 253-259.
145. kochan, Z., Karbowska, J., *Dehydroepiandrosterone up-regulates resistin gene expression in white adipose tissue.* Molecular and Cellular Endocrinology, 2004. **218**: p. 57-64.
146. Hernandz-Morante, J., Milagro, F., Gabaldon, JA., Martinez, JA., Zamora, S., Garaulet, M., *Effect of DHEA-sulphate on adiponectin gene expression in adipose tissue from different fat depots in morbidly obese humans* Euopean Journal of Endocrinology, 2006. **155**: p. 593-600.
147. Tchernof, A., Labrie, F., *Dehydroepiandrosterone, obesity and cardiovascular disease risk: a review of human studies.* European Journal of Endocrinology, 2004. **151**: p. 1-14.
148. Field, A., Colditz, GA., Willet, WC., Longcope, C., McKinlay, JB., *The relation of smoking, age, relative weight, and dietry intake to serum adrenal steroids, sex hormones, and sex hormone-binding globulin in middle-aged men.* Journal of Clinical Endocrinology and Metabolism, 1994. **79**(5): p. 1310-1316.
149. De Pergola, G., Giagulli, VA., Garruti, G., Cospite, MR., Giorgino, M., Cignarelli, M., Giorgino, R. , *Low dehydroepiandrosterone circulating levels in premenopausal obese women with very high body mass index* Metabolism, 1991. **40**(2): p. 187-190.
150. De Pergola, G., Zamboni, M., Sciaraffia, M., Turcato, E., Pannacciulli, N., Armellini, F., Giorgino, F., Perrini, S., Bosello, O., Giorgino, R., *Body fat accumulation is possibly responsible for lower dehydroepiandrosterone circulating levels in premenopausal obese women.* International Journal of Obesity and Related Metababolic Disorders 1996. **20**(12): p. 1105-1110.
151. Barrett-Conner, E., Ferrara, A., *Dehydroepiandrosterone ,dehydroepiandrosterone sulphate, obesity, waist-hip ratio, and noninsulin-dependant diabetes in postmenopausal women: the Rancho Bernardo study* Journal of Clinical Endocrinology and Metabolism, 1996. **81**(1): p. 59-64.
152. Villareal, D., Holloszy, JO., *Effect of DHEA on abdominal fat and insulin action in elderly men and women.* Journal of the American Medical Association, 2004. **292**(18): p. 2243-2248.
153. Ravaglia, G., Forti, P., Maioli, F., Boschi, F., Bernardi, M., Pratelli, L., *The relationship of dehydroepiandrosterone sulphate (DHEAS) to endocrine-metabolic parameters and functional status in the oldest-old. Results from an Italian study on healthy free-living over-ninety-year-olds.* Journal of Clinical Endocrinology and Metabolism, 1996. **81**(3): p. 1173-1178.
154. Maccario, M., Mazza, E., Ramunni, J., Oleandri, SE., Procopio, M., Gauna, G., Ghigo, E. , *Relationship between dehydroepiandrosterone -sulphate and anthropometric, metabolic, and hormonal variables in a large cohort of obese women.* Clinical Endocrinology, 1999. **50**: p. 595-600.

155. Evans, D., Hoffman, RG., Kalkhoff, RK., Kissebah, AH., *Relationship of androgenic activity to body fat topography, fat cell morphology, and metabolic aberrations in premenopausal women.* . Journal of Clinical Endocrinology and Metabolism, 1983. **57**: p. 304-310.
156. De Pergola, G., Triggiani, V., Giorgino, R., Cospite, MR., Garruti, G., Cignarelli, M., Guastamacchi, E., Giorgino, R., *The free testosterone to dehydroepiandrosterone sulphate molar ratio as a marker of visceral fat accumulation in premenopausal obese women.* International Journal of Obesity and Related Metababolic Disorders, 1994. **18**(10): p. 659-664.
157. Ivandić, A., Prpić-Križevac, I., Sučić, M., Jurić, M., *Hyperinsulinemia and sex hormones in healthy premenopausal women: relative contribution of obesity, obesity type, and duration of obesity.* Metabolism, 1998. **47**(1): p. 13-19.
158. Haffner, S., Newcombe, PA., Marcus, PM., Klein, BEK, Klein, R. , *Relation of sex hormones and dehydroepiandrosterone sulfate (DHEA-SO4) to cardiovascular risk factors in postmenopausal women.* American Journal of Epidemiology, 1995. **142**(9): p. 925-934.
159. Williams, D., Boyden, TW., Pamenter, RW., Lohman, TG., Going, SB., *Relationship of body fat percentage and fat distribution with dehyroepiandrosterone sulphate in premenopausal females.* Journal of Clinical Endocrinology and Metabolism, 1993. **77**(1): p. 80-85.
160. Abbassi, A., Duthie, EH., Sheldahl, L., Wilson, C., Sasse, E., Rudman, I,. Mattson, DE. , *Asssociation of dehydroepiandrosterone sulfate, body composition, and physical fitness in independent community-dwelling older men and women.* Journal of the American Geriatric Society, 1998. **46**(3): p. 1173-1178.
161. Herranz, L., Megia, A., Grande, C., González-Gancedo, P., Pallardo, E., *Dehydroepiandrosterone sulphate, body fat distribution and insulin in obese men.* International Journal of Obesity and Related Metababolic Disorders, 1995. **19**(1): p. 57-60.
162. Haffner, S., Mykkänen, L., Valdez, RA., Katz, MS., *Relationship of sex hormones to lipids and lipoproteins in nondiabetic men.* Journal of Clinical Endocrinology and Metabolism, 1993. **77**(6): p. 1610-1615.
163. Haffner, S., Valdez, RA., Stern, MP., Katz, MS., *Obesity, body fat distribution and sex hormones in men.* International Journal of Obesity and Related Metababolic Disorders, 1993. **17**(11): p. 643-649.
164. Haffner, S., Karhapää, P., Mykkänen, L., Laakso, M., *Insulin resistance, body fat distribution, and sex hormones in men* Diabetes, 1994. **43**(2): p. 212-219.
165. Vermeulen, A., Kaufman, JM., Giagulli, VA. , *Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males.* Journal of Clinical Endocrinology and Metabolism, 1996. **81**(5): p. 1821-1826.
166. Pritchard, J., Després, JP., Gagnon, J., Tchernof, A., Nadeau, A., Tremblay, A., Bouchard, C., *Plasma adrenal, gonadal, and conjugated steroids before and after longterm overfeeding in identical twins.* Journal Clinical Endocrinology and Metabolism, 1998. **83**(9): p. 3277-3284.

167. Løvås, K., Gebre-Medhin, G., Trovik, TS., Fougner, KJ., Uhlving, S., Nedrebø, BG., Myling, OL., Kämp, O., Husebye, ES., *Relpacement of dehydroepiandrosterone in adrenal failure: no benefit for subjective health status and sexuality in a 9-month, randomized, parallel group clinical trial.* Journal of Clinical Endocrinology and Metabolism, 2003. **88**(3): p. 1112-1118.
168. Callies, F., Fassnacht, M., van Vlijmen, JC., Koehler, I., Huebler, D., Seibel, MJ., Wiebke Arlt., Alloloi, B., *Dehydroepiandrosterone replacement in women with adrenal insufficiency: effects on body composition, serum leptin, bone turnover, and exercise capacity.* Journal of Clinical Endocrinology and Metabolism, 2001. **86**(5): p. 1968-1972.
169. Nestler, J., Barlascini, CO., Clore, JN., Blackard, WG., *Dehydroepiandrostrerone reduces serum low density lipoprotein levels and body fat but does not alter insulin sensitivity in normal men.* Journal of Clinical Endocrinology and Metabolism, 1988. **66**(1): p. 57-61.
170. Welle, S., Jozefowicz, R., Statt, M., *Failure of dehydroepiandrosterone to influence energy and protein metabolism in humans.* Journal of Clinical Endocrinology and Metabolism, 1990. **71**(5): p. 1259-1264.
171. Flynn, M., Weaver-Osterholtz, D., Sharpe-Timms, KL., Allen, S., Krause, G., *Dehydroepiandrosterone replacement in aging humans.* Journal of Clinical Endocrinology and Metabolism, 1999. **84**(5): p. 1527-1533.
172. Jedrzejuk, D., Medras, M., Milewicz, A., Demissie, M., *Dehydroepiandrosterone replacement in healthy men with age-related decline of DHEA-S: effects on fat distribution, insulin sensitivity and lipid metabolism.* Aging Male, 2003. **6**(3): p. 151-156.
173. Arlt, W., Callies, F., Koehler.I, van Vlijmen, JC., Fassnacht, M., Strasburger, CJ., Seibel, Heubler, D., Ernst, M., Oettel, M., Reincke, M., Schulte, HE., Allolio, B. , *Dehydroepiandroesterone supplementation healthy men with an age-related decline of dehydroepiandrosterone secretion.* Journal of Clinical Endocrinology and Metabolism, 2001. **86**(10): p. 4686-4692.
174. Villareal, D., Holloszy, JO., Kohrt, WM., *Effects of DHEA replacement on bone mineral density and body composition in elderly women and men.* Clinical Endocrinology, 2000. **53**: p. 561-568.
175. Percheron, G., Hogrel, J-Y., Denot-Ledunois, S., Fayet, G., Forette, F., Baulieu, EE., Fardeau, M., Martin, J-F., *Effect of 1-year oral administration of dehydroepiandrosterone to 60- to 80-year-old individuals on muscle function and cross-sectional area.* Archives of Internal Medicine, 2003. **163**: p. 720-727.
176. Diamond, P., Cusan, L., Gomez, JL., Bélanger, A., Labrie, F., *Metabolic effect of 12-month percutaneous dehydroepiandrosterone replacement therarpy in postmenopausal women.* Journal of Endocrinology, 1996. **150 (supplement)**: p. S43-S50.
177. Casson, P., Santoro, N., Elkind-Hirsch, K., Carson, SA., Hornsby, PJ., Abraham, G., Buster, JE., *Postmenopausal dehydroepiandrosterone administration increases free insulin-like growth factor -1 and decreases high-density lipoprotein: a six month trial.* Fertility and Sterility, 1998. **70**(1): p. 107-110.

178. Vogiatzi, M., Boeck, MA., Valchopapadopoulou, E., El-Rashid, R., New, MI., *Dehydroepiandrosterone in morbidly obese adolescents: effects on weight, body composition, lipids and insulin resistance.* Metabolism, 1996. **45**(8): p. 1011-1015.
179. Usiskin, K., Butterworth, S., Clore, JN., Arad, Y., Ginsburg, HN., Blackard, WG., Nestler, JE., *Lack of effect of dehydroepiandrosterone in obese men.* International Journal of Obesity, 1990. **14**(5): p. 457-463.
180. Kawano, H., Yasue, H., Kitagawa, A., Hirai, N., Yoshida, T., Soejima, H., Miyamoto, S., Nakano, M., Ogawa, H., *Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men.* Journal of Clinical Endocrinology and Metabolism, 2003. **88**(7): p. 3190-3195.
181. Mortola, J., Yen, SSC., *The effects of oral dehydroepiandrosterone on endocrine-metabolic parameters in postmenopausal women.* Journal of Clinical Endocrinology and Metabolism, 1990. **71**(3): p. 696-704.
182. Morales, A., Nolan, JJ., Nelson, JC., Yen, SSC., *Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age.* Journal of Clinical Endocrinology and Metabolism, 1994. **78**(6): p. 1360-1367.
183. Ebeling, P., Koiviosto, VA., *Physiological importance of dehydroepiandrosterone.* Lancet, 1991. **343**: p. 1479-1481.
184. Ross, R., *Atherosclerosis - an inflammatory disease.* New England Journal of Medicine, 1999. **340** (24): p. 1928-1929.
185. Vita, J., Keaney, JF., *Endothelial function. A Barometer for cardiovascular risk.* Circulation, 2002. **106**: p. 640-642.
186. Weber, T., . Auer, J,. O'Rourke, MF., Kvas, E., Lassnig, E., Berent, R., Eber, B., *Arterial stiffness, wave reflections, and the risk of coronary artery disease.* Circulation, 2004. **109**: p. 184-189.
187. Williams, M., . Dawood, T., Ling, S., Dai, A., Lew, R., Myles, K., Funder, JW., Krishnankutty, S., Komersaroff, PA., *Dehydroepiandrosterone increases endothelial cell proliferation in vitro and improves endothelial function in vivo by mechanisms independent of androgen and estrogen receptors* Journal of Clinical Endocrinology and Metabolism, 2004. **89**(9): p. 4708-4715.
188. Loscalzo, J., Welch, G. , *Nitric oxide and its role in the cardiovascular system.* Progress in Cardiovascular Disease, 1995. **38**(2): p. 87-104.
189. Cooke, J., Tsao, PS., *Go woth the flow.* Circulation, 2001. **103**: p. 2773-2775.
190. Moreau, M., Garbacki, N., Molinavo, G., Brown ,NJ., Marceau, F., Adam, A., *The kallikrein-kinin system: current and future pharmacological targets* Journal of Pharmacological Science, 2005. **99**: p. 6-38.
191. Goodfriend, T., Elliot, ME., Catt, KJ., *Angiotensin receptors and their antagonists.* New England Journal of Medicine, 1996. **334**(25): p. 1649-1654.
192. Highsmith, R., Blackburn, K., Schmidt, DJ., *Endothelin and calcium dynamics in vascular smooth muscle.* Annual Reveiws in Physiology, 1992. **54**: p. 257-277.
193. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**: p. 801-809.
194. Cox, R., *Mechanics of canine iliac artery smooth muscle in vitro.* American Journal of Physiology 1976. **230**(2): p. 462-470.

195. McEniery, C., Wilkinson, IB., *Large artery stiffness and inflammation* Journal of Human Hypertension, 2005. **19**: p. 507-509.
196. Roman, M., Pini, R., Pickering, TG., Devereux, RB *Non-invasive measurements of arterial compliance in hypertensive compared to normotensive adults* Journal of Hypertension, 1992. **10**(supplement 6 ): p. S115-118.
197. Farrar, D., Green, HD., Bond, MG., Wagner, WD., Gobbee., *Aortic pulse wave velocity, elasticity, and compostion in a nonhuman model of atherosclerosis.* Circulation Research 1978. **43**: p. 52-62.
198. Blacher, J., Guerin, AP., Pannier, B., Marchais, SJ., Safar, M., London, G. , *Impact of aortic stiffness on survival in end-stage renal disease.* Circulation, 1999. **99**: p. 2434-2439.
199. Laurent, S., Boutouyrie, P., Asmar, R., Gautier, I., Laloux, B., Guize, L., Ducimetiere, P., Benetos, A., *Aortic stiffness is an independant predictor of all-cause and cardiovascular mortality in hypertensive patients* Hypertension, 2001. **37**: p. 1236-1241.
200. Cruickshank, K., Riste. L,. Anderson, SG., Wright, JS., Dunn, G., Gosling, RG. , *Aortic pulse-wave velocity and its relationship to mortality in diabetes and glucose intolerance: An integrated index of vascular function? .* Circulation, 2002. **106**: p. 2085-2090.
201. Gribbin, B., Pickering, TG., Sleight, P., *Arterial distensibility in normal and hypertensive man.* Clinical Science, 1979. **56**: p. 413-417.
202. O'Rourke, M., Gallagher, DE., *Pulse wave analysis.* Journal of Hypertension, 1996. **14**(supplement 5): p. S147-S157.
203. Wilkinson, I., Fuchs, SA., Jansen, IM., Spratt, JC., Murray, GD., Cockroft, JR., Webb, DJ. , *Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis.* Journal of Hypertension, 1998. **16**: p. 2079-2084.
204. Feldman, H., Johannes, CB., Araujo, AB., Mohr, BA., Longcope, C., McKinlay, JB., *Low dehydroepiandrosterone and ischaemic heart disease in middle-aged men: prospective results from the Massachusetts Male aging study. .* American Journal of Epidemiology, 2001. **153**(1): p. 79-89.
205. Slowinska-Srzednicka, J., Zgliczynski, S., Soszynski, P., Makowska, A., Zgliczynski, W., Srzednicki, M., Bednarska, M., Chotkowska, E., Woroszylska, M., Ruzyllo, W., *Decreased plasma levels of dehydroepiandrosterone sulphate (DHEA-S) in normolipidaemic and hyperlipoproteinemic young men with coronary artery disease.* Journal of Internal Medicine, 1991. **230**(6): p. 551-553.
206. Slowinska-Srzednicka, J., Malczewska, B., Chotkowska, E., Brzezinska, A., Zgliczynski, W., Ossowski, M., Jeske, W., Zgliczynski, S., Sadowski, Z., *Hyperinsulinemia and decreased plasma levels of dehydroepiandrosterone sulfate in premenopausal women with coronary heart disease.* Journal of Internal Medicine, 1995. **237**(5): p. 465-472.
207. Johannes, C., Stellato, RK., Feldman, HA., Longcope, C., McKinlay, JB., *Relation of dehydroepiandrosterone and dehydroepiandrosterone sulfate with cardiovascular disease risk factors in women: longitudinal results from the*

*Massachusetts Women's Health Study* Journal of Clinical Epidemiology, 1999. **52**(2): p. 95-103.
208. Herrington, D., Gordon, GB., Achuff, SC., Trejo, JF., Weisman, HF., Kwiterovich, PO., Pearson, TA, , *Plasma dehydroepiandrosterone and dehydroepiandrosterone sulphate in patients undergoing diagnostic coronary angiography.* Journal of the American College of Cardiology, 1990. **16**(4): p. 862-870.
209. Herrington, D., Nanjee, N., Achuff, SC., Cameron, DE., Dobbs, B., Baughman, KL., *Dehydroepiandrosterone and cardiac allograft vasculopathy.* Journal of Heart and Lung Transplantation, 1996. **15**: p. 88-93.
210. Gordon, G., Bush, DE., Weisman, HF., *Reduction of atherosclerosis by administration of dehydroepiandrosterone. A study in the hypercholesterolemic New Zealand White Rabbit with aortic intimal injury.* Journal of Clinical Investigation, 1998. **82**: p. 712-720.
211. Liu, D., Dillon, JS. , *Dehydroepiandrosterone activates endothelial cell nitric-oxide synthase by a specific plasma membrane receptor coupled to* $G\alpha_{i\,2,3}$. Journal of Biological Chemistry, 2002. **277**(14): p. 21379-21388.
212. Williams, M., Ling, S., Dawood, T., Hashimura, K., Dai, A., Li, H., Lui, J-P., Funder, JW., Krishnankutty, S., Komersaroff, PA. , *Dehydroepiandrosterone inhibits human vascular smooth muscle cell proliferation independent of ARs and ERs.* Journal of Clinical Endocrinology and Metabolism, 2002. **87**(1): p. 176-181.
213. Beer, N., Jakubowicz, DJ., Matt, DW., Beer, RM., Nestler, JE., *Dehydroepiandrosterone reduces plasma plasminogen activator inhibitor type 1 and tissue plasminogen activator antigen in men.* American Journal of Medical Sciences, 1996. **311**(5): p. 205-210.
214. Jesse, R., Loesser, K., Eich, DM., Qian, YZ., Hess, ML., Nestler, JE., *Dehydroepiandrosterone inhibits human platelet aggregation in vitro and in vivo.* Annals of the New York Academy of Sciences, 1995. **744**(1): p. 281-290.
215. Sudhir, K., Chou, TM., Chatterjee, K., Smith, EP., Williams, TC., Kane, JP., Malloy, MJ., Korach, KS., Rubanyi, GM. , *Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man.* Circulation, 1997. **96**: p. 3774-3777.
216. Smith, E., Boyd, J., Frank, GR., Takahashi, H., Cohen, RM., Specker, B., Williams, TC., Lubahn, DB., Korach, KS. , *Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man.* New England Journal of Medicine, 1994. **331**: p. 1056-1061.
217. Morishima, A., Grumbach, MM., Simpson, ER., Fisher, C., Qin, K. , *Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens.* Journal of Clinical Endocrinology and Metabolism, 1995. **80**: p. 3689-3698.
218. Lew, R., Komesaroff, P., Williams, M., Dawood, T., Sudhir, K. , *Endogenous estrogens influence endothelial function in young men* Circulation Research, 2003. **93**: p. 1127-1133.
219. Hayashi, T., Esaki, T., Muto, E., Kano, H., Asai, Y., Thakur, NK., Sumi, D., Jayachandran, M., Iguchi, A. , *Dehydroepiandrosterone retards atherosclerosis*

*formation through its conversion to estrogen.* Arteriosclerosis Thrombosis and Vascular Biology, 2000. **20**: p. 782-792.
220. Cheng, H., Hu, XJ., Ruan, QR., *Dehydroepiandrosterone anti-atherogenesis effect is not via its conversion to estrogen.* Acta Pharmacol Sin, 2009. **30**: p. 42-53.
221. Stanworth, R., Jones, TH., *Testosterone in obesity, metabolic syndrome and type 2 diabetes.* Frontiers in Hormone Research, 2009. **37**: p. 74-90.
222. Kapoor, D., Goodwin, E., Channer, KS., Jones, TH., *Testosterone replacement therapy imporves insulin resistance, glycaemic control, visceral adiposity and hypercholesterolaemia in hypogonadal men with type 2 diabetes* European Journal of Endocrinology, 2006. **154**: p. 899-906.
223. Kapoor, D., Clarke, S., Stanworth, R., Channer, KS., Jones, TH., *The effect of testosteone replacement therapy on adipocytokines and C-reactive protein in hypogonadal men with type 2 diabetes.* European Journal of Endocrinology, 2007. **156**: p. 595-602.
224. Stanworth, R., Kapoor, D., Channer, KS., Jones, TH., *Androgen receptor CAG repeat polymorphism is associated with serum testosterone levels, obesity and serum leptin in men with type 2 diabetes.* Euopean Journal of Endocrinology, 2008. **159**: p. 739-746.
225. Malkin, C., Pugh, PJ., West, JN., van Beek, EJ., Jones, TH., Channer, KS., *Testosterone therapy in men with moderate severity heart failure: a double-blind randomized placebo controlled trial.* European Heart Journal, 2006. **27**: p. 10-12.
226. Malkin, C., Pugh, PJ., Morris, PD., Kerry, KE., Jones, RD., Jones, TH., Channer, KS., *Testosterone replacement in hypogonadal men with angina improves ishcaemic threshold and qulaity of life.* Heart, 2004. **90**: p. 871-876.
227. Nettleship, J., Jones, TH., Channer, KS., Jones, RD., *Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in Tfm mouse: an effect that is independent of the classical androgen receptor.* Circulation, 2007. **116**: p. 2427-2434.
228. Smith, J., Bennet, S., Evans, LM., Kynaston, HG., Parmer, M., Mason, MD., Cockcroft, JR., Scanlon, MF., Davies, JS., *The effects of induced hypogonadism on arterial stiffness, body composition, and metabolic parameters in males with prostate cancer* Journal Clinical Endocrinology and Metabolism, 2001. **86**: p. 4261-4267.
229. Langer, C., Gansz, B., Goepfert, C., Engel, T., Uehara, Y., von Dehn, G., Jansen, H., Assmann, G., von Eckardstein, A., *Testosterone upregulates scavenger receptor BI and stimulates cholesterol efflux from macrophages.* Biochem. Biophys. Res. Commun, 2002. **296**: p. 1051-1057.
230. Tan, K., Shiu, SW., Kung, AW. *Alterations in hepatic lipase and lipoprotein subfractions with transdermal testosterone replacement therapy.* Clinical Endocrinology, 1999. **51**: p. 765-769.
231. Lam, K., Lo, CY., *A critical examination of adrenal tuberculosis and a 28-year autopsy experience of active tuberculosis* Clinical Endocrinology, 2001. **54**: p. 633-639.

232. Willis, A., Vince, FP. , *The prevalence of Addison's disease in coventry,UK.* Postgraduate medical journal, 1997. **73**: p. 286-288.
233. Laureti, S., Vecchi, L., Santeusanio, F., Faloni, A., *Is the prevalence of Addison's disease underestimated.* Journal of Clinical Endocrinology and Metabolism, 1999. **84**(5): p. 1762.
234. Betterel, C., Volpato, M., Pedini, B., Chen, S., Smith, BR., Furmaniak, J. , *Adrenal-cortex autoantibodies and steroid-producing cells autoantibodies in patients with Addison's disease: comparison of immunofluoresence and immunoprecipitation assays* Journal of Clinical Endocrinology and Metabolism, 1999. **84**(2): p. 618-622.
235. Clark, P., Neylon, I., Raggat, PR., Sheppard, MC., Stewart, PM. , *Defining the normal cortisol response to the short synacthen test: implications for the investigation of hypothalamic-pituitary disorders* Clinical Endocrinology, 1998. **49**(3): p. 287-292.
236. Oelkers, W., Diederich, S., Bähr, V., *Diagnosis and therapy surveillance in Addison's disease: rapid adrenocorticotropin (ACTH) test and measurements of plasma ACTH , renin activity, and aldosterone.* Journal of Clinical Endocrinology and Metabolism, 1992. **75**(1): p. 259-264.
237. Løvås, K., Loge, JH., Huseby, ES. , *Subjective health status in Norwegian patients with Addison's disease.* Clinical Endocrinology, 2002. **56**: p. 581-588.
238. Mason, A., *Epidemiological and clinical picture of Addison's disease.* Lancet, 1968. **2**: p. 744-747.
239. Bergthorsdottir, R., Leonsson-Zachrisson, M., Odén, A., Johannsson, G., *Premature mortality in patients with Addison's disease: A population based study.* Journal of Clinical Endocrinology and Metabolism, 2006. **91**(12): p. 4849-4853.
240. Gurnell, E., Hunt, PJ., Curran, SE., Conway, CL., Pullenayegum, EM., Huppert, FA., Compston, JE., Herbert, J., Chatterjee, VKK., *Long term DHEA replacement in primary adrenal insufficiency: A randomised controlled trial.* Journal of Clinical Endocrinology and Metabolism, 2008. **93**(2): p. 400-409.
241. Christiansen, J., Anderson, NH., Sørensen, KE., Pedersen, EM., Bennets, P., Anderson, M., Christansen, JS., Jørgensen, JOL., Gravholt, CHG., *Dehydroepiandrosterone substitution in female adrenal failure: no impact on endothelial function and cardiovascular parameters despite normalization of androgen status* Clinical Endocrinology, 2007. **66**: p. 426-433.
242. Nilsson, B., Gustavasson-Kadaka, E., Bengtsson, BA., Jonsson, B. , *Pituitary adenomas in Sweden between 1958 and 1991: incidence, survival, and mortality.* Journal of Clinical Endocrinology and Metabolism, 2000. **85**(4): p. 1420-1425.
243. Regal, M., Paramo, C., Sierra, SM., Garcia-Mayor, RV. , *Prevalence and incidence of hypopituitarism in an adult population in northwestern Spain.* Clinical Endocrinology, 2001. **55**: p. 735-740.
244. Rosén, T., Bengtsson, BA., *Premature mortality due to cardiovascular disease in hypopituitarism.* Lancet, 1990. **336**: p. 285-288.
245. Tomlinson, J., Holden, N., Hills, RK., Wheatley, K., Clayton, RN., Bates, AS., Sheppard, MC., Stewart, PM., *Association between premature mortality and*

*hypopituitarism. West Midlands Prospective Hypopituitary study Group.* Lancet, 2001. **357**: p. 425-431.
246. Mills, J., Schonberger, LB., Wysowski, DK., Brown, P., Durako, SJ., Cox, C., Kong, F., Fradkin, JE., *Long-term mortality in the United States cohort of pituitary-derived growth hormone recipients.* Journal of Paediatrics, 2004. **144**: p. 430-436.
247. Bates, A., Hoff, WV., Jones, PJ., Clayton, RN., *The effect of hypopituitarism on life expectancy.* Journal of Clinical Endocrinology and Metabolism, 1996. **81**(3): p. 1169-1172.
248. Yamaji, T., Ishibashi, M., Takaku, F., Itabashi, A., Katayama, S., Ishii, J., *Serum DHAS concentrations in secondary adrenal insufficiency.* Journal of Clinical Endocrinology and Metabolism, 1987. **65**: p. 448-451.
249. Johannsson, G., Burman, P., Wirén, L., Engström, BE., Nilsson, AG., Ottosson, M., Jonsson, B., Bengtsson, B., Karlsson, FA. , *Low dose dehydroepiandrosterone affetcs behaviour in hypopituitary androgen deficient women: a placebo-controlled trial* Journal of Clinical Endocrinology and Metabolism, 2002. **87**(5): p. 2046-2052.
250. Ailhaud, G., Grimaldi, P., Négrel, R., *Cellular and molecular aspects of adipose tissue development.* Annual Reviews Nutrition, 1992. **12**: p. 207-233.
251. Pairault, J., Green, H., *A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as a differentiation marker.* Proceedings of the National Academy for Science U.S.A, 1979. **76**(10): p. 5138-5142.
252. Grégoire, F., Genart, C., Hauser, N., Remacle, C., *Glucocorticoids induce a drastic inhibition of proliferation and stimulate differentiation of adult rat fat cell precursors.* Experimental and Cellular Research, 1991. **196**(2): p. 270-278.
253. Entenaman, G., Hauner, H., *Relationship between replication and differentiation in cultured human adipocyte precursor cells.* American Journal of Physiology - Cellular Physiology, 1996. **270**: p. C1011-1016.
254. Ailhaud, G., *Extracellular factors, signalling pathways and differentiation of adipose cell precursors.* Current Opinion in Cellular Biololgy, 1990. **2**: p. 1043-1049.
255. Guller, S., Corin, RE., Mynarcik, DC., London, BM., Sonenberg, M. , *Role of insulin in growth hormone-stimulated 3T3 cell adipogenesis.* Endocrinology, 1988. **122**: p. 2084-2089.
256. Girard, J., Perdereau, D., Foufelle, F., Prip-Buus, C., Ferré, P., *Regulation of lipogenic enzyme gene expression by nutrients and hormones.* FASEB J, 1994. **8**: p. 36-42.
257. Shantz, L., Talalay, P., Gordon, GB. , *Mechanism of inhibition of growth of 3T3-L1 fibroblasts and their differentiation to adipoctyes by dehydroepiandrosterone and related steroids: Role of glucose-6-phosphate dehydrogenase.* Proceedings of the National Academy for Science U.S.A, 1989. **86**: p. 3852-3856.
258. McIntosh, M., Lea-Currie, YR., Geigerman, C., Patseavouras, L., *Dehydroepiandrosterone alters the growth of stromal vascular cells from human adipose tissue.* International Journal of Obesity 1999. **23**: p. 595-602.

259. Kopecký, J., Baudyšová, M., Zanotti, F., Janiková, D., Pavelka, S., Houštěk., *Synthesis of mitochondrial uncoupling protein in brown adipocytes differentiated cell culture.* Journal of Biological Chemistry, 1990. **265**(36): p. 22204-22209.
260. Rosen, E., Walkey, CJ., Puigserver, P., Speigelman, BM., *Transcriptional regulation of adipogenesis.* Genes and Development, 2000. **14**: p. 1293-1307.
261. Hansen, J., Jørgensen, C., Peterson, RK., Hallenborg, P., De Matteis, R., Bøye, HA., Petrovic, N., Enerbäck, S., Nedergaard, J., Cinti, S., Riele, Ht., Kristiansen, K., *Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiaiton.* Proceedings of the National Academy for Science U.S.A, 2004. **101**(12): p. 4112-4117.
262. Scimé, A., Grenier, G., Huth, MS., Gillespie, MA., Bevilaccqua, L., Harper, ME., Rudnicki, MA., *Rb and p107 regulate preadipocyte differentiaiton into white versus brown fat through repression of PGC-1alpha* Cell Metabolism, 2005. **2**(5): p. 283-295.
263. Rosen, E., Spiegelman, BM., *Molecular regulation of adipogenesis.* Annual Reviews in Cellular and Developmental Biology, 2000. **16**: p. 145-171.
264. Zilberfarb, V., Piétri-Rouxel, F., Jockers, R., Krief, S., Delouis, C., Issad, T., Strosberg, D., *Human immortalized brown adipocytes express functional β3-adrenoceptor coupled to lipolysis.* Journal of Cell Science 1997. **110**: p. 801-807.
265. Todaro, G., Green, H., *Quantitative studies of the growth of mouse embryo cells in culture and their develpoment into established lines.* The Journal of Cell Biology, 1963. **17**: p. 299-313.
266. Green, H., Kehinde, O., *Sublines of Mouse 3T3 cells that accumulate lipid.* Cell, 1974. **1**(3): p. 113-116.
267. Green, H., Meuth, M., *An established pre-adipose cell line and its differentiation in culture.* Cell, 1974. **3**(2): p. 127-133.
268. Crisp, M., Starkey, KJ., Lane, C., Ham, J., Lugdate, M., *Adipogenesis in thyroid eye disease.* Investigative Ophthalmology & Visual Science, 2000. **41**: p. 3249-3255.
269. Zhang, L., Baker, G., Janus, D., Paddon, CA., Fuhrer, D., Ludgate, M., *Biological effects of thyrotropin receptor activation on human orbital preadipocytes.* Investigative Ophthalmology & Visual Science, 2006. **47**(12): p. 5197-5203.
270. Smith, J., Martin, L., *Do cells cycle.* Proceedings of the National Academy for Science U.S.A, 1973. **70**(4): p. 1263-1267.
271. Elledge, S., *Cell cycle check points: Preventing an identity crisis.* Science, 1996. **274**: p. 1664-1672.
272. Nigg, E., *Cyclin-dependent protien kinases: key regulators of the eukaryotic cell cycle* Bioessays, 1995. **17**(6): p. 471-480.
273. Mattern, C., Brackett, FS., Olson, BJ. , *Determination on number and size of particles by electrical gating: blood cells.* Journal of Applied Physiology, 1957. **10**: p. 56-70.
274. Gravreli, Y., Sherman, Y., Ben-Sasson, SA. , *Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation.* Journal of cell Biology, 1992. **119**(3): p. 493-501.

275. Burnette, W., . *"Western blotting": Electrophoretic transfer of proteins from sodium dodecyl-sulfate-polyacrylamide gels to unmodified nitocellulose and radiographic detection with antibody and radioiodinated protein A* Annals in Biochemistry, 1981. **112**: p. 195-203.
276. Mandell, J., *Phosphorylation state-specific antibodies applications in investigative and diagnostic pathology.* American Journal of Pathology, 2003. **163**(5): p. 1687-1698.
277. Saiki, R., Scharf, S., Faloona, F., Mullis, KB., Horn, GT., Erlich, HA., Arnheim, N., *Enzymic amplification of β-globulin genomic sequence and restriction site analysis for diagnosis of sickle cell anaemia.* Science, 1985. **230**: p. 1350-1354.
278. Cross, N., *Quantitative PCR techniques and applications* British Journal of Haematology, 1995. **89**(4): p. 693-697.
279. Wiesner, R., *Direct quantification of picomolar concentrations of mRNAs by mathmatical analysis of a reverse transcription/exponential polymerase cahin reaction assay.* Nucleic Acids Research, 1992. **20**(21): p. 5863-5864.
280. Stockelman, M., Lorenz, JN., Smith, FN., Biovin, GR., Sahota, A., Tischfield, JA., Stambrook, PJ., *Chronic renal failure in a mouse of human adenine phosphoribosyltransferase deficiency.* American Journal of Physiology - Renal Physiology, 1998. **275**: p. F154-F163.
281. Bryant, S., *DMSO inhibits the induction of adipogenesis in 3T3L1 cells*, in *California state science fair.* 2004.
282. Darimont, C., Zbinden, I., Avanti, O., Leone-Vautravers, P., Giusti, V., Burckhardt P., Pfiefer, AMA., Macé, K., *Reconstitution of telomerase activity combined with HPV-E7 expression allow human preadipocytes to preserve their differentiation capacity after immortalization.* Cell Death and Differentiaiton, 2003. **10**: p. 1025-1031.
283. Björntorp, P., *Metabolic implications of body fat distribution.* Diabetes Care, 1991. **14**(12): p. 1132-1143.
284. Després, J., Allard, C., Tremblay, A., Talbot, I., Bouchard, C., *Evidence for a regional component of body fatness in association with serum lipids in men ane women.* Metabolism, 1985. **34**(10): p. 967-973.
285. Lapidus, L., Bengtsson, C., Larsson, B., Pennert, K., Rybo, E., Sjöstrom, L. , *Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden* British Medical Journal, 1984. **289**: p. 1257-1261.
286. Isomaa, B., . Almgran, P,. Tuomi, T., Forsén, B., Lahti, K., Nissén, M., Taskinen, M-R., Groop, L. , *Cardiovascular morbidity and mortality associated with the metabolic syndrome.* Diabetes Care, 2001. **24**(4): p. 683-689.
287. Kaplan, N., *The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia and hypertension.* Archives of Internal Medicine, 1989. **149**(7): p. 1514-1520.
288. Haffner, S., Cassells, HB., *Metabolic syndrome - a new risk factor of coronary heart disease.* Diabetes, Obesity and Metabolism, 2003. **5**: p. 359-370.
289. McIntosh, M., Hauseman, D., Martin, R., Hauseman, G., *Dehydroepiandrosterone (DHEA) attenuates preadipocyte proliferation and*

*differentiation in primary cultures of stromal-vascular cells* American Journal of Physiology - Endocrinology and Metabolism, 1998. **275**: p. E285-E293.
290. Morales, A., Haubrich, RH., Hwang, JY., Asakura, H., Yen, SS. , *The effects of six months treatment with a 100 mg daily dose of dehydroepiandrosterone (DHEA) on circulating sex steroids, body composition and muscle strength in age-advanced men and women.* Clinical Endocrinology, 1998. **49**: p. 421-423.
291. Mendelsohn, M., Karas, RH. , *The protective effects of estrogen on the cardiovascular system.* New England Journal of Medicine, 1999. **340**(23): p. 1801-1811.
292. Bilezikian, J., Morishima, A., Bell, J., Grumbach, MM. , *Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency.* New England Journal of Medicine, 1998. **339**(9): p. 559-603.
293. Kimura, M., Sudhir, K., Jones, M., Simpson, E., Jefferis, A-M., Chin-Dusting, JPF. , *Impaired acetylcholine-induced release of nitric oxide in the aorta of male aromatase-knockout mice: regulation of nitric oxide production by endogenous sex hormones in males.* Circulation Research, 2003. **93**: p. 1267-1271.
294. Baulieu, E., . Thomas, G., Legrain, S., Lahlou, N., Roger, M., Debuire, B., Faucounau, V., Girard, L., Hervy, M., Leaud, M., Mokrane, A., Pitti-Ferrandi, H., Trivalle, C., Lacharrière, O., Nouveau, S., Rakoto-Arison, B., Souberbielle, J., Raison, J., Le Bouc, Y., Raynaud, A., Girerd, X., Forette, F. , *Dehydroepiandrosterone (DHEA), DHEA sulfate, and aging: contribution of the DHEage study to a sociobiomedical issue.* Proceedings of the National Academy for Science U.S.A, 2000. **97**(8): p. 4279-4284.
295. Willerson, J., Ridker, PM., *Inflammation as a cardiac risk factor.* Circulation, 2004. **109**: p. II-2-II-10.
296. Chudek, J., Więcek, A., *Adipose tissue inflammation and endothelail dysfunction.* Pharmacological Reports, 2006. **58**: p. 81-88.
297. Bonora, E., Targher, G., Alberiche, M., Bonadonna, RC., Saggiani, F., Zenere, MB., Monauni, T., Muggeo, M. , *Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity. Studies in subjects with various degrees of glucose tolerance and insulin sensitivity.* Diabetes Care, 2000. **23**(1): p. 57-63.
298. Wilkinson, I., Hall, IR., MacCallum, H., Mackenzie, IS., McEniery, CM., van der Arend, BJ., Shu, Y-E., Mackay, LS., Webb, DJ., Cockroft, RJ. , *Pulse wave analysis: clinical evaluation of a non-invasive, widely applicable method for assessing endothelial function.* Arteriosclerosis Thrombosis and Vascular Biology, 2002. **22**: p. 147-152.
299. Pauca, A., O'Rourke, MF., Kon, ND., *Prospective evaluation of a method for estimating ascending aortic pressure from the radial artery pressure waveform.* Hypertension, 2001. **38**: p. 932-937.
300. Chen, C., Nevo, E., Fetis, B., Pak, P.,Yin, FCP.,Maughan, L., Kass, DA. , *Estimation of central aortic pressure waveform by mathmatical transformation of radial tonometry pressure. Validation of generalized transfer function* Circulation, 1997. **95**: p. 1827-1836.

301. Karamanoglu, M., O'Rourke, MF., Avolio, AP., Kelly, RP., *An analysis of the relationship between central aortic and peripheral upper limb pressure waves in man* European Heart Journal, 1993. **14**: p. 160-167.
302. Fetics, B., Nevo, E., Chen, CH., Kass, DA., *Parametric model derivation of transfer function for noninvasive estimation of aortic pressure by radial tonometry.* IEEE Transactions on Biomedical Engineering, 1999. **46**(6): p. 698-706.
303. Hope, S., Tay, DB., Meredith, IT., Cameron, JD., *Use of arterial transfer functions for the derivation of central aortic waveform characteristics in subjects with type 2 diabetes and cardiovascular disease.* Diabetes Care, 2004. **27**(3): p. 746-751.
304. Hope, S., Tay, DB., Meredith, IT., Cameron, JD., *Comparison of generalized and gender-specific transfer functions for the derivation of aortic wave forms.* Am J Physiol Heart Circ Physiol, 2002. **283**: p. H1150-H1156.
305. Westerhof, N., O'Rourke, MF., *Haemodynalmic basis for the development of left ventricular in systolic hypertension and for its logical treatment.* Journal of Hypertension, 1995. **13**(9): p. 943-952.
306. O'Rouke, M., Kelly, RP., *Wave reflection in the systemoc circulation and its implications in ventricular function.* Journal of Hypertension, 1993. **11**: p. 327-337.
307. London, G., Guerin, AP., Pannier, B., Marchais, SJ., Simpel, M., *Influence of sex on arterial haemodynamics and blood pressure. Role of body height.* Hypertension, 1995. **26**: p. 514-519.
308. Wilkinson, I., MacCallum, H., Flint, L., Cockcroft, JR., Newby, DE., Webb, DJ., *The influence of heart rate on augmentation index and central arterial pressure in humans* Journal of Physiology, 2000. **525**(1): p. 263-270.
309. London, G., Blacher, J., Pannier, B., Guérin, AP., Marchais, SJ., Safar, ME. , *Arterial wave reflections and survival in end-stage renal failure.* Hypertension, 2001. **38**: p. 434-438.
310. Hickler, R., *Aortic and large artery stiffness:current methodolgy and clinical correlations.* Clinical Cardiology, 1990. **13**(5): p. 317-322.
311. McVeigh, G., Brennan, G., Hayes, R., Cohn, J., Finkelstein, S., Johnston, D., *Vascular abnormalities in non-insulin-dependent diabetes mellitus identified by arterial waveform analyisis.* American journal of Medicine, 1993. **95**: p. 424-430.
312. Benetos, A., Laurent, S., Hoeks, AP., Boutouyrie, PH., Safar, ME., *Arterial alterations with aging and high blood pressure. A noninvasive study of carotid and femoral arteries.* Arteriosclerosis Thrombosis and vascular biology, 1993. **13**: p. 90-97.
313. Safar, M., Blacher, J., Pannier, B., Guerin, AP., Marchais, SJ., Guyonarc'h, P-M., London, GM., *Central pulse pressure and mortality in end-stage renal disease.* Hypertension, 2002. **39**: p. 735-738.
314. Hills, M., Armitage, P. , *The two-period cross-over clinical trial.* British Journal of Clinical Pharmacology, 1979. **8**: p. 7-20.
315. Koch, G., *The use of non-parametric methods in the statistical analysis of the two period change-over design.* Biometrics, 1972. **28**: p. 577-584.

316. Hernández-Morante, J., Pérez-de-Heredia, F., Luján, J., Zamora, S., Garaulet, M. , *Role of DHEA-S on body fat distribution: gender- and depot-specific stimulation of adipose tissue lipolysis.* Steroids, 2008. **73**: p. 209-215.
317. Kanazawa, I., Yamaguchi, T., Yamamoto, M., Yamauchi, M., Kurioka, S., Yano, S., Sugimoto, T., *Serum DHEA-S is associated with the presence of atherosclerosis in postmenopausal women with type 2 diabetes mellitus.* Endocrine Journal, 2008. **(Eupub ahead of print)**.
318. Fukui, M., Ose, H., Kitagawa, Y., Yamazaki, M., Hasegawa, G., Yoshikawa, T., Nakamura, N., *Relationship between low serum endogenous androgen concentations and arterial stiffness in men with type 2 diabetes.* Metabolism Clinical and Experimental, 2007. **56**: p. 1161-1173.
319. Donald, A., Charakida, M., Cole, TJ., Friberg, P., Chowienczyk, PJ., Millasseau, SC., Deanfield, JE., Halcox, JP., *Non-invasive assessment of endothelial function: which technique?* Journal of American Collage of Cardiology, 2006. **48**(9): p. 1846-1850.
320. Barrett-Conner E, K., KT., Yen, S., *A prospective study of dehydroepiandrosterone sulphate, mortality, and cardiovascular disease.* . New England Journal of Medicine, 1986. **315**: p. 1519 to 1524.
321. Tilvis, R., Kähönen, M., Härkönen, M. , *Dehydroepiandrosterone sulphate, diseases and mortality in a general aged population.* Aging Clinical and Experimental Research 1999. **11**: p. 30-34.
322. Yorek, M., Coppey, LJ., Gellett, JS., Davidson, EP., Bing, X., Lund, DD., Dillon, JS., *Effect of treatment of diabetic rats with dehydroepiandrosterone on vascular and neural function.* American Journal of Physiology - Endocrinology and Metabolism, 2002. **283**: p. E1067-E1075.
323. Eich, D., Nestler, JE., Johnson, DE., Dworkin, GH., Ko, D., Wechsler, AS., Hess, ML., *Inhibition of accelerated coronary atherosclerosis with dehydroepiandrosterone in the heterotopic rabbit model of cardiac transplantation.* . Circulation, 1993. **87**: p. 261-269.
324. Yoneyama, A., Kamiya, Y., Kawaguchi, M., Fujinami, T., *Effects of dehydroepiandrosterone on proliferation of human aortic smooth muscle cells.* Life Sciences, 1997. **60**: p. 833-838.
325. Simoncini, T., Mannella, P., Fornari, L., Varone, G., Caruso, A., Genazzani, AR., *Dehydroepiandrosterone modulates endothelial nitric oxide synthesis via direct genomic and nongenomic mechanisms.* Endocrinology, 2003. **144**: p. 3449-3455.
326. Formoso, G., Chen, H., Kim, JA., Montagnani, M., Consoli, A., Quon, MJ., *Dehydroepiandrosterone mimics acute actions of insulin to stimulate production of both nitric oxide and endothelin 1 via distinct phosphatidylinositol 3-kinase- and mitogen-activated protein kinase-dependent pathways in vascular endothelium.* Molecular Endocrinology, 2006. **20**: p. 1153-1163.
327. Liu, D., Si, H., Reynolds, KA., Zhen, W., Jia, Z., Dillon, JS., *Dehydroepiandrosterone protects vascular endothelial cells against apoptosis through a Gα i protein-dependent activation of phosphatidylinosirol 3-kinase/Akt and regulation of antiapoptotic Bcl-2 expression.* Endocrinology, 2007. **148**: p. 3068-3076.

328. Liu, D., Iruthayanathan, M., Homan, LL., Wang, Y., Yang, L., Wang, Y., Dillon, JS. , *Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms.* Endocrinology, 2008. **149**: p. 889-898.
329. Chen, H., Lin, AS., Li, Y., Reiter, CE., Ver, MR., Quon, MJ. , *DHEA stimulates phosphorylation of FoxO1 in vascular endothelial cells via PI3-kinase- and PKA-dependent signaling pathways to regulate ET-1 synthesis and secretion.* Journal of Biological Chemistry, 2008. **[Epub ahead of print]**.
330. Smith, J., Evans, LM., Wilkinson, I., Goodfellow, J., Cockcroft, JR., Scanlon, MF., Davies, JS., *Effects of GH replacement on endothelial function and large artery stiffness in GH-deficienct adults: a randomized, double-blind, placebo-controlled study.* Clinical Endocrinology, 2002. **56**: p. 493-501.
331. Libe, R., Barbetta, L., Dall'Asta, C., Salvaggio, F., Gala, C., Beck-Peccoz, P., Ambrosi, B., *Effects of dehydroepiandrosterone (DHEA) supplementation on hormonal, metabolic and behavioural status in patients with hypoadrenalism.* Journal of Endocrinological Investigation, 2004. **27**: p. 736-741.
332. Karbowska, J., Kochan, Z. , *Effect of DHEA on endocrine functions of adipose tissue, the involvement of PPAR gamma.* . Biochemical Pharmacology, 2005. **70**: p. 249-257.
333. von Eckardstein, A., Nofer, JR., Assmann, G., *HDL and coronary heart disease: role of cholesterol efflux and reverse cholesterol transport.* Arteriosclerosis Thrombosis and Vascular Biology, 2001. **20**: p. 13-27.
334. Ryu, J.-W., Kim, MS., Kim C-H., Song, K-H., Park, J-Y., Lee, J-D., Kim, J-B., Lee, K-U., *DHEA administration increase brown fat uncoupling protein 1 levels in obese OLEFT rats.* Biochemical Biophysical Research Communications, 2003. **303**: p. 726-731.
335. Sánchez, J., Pérez-de-Heredia, F., Priego T., Portillo, M., Zamora, S., Garaulet, M., Palou, A., *Dehydroepinadrosterone prevents age-associated alterations, increasing insulin sensitivity.* Journal of Nutritional Biochemsitry, 2008. **(Epub ahead of print)**.
336. Pérez-de-Heredia, F., Hernández-Morante, J., Priego T., Nicolás, F., Portillo, M., Palou, A., Zamora, S., Garaulet, M., *Adiponectin is involved in the protective effect of DHEA aginst metabolic risk in aged rats.* Steroids, 2008. **(Epub ahead of print)**.
337. Charlton, M., Angulo, P., Chalasani, N., Merriman, R., Viker, K., Charatcharoenwittaya, P., Sanderson, S., Gawrieh, S., Krishnan, A., Lindor, K. , *Low circulating levels of dehydroepiandrosterone in histologically advanced nonalcoholic fatty liver disease.* Hepatology, 2008. **47**(2): p. 484-492.
338. Basu, R., Dalla Man, C., Campioni, M., Basu, Ananda., Nair, S., Jensen, M., Khosla, S., Klee, G., Toffolo, G., Cobelli, C., Rizza, R., *Two years of treatment with dehydroepiandrosterone does not improve insulin secretion, insulin action, of postprandial glucose turnover in elderly men and women.* Diabetes, 2007. **56**: p. 753-766.
339. Davoodi, G., Amirezadegan, A., Borumand, M., Dehkordi, M., Kazemisaeid, A., Yaminisharif, A., *The relationship between level of androgenic hormones and*

*coronary artery disease in men.* Cariovascular Journal of Africa, 2007. **18**(6): p. 362-366.

340. Akishita, M., Hashimoto, M., Ohike, Y., Ogawa, S., Iijima, K., Eto, M., Ouchi, Y., *Association between plasma dehyroepiandrosterone-sulphate levels with endothelial function in postmenopausal women with coronary risk factors.* Hypertension Research, 2008. **31**(1): p. 68-74.
341. Page, J., Ma, J., Rexrode, K., Rifai, N., Manson, J, Hankinson, S., *Plasma dehyroepiandrosterone and risk of myocardial infarction in women.* Clinical Chemistry, 2008. **54**(7): p. 1190-1196.
342. Kawano, H., Nagayoshi, Y., Soejima, H., Tanaka, Y., Yamabe, H., Kinoshita, Y., Ogawa, H. , *Dehydroepiandrosterone levels vary according as heart failure condition in patients with idiopathic dilated cardiomyopathy.* International Journal of Cardiology, 2008. **125**: p. 277-279.
343. Spalding, K., Arner, E., Westermark, PO., Bernard, S., Buchholz, BA., Bergmann, O., Blomqvist, L., Hoffstedt, J., Näslund, E., Britton, T., Concha, H., Hassan, M., Rydén, M., Frisén, J & Arner, P., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**: p. 783-787.
344. Nedergaard, J., Bengtsson, T., Cannon, B., *Unexpected evidence for active brown adipose tissue in adult humans.* American Journal of Physiology - Endocrinology and Metabolism, 2007. **293**: p. E444-E452.