

**PRODUCTION AND INTERCONVERSION OF STEROID
HORMONES IN OBESITY AND POLYCYSTIC OVARY
SYNDROME**

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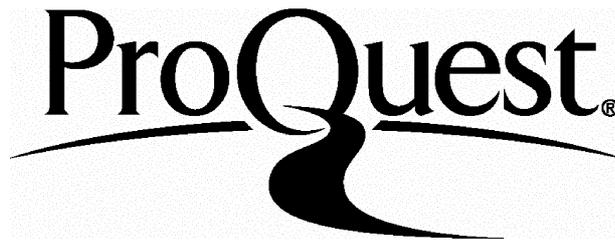
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ABSTRACT

This thesis examines alterations in the metabolism of steroid hormones in two conditions - obesity and polycystic ovarian syndrome (PCOS).

Whilst few authors doubt that alterations in steroid hormone metabolism occur in obesity and PCOS, there is considerable debate in the literature as to the exact nature of these perturbations.

The metabolism of two groups of steroid hormones – corticosteroids and sex steroids – is assessed in obesity and PCOS. Chapters 1 and 2 introduce the thesis and describe the methods, respectively. The initial study (Chapter 3) assesses the integrity of the hypothalamo-pituitary-adrenal axis (HPAA) in obesity. Some authors propose that a hyperactive HPAA gives rise to central obesity. Evidence is presented in favour of a hyperactive HPAA in centrally obese men.

The equilibrium of the cortisol-cortisone shuttle in adipose tissue is investigated. An *in vivo* study of arteriovenous sampling is described in Chapter 4, demonstrating that subcutaneous abdominal adipose tissue produces cortisol from cortisone, accounting for 4% of daily cortisol production. Adipose tissue thus produces its own growth factor – cortisol – which may exacerbate obesity in a vicious cycle.

Androstenedione conversion to testosterone in adipose tissue is said to account for 60% of testosterone production. Does peripheral testosterone production account for the hyperandrogenism seen in obese subjects with PCOS? In Chapter 5, total body fat testosterone production is calculated to be equal in PCOS and non-PCOS obese women, representing a lower proportion (4-10%) of systemic production than previously described. However, acute hyperinsulinaemia in the euglycaemic clamp increases peripheral production of testosterone (Chapter 6) without altering systemic testosterone levels (Chapter 7).

This thesis extends scientific knowledge on abnormalities of steroid hormone metabolism in obesity and the role of adipose tissue as an endocrine organ.

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Glossary of Abbreviations

A	androstenedione
A-V	arteriovenous
11-BHSD	11- β hydroxysteroid dehydrogenase
17-BHSD	17- β hydroxysteroid dehydrogenase
BMI	body mass index
DEXA	dual energy x-ray absorptiometry
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
E1	oestrone
E2	oestradiol
FGS	Ferriman and Gallwey Score
FM	Forearm muscle
HPAA	hypothalamo-pituitary-adrenal axis
LBM	lean body mass
PCO	polycystic ovaries on ultrasound
PCOS	polycystic ovary syndrome
SAAT	subcutaneous abdominal adipose tissue
SHBG	sex hormone binding globulin
T	Testosterone
TBF	Total body fat
WHR	waist: hip ratio

CHAPTER 1

INTRODUCTION

Scientific Background and Aims

1-1) Aims of Chapter 1

This chapter introduces the thesis. The common theme that runs throughout this thesis is examination of abnormalities of steroid hormone metabolism, as applied to obesity and polycystic ovary syndrome (PCOS). The aim of the introduction is to summarise the research undertaken previously which is relevant to this thesis and is not intended as a comprehensive review of the entire endocrinology of obesity. It is logical to begin by defining the terms “obesity” and “central obesity” which are discussed in 1-2 (a) and 1-2(b).

Section 3(a) of the introduction discusses knowledge of abnormalities of the HPAA axis in obese premenopausal women, relating to efforts to delineate this relationship in men and postmenopausal women (Chapter 3). The remainder of section 3 discusses current knowledge of peripheral corticosteroid metabolism. The relevance of the cortisol-cortisone shuttle in adipose tissue is discussed in section 3(b) and this sets the scene for the *in vivo* study of the cortisol-cortisone shuttle in adipose tissue described in Chapter 4 of this Thesis.

The hydroxysteroid dehydrogenase enzymes are key to an understanding of adipose tissue steroid hormone metabolism. Whilst section 3 of this introductory chapter includes discussion of the role of 11-beta hydroxy

steroid dehydrogenase (11-BHSD), section 4 focuses on the related enzyme 17-beta hydroxysteroid dehydrogenase (17-BHSD), the various isoforms of which control (i) interconversion of androstenedione (A) and testosterone (T) and (ii) interconversion of oestrone (E1) and oestradiol (E2). Aromatase activity, converting A to E1 and T to E2 is also discussed in section 4. This section relates to Chapter 5 of this Thesis, where *in vivo* studies of peripheral sex steroid metabolism in obese women (with and without PCOS) are described, qualifying the net direction of 17-BHSD and aromatase enzyme action and quantifying peripheral production rates of sex steroids .

Section 5 of the introduction outlines current controversies on the definition of PCOS and summarises the knowledge to date on the inter-relationships of PCOS, obesity and insulin resistance. This section introduces the debate on the influence of hyperinsulinaemia on hyperandrogenism. Later in the Thesis (Chapters 6 and 7) the influence of acute hyperinsulinaemia on peripheral and systemic sex steroid production is assessed.

Section 6 of the introduction describes the central hypothesis of the Thesis. Section 7 describes how each of the remaining chapters aims to test different aspects of the central hypothesis.

1-2) Obesity

1-2 (a) Definition of Obesity

As we enter the new millenium, obesity is emerging as a major public health problem, with a dramatic increase in obesity rates in western populations in the last fifty years (1;2). Whilst there has been a great deal of scientific interest in genetic factors predisposing to obesity, environmental factors - the consumption / availability of high-fat convenience food, motorised transport and television viewing – are of paramount importance (1;3;4). Obesity has a plethora of health consequences and represents, after smoking, the second most important modifiable risk factor for the prevention of cerebrovascular disease, ischaemic heart disease, hypertension, breast and endometrial cancers (5;6).

Obesity reflects a disorder of energy imbalance, where caloric intake in excess of energy requirements is stored in adipose tissue (7).

Obesity is defined as an excess of body fat, but indirect measures are often used as body fat may be difficult to measure. Body mass index (BMI), which is the weight (in kilograms) divided by the height² (in metres), is a widely accepted measure of “fatness” (8). The World Health

Organisation (WHO) define obesity as a BMI > 30 kg/m² (2). The U.S. Metropolitan life insurance data from the 1959 and 1983 (9;10) plus Framingham (11) data provide evidence that high BMI is associated with increased morbidity and mortality. There is a J-shaped curve with low BMIs also demonstrating adverse events. Table 1.1 depicts the BMI strata. Definitions of obesity based on BMI are the most widely applied clinical criteria but fail to take into account lean body mass.

Table 1.1 Classification of obesity by BMI (2)

BMI (kg/m ²)	Obesity category
<20	Underweight
20-25	Healthy weight
25.1- 29.9	Overweight
30-40	Obesity
>40	Morbid obesity

Fat-free mass and lean body mass are defined by the following equations:

Fat-free mass = Body weight – Total Body Fat

Lean Body Mass = Fat-free mass – skeletal mass

Skeletal mass is difficult to measure without dual photon absorptiometry and lean body mass (LBM) is generally taken to be 50% of the fat-free mass (12;13). When measuring obesity by weight or BMI, muscle mass is a key confounder. Athletes may have a high muscle mass, hence a high lean body mass and BMI, with normal or even low fat mass (8). Thus a BMI of 30 in a swimmer is not unusual and would not be a measure of increased risk of morbidity. So measures other than BMI need to be employed to characterise obesity and total body fat may be measured by bioimpedance (14) or dual photon absorptiometry (15).

1-2 (b) Central obesity

The association of central obesity with insulin resistance, dyslipidaemia and high blood pressure was recognised over 40 years ago (16), but has received greater attention in recent years (17). Central obesity is an independent risk factor for the development of cardiovascular morbidity and mortality (11;18;19). Central obesity has been variously termed android or male adiposity, upper body or abdominal obesity, truncal obesity and visceral obesity – these terms have been used interchangeably in the literature. Fat distribution may be measured by calculating the ratio of waist : hip circumference (WHR). Central obesity is defined as a WHR >0.9 in men and >0.8 in women (7). Subscapular: triceps skinfold thickness ratios, DEXA scanning, MRI or CT imaging in a single transverse abdominal section have all been used to measure the distribution of body fat (20). The advantages and limitations of each technique will be discussed in the methods section (Chapter 2).

One cause of central obesity is cortisol excess. Cushing's syndrome is the term given to the symptoms and signs generated by chronic elevation of circulating free cortisol (21). Cushing's syndrome results from extra-pituitary ACTH or cortisol secreting tumours or, more commonly, corticosteroid therapy. Cushing's disease refers to the pituitary ACTH-dependent subtype. The clinical features of Cushing's syndrome include

central obesity, hypertension, glucose intolerance and depression (21).

Whilst Cushing's syndrome, in the absence of exogenous cortisol administration, is rare, some authors postulate that central obesity and its associated metabolic abnormalities represent a subclinical form of Cushing's syndrome. In other words, there is a large subgroup of patients who exhibit hyperactivity of the HPAA, but suppress cortisol production when tested with standard clinical dexamethasone suppression tests (21).

I will now address the developing literature on links between hyperactivity of the HPAA and central obesity.

1-3) Corticosteroid metabolism in obesity.

1-3 (a) The HPA-axis in Obesity

The pituitary and adrenal response to provocation tests in obese premenopausal women has been extensively studied (22-27). However, there is some controversy regarding the influence of body mass index (BMI) on the adreno-corticotrophic hormone (ACTH) response to corticotrophin releasing hormone (CRH). The ACTH response to CRH in obese premenopausal women has been variously reported as blunted (25), normal (22;24) or enhanced (27). The cortisol response to CRH in obese women has been reported as normal (24;27) or reduced (22;25). None of these studies controlled for the influence of depression, which is

more prevalent in obesity (28). Depression is associated with elevated mean 24hr plasma cortisol levels (29), a blunted ACTH and a normal cortisol response to CRH (30;31).

Other studies (23;32) have demonstrated that distribution of body fat rather than BMI predicts the pituitary and adrenal response to stimulation tests. In centrally obese premenopausal women, there is an enhanced ACTH response to CRH (23) and an enhanced cortisol response to stimulation including the CRH test (23;32). Some authors have extrapolated from these data to assert that a hyperactive hypothalamo-pituitary-adrenal (HPA) axis results in increased cortisol production rates and that this leads to central obesity and insulin resistance (33). The data in this area is almost entirely drawn on studies of premenopausal women and, in Chapter 3, I will assess the integrity of the HPAA in relation to adiposity in men and postmenopausal women.

The study of cortisol metabolism in obesity or central obesity has to take into account more than simply an assessment of the activity of the HPAA. There has been a great deal of discussion as to the direction of the cortisol-cortisone shuttle in adipose tissue, with some evidence that visceral adipose tissue produces cortisol from cortisone. In 1997, one group declared evidence for “Cushing’s disease of the omentum” (34). I

will now summarise the debate on the cortisol-cortisone shuttle in adipose tissue.

1-3 (b) The cortisol – cortisone shuttle in adipose tissue

The nature of peripheral corticosteroid metabolism in obesity is controversial. Traditionally, it has been assumed that cortisol is converted to its inactive metabolite cortisone in adipose tissue. The *in vitro* studies on which this assumption was based (35) have been challenged by recent studies demonstrating oxo-reduction of cortisone to cortisol in visceral adipose stromal cell cultures (34). The cortisol-cortisone shuttle is controlled by the two isoforms of 11 β -Hydroxysteroid Dehydrogenase, 11-BHSD1 and 11-BHSD2 (36;37). Although both isoforms may dehydrogenase cortisol at the 11 β -position to form cortisone, the type 2 isoform has the higher affinity for this reaction. The type 1 isoform is a low affinity enzyme, which also promotes the reverse reaction - oxo-reduction of cortisone to cortisol. Early studies on subcutaneous abdominal fat homogenates (35) suggested dehydrogenation of cortisol to cortisone occurred in adipose tissue. However, net oxo-reduction of cortisone to cortisol has been demonstrated in breast adipose tissue (38). More recent studies detected 11-BHSD1, but not 11-BHSD2, mRNA in visceral and subcutaneous adipose tissue (34). Furthermore visceral

adipose stromal cell cultures demonstrated predominant oxo-reductase activity (34).

There have been few studies of the cortisol-cortisone shuttle in adipose tissue *in vivo*. A previous study of cortisol metabolism utilising A-V difference techniques demonstrated no significant gradient of plasma cortisol across deep (largely muscle) and superficial (skin and adipose tissue) forearm compartments (39). However, superficial forearm veins only drain limited amounts of adipose tissue. In chapter 2, we shall describe the technique of measuring A-V differences in hormones across SAAT.

The above *in vitro* and *in vivo* studies examine adipose tissue directly, but the subject of peripheral corticosteroid metabolism has also been studied by measuring levels of cortisol metabolites in urine. In the next section, I outline the literature on urine corticosteroid profiles in obesity.

1-3 (c) Urine cortisol metabolite studies in obesity

Total urine cortisol metabolite levels (TCM) can be utilised to reflect (or indicate) cortisol production rates. Correction for creatinine controls for the influence of lean body mass (40). Increased TCM have been reported in obesity (41), but two recent studies (42;43) demonstrated no significant relationship between BMI and TCM in men and women.

The ratio of urine cortisol:cortisone metabolites (Fm/Em) provides a measure of net whole body 11-BHSD activity and, thereby, the net direction of the cortisol-cortisone shuttle. There are conflicting data on whether the ratio of Fm/Em is influenced by the distribution of body fat. Weaver *et al* studied 43 hypopituitary patients on hydrocortisone replacement therapy (44) and suggested that women on conventional hormone replacement therapy (HRT) had a lower urinary Fm/Em ratio. Increasing obesity, whether android or gynoid, was associated with a lower urinary Fm/Em ratio, but stepwise regression analysis confirmed gynoid fat to be most important in this regard (44). Andrew *et al* analysed urine steroid profiles in 68 men and women and they found a positive correlation of the Fm/Em ratio with both hip circumference and waist circumference in men (42). However, they do not present their data on waist-hip ratio and have no other index of central obesity (42). Meanwhile, Stewart found that the Fm/Em ratio is inversely related to

android (central) fat distribution, (43), which is contrary to his group's *in vitro* data on visceral adipose tissue (34). Urine steroid profiles and CRH responses have not as yet been analysed in a single cohort and this is the subject of chapter 3.

1-4) Sex Steroid metabolism in Obesity

1-4 (a) Androgen metabolism in obesity and adipose tissue

In men, the testes are the principal source of testosterone and absence of gonadal function results in a failure to develop mature secondary sexual characteristics, as in the inherited disorder Klinefelter's syndrome (45). In women, the adrenals are an important source of androstenedione, which may be converted peripherally to oestrone (aromatisation) or testosterone (17-BHSD activity). In premenopausal women, testosterone is produced both by the ovaries and by peripheral conversion of androstenedione (46-48). Dihydrotestosterone (DHT) is produced entirely in the periphery, by 5- α reductase action on testosterone (47). In the circulation, sex steroids are bound with high affinity to SHBG (49) and fluctuations in SHBG levels alter the levels of "free" hormone available for peripheral conversion and hepatic metabolism. As with corticosteroids, sex steroid metabolism in obesity can be assessed by looking at circulating levels of hormones, whole body metabolic clearance studies and assessing directly (by *in vivo* or *in vitro* techniques) how adipose tissue handles sex steroids. I will now review the subject of androgen metabolism in obesity, starting with studies on circulating levels of androgens in obesity. Most studies have shown total androgen levels in obese eumenorrhoeic women are either normal or low (6;50;51). However, Kopelman found

that, in massively obese women, total A and T were increased, levels normalising after weight loss of 40kg (52). In another study by Turcato *et al*, 26 premenopausal and 15 postmenopausal women were hospitalised and put on a “very low energy diet”, with no effect of weight loss (mean weight loss of 7.8 kg or 8% of initial weight) on total testosterone levels in pre- or post-menopausal women (53). However, the weight loss in the Turcato study was not as dramatic as that witnessed in the Kopelman study and changes in testosterone levels may only become significant after massive weight gain / reduction.

There is controversy regarding the levels of free (unbound) testosterone in obesity. It has been clearly shown that SHBG is reduced in obesity (54). Whilst some studies show free testosterone levels are increased (50;54) in obese women, falling after weight loss (53;55), other studies conclude that free T levels are normal in obese premenopausal (56) and postmenopausal (57) women. Thus there is some evidence that testosterone or free testosterone levels are increased in obesity, but additional information may be found in whole body metabolic clearance studies:

Samojlik found an increased metabolic clearance rate (MCR) of T and DHT in obese eumenorrheic women (51). The MCR of T was 1,256 +/- 145 L/day in obese women compared to 740 +/- 40 L/day in normal weight controls. Adrenal and ovarian androgen production rates were also increased in obesity (51). An increased MCR of A and DHEA was found in another study of obese eumenorrheic women, despite normal circulating levels of these androgens (58). Both the production rate and MCR of A correlated with WHR, suggesting an association of central obesity with increased A production and clearance (58). The cause of the increased MCR of androgens in obesity has not been established, but two main arguments have been put forward (59).

1) Decreased SHBG in obesity leaves increased free T available for hepatic extraction and clearance (60). However this does not explain the increased MCR of A, DHEA, DHT which are not bound significantly to SHBG in plasma.

2) Hyperinsulinaemia may lead directly to increased production of androgen in ovaries and adrenals, decreased hepatic SHBG production and thereby requires an increased MCR to maintain normal plasma levels (61).

The latter argument is the more plausible of the two as one would not expect SHBG levels to influence systemic testosterone clearance in steady state conditions. Having assessed circulating androgen levels and whole body metabolic clearance studies in obesity, we now turn to consider what is known of the role of adipose tissue in androgen metabolism. A summary of *in vivo* and *in vitro* studies on androgen metabolism in adipose tissue follows to complete this review of androgen metabolism in obesity.

There is *in vitro* evidence to suggest active sex steroid metabolism in adipose tissue, with concentrations of sex steroids in adipose tissue explants being higher than plasma levels (62). Deslypere demonstrated aromatase and 17-BHSD activity in these adipose tissue explants. More recently, 17-BHSD has been subtyped into 7 enzymes (63-65). Type 3 17-BHSD is responsible for catalysing the conversion of A to T whilst type 2 17-BHSD catalyses the reverse reaction. Both conversion of A to T (66) and the reverse reaction (67) have been demonstrated in human adipose tissue homogenates *in vitro*, with controversy as to which reaction predominates. Both type 2 and type 3 are expressed in both SAAT and visceral adipose tissue (64) and the question remains as to which enzyme action is predominant *in vivo*.

In vivo studies have sought to examine the contribution of peripheral tissues to androgen metabolism. In 1975, Chang and Abraham measured arterial and venous concentration gradients across the deep forearm compartment and found no A-V difference in DHEA, DHEAS, A, T and DHT (68). In 1985, Longcope combined the A-V difference technique with infusions of radiolabelled A and T to measure DHT production in the forearm (69). He found that 14% of radiolabelled A and 6% of T were converted to DHT in the superficial compartment (adipose tissue) of the forearm. Longcope's studies, whilst elegant and methodologically sound, only assessed the forearm. This is problematic when you consider that the volume of adipose tissue in the superficial compartment of the forearm can be very small. In forearm studies, it is difficult to be certain that what is being measured is adipose tissue activity as there is a significant contribution from skin or, via anastomoses, with muscle in the deep compartment of the forearm.

A-V difference studies of abdominal adipose tissue were undertaken in 1992 by Boulton *et al* (70), when, in addition to arterialisation of a vein, the superficial epigastric vein was cannulated in each subject to measure concentrations of hormones in the venous drainage of subcutaneous abdominal adipose tissue (SAAT). This technique yielded a measure of A-V difference of sex steroids across SAAT in 8 men and 7 women. The

study demonstrated that in SAAT there was testosterone clearance in men, testosterone output in women, and E1/E2 output in men and women (70). This technique represents an advance in that more fat is stored in SAAT than the forearm, making this a more representative tissue bed to study. The problems with this study are that numbers of male and female subjects were small and the authors relied on arterialised, rather than arterial, samples which can lead to inaccuracy when assessing small AV differences.

1-4 (b) Oestrogens in obesity and adipose tissue

Oestrogen metabolism in obesity has been widely studied (6;71). The association of obesity with an increased risk of endometrial and breast cancer has led to a search for an endocrine basis for this relationship. There is a linear relationship between excess body weight and risk of endometrial cancer (72;73) in women over the age of 50 years. Obesity is not only a risk factor for the development of breast cancer (74) but also for the recurrence of treated breast cancer (75). Although there are a number of possible explanations - ranging from dietary to oncogenic - for the link between obesity and cancer in women, the fact that unopposed oestrogen action is already causally linked to both breast and endometrial cancer has provoked a search for evidence of oestrogen excess in obesity.

Oestrogen levels in obesity

Indirect evidence comes from studies of osteoporosis. Whilst oestrogen deficiency in the menopause is associated with osteoporosis, obese women have a lower incidence of osteoporosis (6). Studies have assessed levels of both plasma oestradiol (E2) and its inactive precursor oestrone (E1). The most accurate studies involve venous sampling every 20 minutes for 24 hours and measurement of plasma E1 and E2 – no difference was found between obese and non-obese premenopausal

women (50). However SHBG levels are lower in obesity and free oestradiol levels may be increased. In postmenopausal women- and it is in this group that the excess risk of cancer is found - there is a correlation between total and free oestradiol levels and degree of obesity or fat mass (76), although total E1 and E2 levels fall progressively with advancing age in obese women (77).

Oestrogen metabolism - in vitro studies

Peripheral conversion of sex steroids can result in oestrogen production by aromatisation of A to E1, aromatisation of T to E2 and finally 17BHS conversion of E1 to E2. These reactions have been studied both *in vitro* and *in vivo*. Deslypere *et al* demonstrated 17BHS and aromatase activity in fat tissue removed from 129 women at surgery (62) as outlined in 1-4 (a). However, *in vitro* enzyme studies do not necessarily reflect reactions that take place *in vivo* and do not provide a quantitative index of oestrogen production in adipose tissue.

Radiolabelled A and E2 infusions (2 label technique) have been used to quantify peripheral conversion of sex steroids. Using this technique, Macdonald and Sitteri demonstrated a positive correlation between body weight and the rate of aromatisation of A to E1 (78). Longcope found a positive correlation between body weight and conversion of T to E2 (79).

These studies examined systemic oestrogen metabolism and could not locate the sites at which aromatisation was taking place. Sitteri himself concluded, after 20 years of research in the field, that the exact site of peripheral aromatisation and the factors controlling the process had not been defined clearly (80;81).

Direct study of sex steroid metabolism in adipose tissue was undertaken by Boulton *et al*, who demonstrated with the AV difference technique output of E1 and E2 across SAAT *in vivo*. The limitations of these studies were discussed in 1-4(a).

1-5) Polycystic Ovary Syndrome

1-5 (a) Definitions of PCOS

A definition of polycystic ovary syndrome is not readily achieved in a single sentence. There is no single globally accepted definition. Many reproductive endocrinologists in the United Kingdom define polycystic ovary syndrome (PCOS) as the presence of polycystic ovaries (PCO) on ultrasound (82) and, in addition, one or more of the clinical features of hirsutism, acne, menstrual disturbance, infertility or alopecia (83). The prevalence of PCO on ultrasound in population-based studies of premenopausal women is consistently 22-23% (84;85) and is frequently not associated with any clinical symptoms¹. This has led investigators in the United States to base diagnostic criteria on features other than ovarian morphology on ultrasound. Thus, PCOS has been defined as the combination of “hyperandrogenism with ovulatory dysfunction with the exclusion of specific disorders such as nonclassic 21-hydroxylase deficiency, hyperprolactinaemia or androgen-secreting neoplasms. The polycystic ovary morphology is consistent with, but not essential for, the diagnosis of the *syndrome...*” (86). The inconsistency in these definitions reflects the gaps in our knowledge of PCOS as a pathological entity.

¹ The Clayton study found that 43 (22%) of the 208 women studied had PCO on ultrasound. Within the PCO group, 6 (14%) had hirsutism, 9 (21%) had acne, 10 (30%) had oligo/amenorrhoea. Hirsutism was the only variable significantly higher in the PCO vs non-PCO group.

Furthermore, the lack of a common definition makes comparisons between different clinical studies difficult.

Clinical features of PCOS

Taking the broader definition of PCOS to include women with ultrasound appearances of PCO with one or more clinical symptoms of hyperandrogenism, Conway *et al* have described the clinical and biochemical heterogeneity of PCOS in a cohort of 556 patients attending a reproductive endocrine clinic (83). The commonest clinical features were menstrual irregularity (oligomenorrhoea in 45%, amenorrhoea in 26%) followed by hirsutism (61%), infertility (29%), acne (24%), alopecia (8%) and acanthosis nigricans (2%). Selection bias in a clinic setting means that these figures are not universally applicable, but give a good idea of the array of symptoms in PCOS found in a tertiary referral centre.

Biochemical abnormalities in PCOS

When compared to controls (n=23), women with PCOS have higher LH and testosterone levels (83). High levels of LH (>10 IU/l) were found in 44% of PCOS subjects and these “high LH” subjects had higher levels of testosterone, larger ovaries and a greater prevalence of infertility than the “normal LH” PCOS group. Hirsutism and obesity within the PCOS

cohort were independently associated with higher mean serum testosterone levels (83). This cohort had a single LH measurement. However, LH is secreted in pulses with a circadian rhythm under the influence of the gonadotrophin releasing hormone (GnRH) pulse generator. A detailed evaluation, with 10 minute serum sampling for 24 hours, demonstrated increased LH pulse frequency and amplitude in anovulatory PCOS women, compared to controls (87). Lower serum HDL cholesterol levels have been shown in PCOS (88). There is evidence to suggest cardiovascular risk is increased in patients with PCOS. This is based on cross-sectional analysis of patients attending cardiac catheterisation (89) and cardiovascular event rates in women who previously had wedge resection of the ovaries (90), but this latter study has not been replicated² and there is a lack of prospective data in this important area.

2

Prof H.S. Jacobs carried out such a study, looking at death certificates of women who had wedge resections in the 1950-60's, but their cardiovascular mortality was not increased. (personal communications).

1-5 (b) Obesity and the Polycystic Ovary Syndrome

Obesity is strongly associated with PCOS, with 35% of PCOS women being overweight (BMI>25) in one series (83). I will discuss in section 1-5(d) the association of menstrual irregularity with insulin resistance in lean and obese PCOS women. Insulin resistance is heightened when obesity is present in addition to menstrual irregularity (86). Obese, but not lean, anovulatory PCOS women display abnormal glucose tolerance with 30-40% meeting WHO criteria for impaired glucose tolerance compared to approximately 10% for age and weight-matched non-PCOS women (86). PCOS strongly predisposes the individual to the development of NIDDM with a 15% prevalence of NIDDM following the menopause in women who previously underwent wedge resection of the ovaries (91).

Obese PCOS subjects in addition to greater insulin resistance (86), also have higher levels of total T and increased hirsutism(83) when compared to lean PCOS females. In obese patients with anovulatory PCOS, weight loss of greater than 5% significantly reduces fasting insulin levels and restores ovulatory menstrual cycles (92). Whilst weight loss in obese PCOS subjects has undoubted metabolic benefits, low body weight (BMI <20) may exacerbate oligomenorrhoea and other symptoms. The bulk of the evidence suggests that obesity and oligo-/amenorrhoeic PCOS

combined produce a markedly insulin resistant state, with deleterious consequences for gonadotrophin secretion, adrenal and ovarian steroidogenesis.

The mechanism by which obesity gives rise to insulin resistance and hyperandrogenism in PCOS is not clear. At one stage, there was great interest in leptin as the missing link between adipose tissue and the hypothalamo-pituitary-adrenal/gonadal axes. Indeed, there is animal data to support this (93). However, although leptin is implicated as the signal of critical fat mass in normal puberty (94), carefully controlled studies in anovulatory PCOS subjects have not shown any left or right shift in the established relationship between leptin and BMI (95-97).

1-5 (c) The contribution of peripheral tissues to testosterone production rates in PCOS

In 1966, Horton and Tait measured metabolic clearance rates of infused radiolabelled A and T in eight women with regular menstrual cycles (48).

They estimated the production rate of testosterone to be 0.34mg/day.

More recently, this figure has been replicated with 24 hr sampling using a stable isotope dilution technique (98) where healthy women were found to have a testosterone production rate of 0.4 +/- 0.1 mg/day, compared to 3.7 +/- 2.2mg/day in men. In women with ovarian hyperthecosis

(anovulatory PCOS with marked virilisation) the testosterone production rate has been estimated as 2.1 mg per day, as compared to 0.3 mg/day in controls. There is a lack of data on testosterone production rates in PCOS subjects with less aggressive virilisation and this may only be 1mg/day.

What proportion of the daily testosterone production is derived from peripheral conversion of androstenedione? Horton and Tait estimated 60% of circulating T was derived from 17-BHSD conversion of androstenedione in the periphery (48). Vermeulen (6;47) estimated that 55% of testosterone in premenopausal women is derived from peripheral conversion with only 25% coming directly from the ovaries.

Boulton *et al*, using the SAAT A-V difference technique, estimated TBF production of testosterone in women to be 0.04mg/day, which would represent 10% of testosterone production, much smaller than the figures suggested by previous studies (70). One of the seven women in the Boulton study had PCOS – the question remains whether peripheral conversion of androstenedione contributes significantly to testosterone production rates in women with PCOS.

1-5 (d) Insulin resistance in PCOS

The clinical association of diabetes with hirsutism was established in 1921 by Achard and Thiers (99). It was not until 1980 that the biochemical association of hyperinsulinaemia with hyperandrogenism in PCOS was established by Burghen (100). Later, it became clear that insulin resistance was a feature of oligo-/amenorrhoea rather than hyperandrogenism *per se* (88;101;102). Women with PCO and a normal menstrual cycle have normal insulin sensitivity (102). Studies of PCOS have shown higher fasting insulin levels when menstrual irregularity is present (88;102). Dunaif demonstrated a significantly enhanced insulin response to a glucose load in both lean and obese women with ovulatory dysfunction and hyperandrogenism³ as compared to both hyperandrogenic ovulatory women and controls (101). Glucose intolerance or frank NIDDM was demonstrated in 20% of obese hyperandrogenic anovulatory women but was not a feature of lean subjects (86;101).

³ Dunaif definition of PCOS being hyperandrogenic anovulation, excluding ovulation in oligomenorrhoeic women with day21 progesterone measurements; ovulatory hyperandrogenic women could include idiopathic hirsutism, PCO on ultrasound with hirsutism, PCO with high androgens and no symptoms..a heterogenous group.

Further studies have demonstrated intrinsic defects in insulin action in PCOS at receptor or post-receptor level (103). Investigations of the molecular basis of insulin resistance in PCOS suggest that serine phosphorylation of the insulin receptor is one factor, although 50% of PCOS women demonstrate no abnormality in insulin receptor phosphorylation (86). Post-receptor signalling defects, including serine phosphorylation of insulin receptor substrate-1 (IRS-1) have been described (86).

There have been a number of clinical studies assessing the impact of manipulating insulin levels or insulin sensitivity in PCOS. They fall into two broad categories: firstly, assessing the impact of several weeks administration of both insulin sensitising agents (metformin, troglitazone) and agents influencing insulin secretion (diazoxide, octreotide); secondly examining the effects of intravenous insulin infusions for 2 to 16 hours by the euglycaemic clamp method.

1-5 (e) Modifying insulin resistance in PCOS

Clinical studies in which reductions in insulin secretion or insulin resistance were achieved by pharmacological intervention have produced significant reductions, without normalisation, in circulating plasma androgen levels in obese women with PCOS. Insulin secretion has been inhibited with diazoxide (104) and somatostatin (105) for up to 3 months in PCOS subjects, with a concomitant fall in androgen levels.

Somatostatin treatment, but not diazoxide, produced a reduction in LH pulse amplitude and frequency (104;105). Ehrmann has reviewed 12 studies demonstrating that metformin reduces insulin resistance, lowers androgen levels and increases menstrual regularity in obese PCOS women (106). Whilst some of the benefits of metformin have been attributed to weight loss, at least one major study (107) showed benefits of metformin without weight loss: administration of metformin 500mg tds to 11 obese PCOS subjects resulted in significant lowering of insulin resistance (as measured by OGTT with insulin/glucose levels), LH and androgen levels with a significant increase in SHBG (107).

Two major studies on increasing insulin sensitivity in obese PCOS women (108;109) gave obese anovulatory PCOS women troglitazone 400mg daily for 12 weeks. Troglitazone increased insulin sensitivity and reduced free testosterone, DHEAS and oestradiol levels. There was a

trend in the above studies to increased rate of menses during troglitazone treatment in oligo-/amennorrhoeic PCOS women, but larger studies with longer follow-up would be needed to assess whether this is statistically significant. Taken together these studies suggest that insulin resistance in anovulatory PCOS affects the HPA/HPG axis at pituitary, adrenal and ovarian levels. Increased insulin sensitivity/reduced insulin levels result in reductions in levels of LH, SHBG, adrenal androgens (DHEAS) and testosterone. However androgen levels are not normalised and the clinical benefits in terms of ameliorating hirsutism, acne and menstrual irregularity would require longer trials of therapy and there is a lack of published data in this area. Equally, 50% of PCOS subjects are lean and the effects of insulin sensitising agents in these individuals – whether ovulatory or not - has not been assessed.

The above studies have all addressed the issue of ameliorating insulin resistance and lowering insulin levels. Conversely, other investigators have assessed the impact of acute hyperinsulinaemia by the euglycaemic clamp method and this will be outlined in the next section.

1-5 (f) Hyperinsulinaemic euglycaemic clamp studies in PCOS

Fox *et al* infused insulin at a near physiological post-prandial rate of 40 $\mu\text{u}/\text{m}^2/\text{min}$ for 2 hours to lean and obese PCOS subjects with appropriate controls, there was no alteration in A or T levels in PCOS subjects (110). Obese controls showed a small rise in A, but levels were still well below the pre-clamp levels in PCOS subjects (110).

Micic *et al* infused insulin for 270 minutes at 3 stepwise incremental rates (achieving peak insulin concentrations of 600 $\mu\text{u}/\text{L}$, double that in the previous study by Fox *et al*) in a group of 6 obese PCOS subjects with no controls (111). They found a significant increase in T from 4.8 nmol/l at baseline to 8.1 nmol/l at the end of the study (111).

Nestler *et al* performed euglycaemic clamp studies on five normal women and only one subject with PCOS, continuing insulin at a high rate for 16 hours (steady state insulin concentrations of 1800 $\mu\text{u}/\text{L}$ (112). This study demonstrated no effect of insulin on testosterone or hourly LH levels, but there was a fall in DHEA-S in the normal women, independent of circadian rhythm; A was not measured and it is possible DHEA-S was converted to A.

The effect of acute hyperinsulinaemia on SHBG was assessed by Fendri *et al* who studied a group of 14 obese PCOS women and found that SHBG levels fell after a 6 hour insulin infusion, plasma insulin concentrations peaking at 1,100 $\mu\text{u/L}$ ⁴ (113). Taken together these studies imply that obese PCOS women when studied in sufficient numbers with insulin infusion continuing for at least 4 hours, do demonstrate an increase in T and a fall in SHBG. One limitation of these studies are that no single study has yet analysed simultaneously SHBG, T, A ,E2 and LH /FSH levels in response to insulin and this is a subject we address in Chapter 7.

⁴ This figure has been calculated using a conversion factor of 7.175, the original figure quoted being 8000 pmol/l

1-6) Hypotheses

The central hypothesis of this thesis is that the production and interconversion of steroid hormones are altered in obesity and PCOS. Furthermore, steroid hormone metabolism is related to body fat distribution, with central obesity reflecting a physiology overlapping with, but distinct from, peripheral or generalised obesity. This central hypothesis is tested from a variety of angles in this thesis, through a range of studies:

Firstly, the production of corticosteroids in a group of men and postmenopausal women with a range of body fat distribution is assessed. The hypothesis that centrally obese men and postmenopausal women have increased HPAA activity, independent of the influence of depression is tested.

The physiology of the endocrine function of adipose tissue with respect to corticosteroid and sex steroid metabolism is assessed. Steroid hormone interconversion occurs in adipose tissue, but what is the relative contribution of adipose tissue to daily corticosteroid and sex steroid production and how is this altered in PCOS?

The cortisol-cortisone shuttle in adipose tissue is studied *in vivo*. The hypotheses that (i) adipose tissue is a net converter of cortisone to cortisol and (ii) peripheral cortisol production is increased in centrally obese subjects are tested.

In terms of sex steroid interconversion, this thesis tests the hypotheses that (i) adipose tissue is a net producer of testosterone and oestradiol (Chapter 5) and (ii) peripheral testosterone production is increased in PCOS, contributing to the hyperandrogenism seen in this condition.

The influence of insulin on the endocrine function of adipose tissue is examined in the specific context of sex steroid metabolism in PCOS. This thesis tests the hypothesis that insulin infusion by the euglycaemic clamp method in PCOS subjects increases peripheral testosterone production, increases systemic levels of testosterone and lowers levels of SHBG.

The next section, entitled “Aims of the Thesis”, outlines how each of the subsequent chapters examines the central hypothesis of this thesis, which is that the production and interconversion of steroid hormones is altered in obesity and PCOS.

1-7) Aims of the Thesis

- 1) Describe the methods, which have been used in the thesis (Chapter 2).
- 2) The relationship of body fat distribution to production of corticosteroids by the HPAA is assessed first. The activity of the HPAA in a group of men and postmenopausal women with a range of distribution of body fat is assessed, to determine whether central obesity is associated with increased HPAA activity, independent of depression (Chapter 3).
- 3) The endocrine function of adipose tissue with respect to steroid hormone metabolism is assessed. The cortisol-cortisone shuttle is studied *in vivo* in subcutaneous abdominal adipose tissue and forearm muscle utilising A-V difference techniques in order to quantify daily peripheral cortisol production and its relationship to body fat distribution (Chapter 4).
- 4) Sex steroid interconversion in adipose tissue is studied in a group of obese women with and without PCOS. The net clearance / production rate of androstenedione, testosterone and oestradiol is measured *in vivo* in adipose tissue and forearm muscle in women with and without

polycystic ovary syndrome utilising the A-V difference technique (Chapter 5).

- 5) In order to assess how acute hyperinsulinaemia influences steroid hormone metabolism in PCOS, the hyperinsulinaemic euglycaemic clamp method is applied in PCOS subjects following baseline A-V difference studies. The effect of insulin on peripheral sex steroid production (Chapter 6), systemic sex steroid and SHBG levels (Chapter 7) is measured.

- 6) Finally, in Chapter 8, conclusions are drawn regarding the role of adipose tissue as an endocrine organ. The novel findings in this thesis regarding the production and interconversion of steroid hormones in obesity and PCOS are summarised. Finally, ideas for further research in this area are discussed.

Chapter 2

METHODS

2-1) Aims of Chapter 2

This chapter describes the methods of the research which forms the substance of the following five chapters (3-7) of this thesis. The recruitment of volunteers is described. The methods employed in measuring adiposity (obesity and distribution of body fat) are discussed along with a consideration of other available methods. The protocols for the CRH tests and dexamethasone suppression tests employed in Study I are described here. The A-V difference techniques and measures of blood flow across the deep compartment of the forearm and SAAT are described. Assay methods for measuring steroid hormone and cytokine concentrations are outlined. The limitations of the A-V difference technique with particular reference to assay CV are discussed.

2-2)Hospital ethical approval

The local ethical committee approved the study and informed consent was obtained from all volunteers. The methods of recruiting patients (discussed below) and the information sheets handed or posted to prospective volunteers were submitted as part of the application to the Whittington Hospital Ethical Committee. A copy of the ethical approval letter is shown in the appendix to this thesis.

2-3) Subjects

Recruitment

Further details about the subjects who attended for the various studies are to be found in chapters 3 - 7. The subjects were recruited from three general sources: hospital out-patient clinics, general practice and the local community. Obese patients (for all studies) were recruited from Dr S.W.Coppack's obesity clinic at the Whittington Hospital. Patients for the female sex steroid studies (chapters 5-7) were recruited from Dr P. Moul't's endocrinology clinic and the obesity clinic at the Whittington Hospital. In addition, patients for the corticosteroid studies (chapters 3-4) were recruited from a general practice – written invitations were sent to patients age 25 – 65 not taking hormone medication. Volunteers from the local community (largely controls for the studies) responded to

advertisements placed (with permission of the relevant agencies) in the Whittington Hospital, public libraries, police and fire stations. Due to the invasive nature of most of the studies, it was extremely difficult in practice to recruit control subjects in large numbers. Obese people were more interested in the study and were more keen to contribute to this research, which investigates adipose tissue (or peripheral) metabolism of hormones and aspects of the endocrinology of obesity.

Exclusion criteria

A full medical history was taken. Subjects who had recently experienced symptoms from cardiac or respiratory disease were excluded. Patients with heart valve replacements were excluded from arteriovenous difference studies due to the risk of infective endocarditis. Subjects taking corticosteroids (inhaled, oral or topical) were excluded from the corticosteroid studies. Women taking oestrogen or progesterone-containing drugs or medication that would interfere with the hypothalamo-pituitary-gonadal axis or sex steroid metabolism were excluded.

Subjects were asked to fast overnight and drink only water prior to all studies. The methodology of the procedures within the studies is discussed in detail below.

2-4) Measures of adiposity

a) Indices of obesity

(i) Body mass index (BMI), which is the weight (in kilograms) divided by the height² (in metres), is a widely accepted measure of “fatness” (2) and has been advocated by the W.H.O. Weight was measured using a digital weighing machine (Seca, Marsden, UK) to the nearest 0.1 kg. Height was measured using a Harpenden stadiometer (Holtain Ltd., Crymych, UK).

(ii) Total body fat (TBF) was measured by bioimpedance with the subjects recumbent and biostat electrodes (Bodystat 1500, Bodystat Ltd, Douglas, UK) attached to the dominant side (114).

b) Indices of distribution of body fat

(i) Waist-Hip ratio (WHR) : Waist and hip circumference were both measured in a transverse plane at the level of the umbilicus and greater trochanters respectively (115). Measurements were carried out using a metal tape by two observers (myself and one trained assistant) with the

patient standing. In massively obese subjects, an assistant was employed to raise the apron of abdominal fat so that waist circumference could be measured with the umbilicus in a transverse plane. Central obesity was defined as a WHR >0.9 in men and >0.8 in women (7).

(ii) Subscapular : triceps skinfold thickness ratio (STR). Skinfold thicknesses were measured with a pair of calipers (Holtain Ltd., Crymych, UK) by a single observer (myself) with the patient standing. Subscapular skinfold thickness was measured immediately below the tip of the scapula with the skinfold at an angle of 45° to the vertical. The triceps skinfold was measured in a vertical plane at the mid-point between the acromion process of the scapula and the tip of the olecranon process.

Skinfold thickness is one measure of subcutaneous fat deposition and presents problems of inter-observer variability and a lack of normative values (6;7), but the ratio of subscapular: triceps skinfold thickness provides a practical measure of central (truncal) obesity.

(iii) Measures not used, with explanations: MRI and CT imaging in a single transverse abdominal section and DEXA scanning have all been used to measure the distribution of body fat (20). Whilst each of these methods has technical advantages over skinfold measurements in the

accuracy of determining body fat distribution, they are all impractical in massively obese individuals who simply will not fit on/in the machines. Specially adapted MRI / CT scanners would overcome this barrier, but were not available to us. CT - scanning of the abdomen results in a significant radiation dose, beyond the administration of Xenon to subjects having SAAT blood flow studies. DEXA scanning was not employed for practical and cost considerations - we did not have DEXA on site and would have had to purchase scans from another trust. Again, massively obese subjects would have had problems fitting on a standard DEXA table. Travelling to another hospital would have presented a further task - in an already busy protocol – to patients and may have been a barrier to recruitment.

(c) Lean body mass

Lean body mass is defined as fat-free mass minus skeletal mass. Since skeletal mass is difficult to measure, lean body mass is generally taken to be 50% of the fat-free mass.

2-5) CRH and dexamethasone suppression tests-Study I

At 08.15hrs the day before the CRH test, all subjects attending for Study I commenced a 24 hr urine collection for cortisol metabolites (116).

Subjects fasted overnight and returned to the department at 08.00hrs the next day for a standard CRH test (117). A cannula was inserted under local anaesthesia in the antecubital fossa. They rested supine for at least 30 minutes before the first blood sample was taken. They remained supine for the duration of the test. At 09.00hrs CRH (100 micrograms human CRH, Ferring Pharmaceuticals Ltd.) was injected intravenously. Serum and EDTA samples were collected at -15, 0, 15, 30, 45, 60, 90 and 120 minutes after CRH administration. EDTA samples for ACTH, leptin and IL-6 analysis were stored on ice, centrifuged (4⁰C) and frozen (-20⁰C) within an hour. Serum samples were centrifuged at room temperature and stored (-20⁰C).

At least 2 weeks after the CRH test, an overnight dexamethasone suppression test was carried out (117). All subjects took 2mg of dexamethasone at 11pm. The following morning between 0800 and 0900hrs, a serum sample was taken from each subject for cortisol assay. To exclude Cushing's syndrome, the early morning cortisol should suppress to <50 nmol/l.

2-6) The A-V difference technique –Studies II,III,IV

All subjects were studied in the morning after an overnight fast. Only water was permitted during the studies to avoid any influence of caloric or nicotine intake on peripheral corticosteroid metabolism.

2-6 (a) Cannulae and sampling

Arterial and abdominal venous sampling

Lignocaine 1% was used for local anaesthesia prior to cannulation.

Firstly, a radial artery cannula (Insyte-20g, 30mm) was inserted. Then, in 34 subjects, a polyurethane catheter (Ohmeda Hydrocath 20G 20cm) was passed into the superficial epigastric vein (see Figure 2.1), draining the subcutaneous adipose tissue of the anterior abdominal wall (118). The blood collected from this abdominal vein represents the drainage from adipose tissue with a minor contribution from skin and none from muscle (118). Pairs of arterial and adipose venous serum samples were taken simultaneously during the late morning (11.00-12.00hrs) for measurement of steroid hormone concentrations.

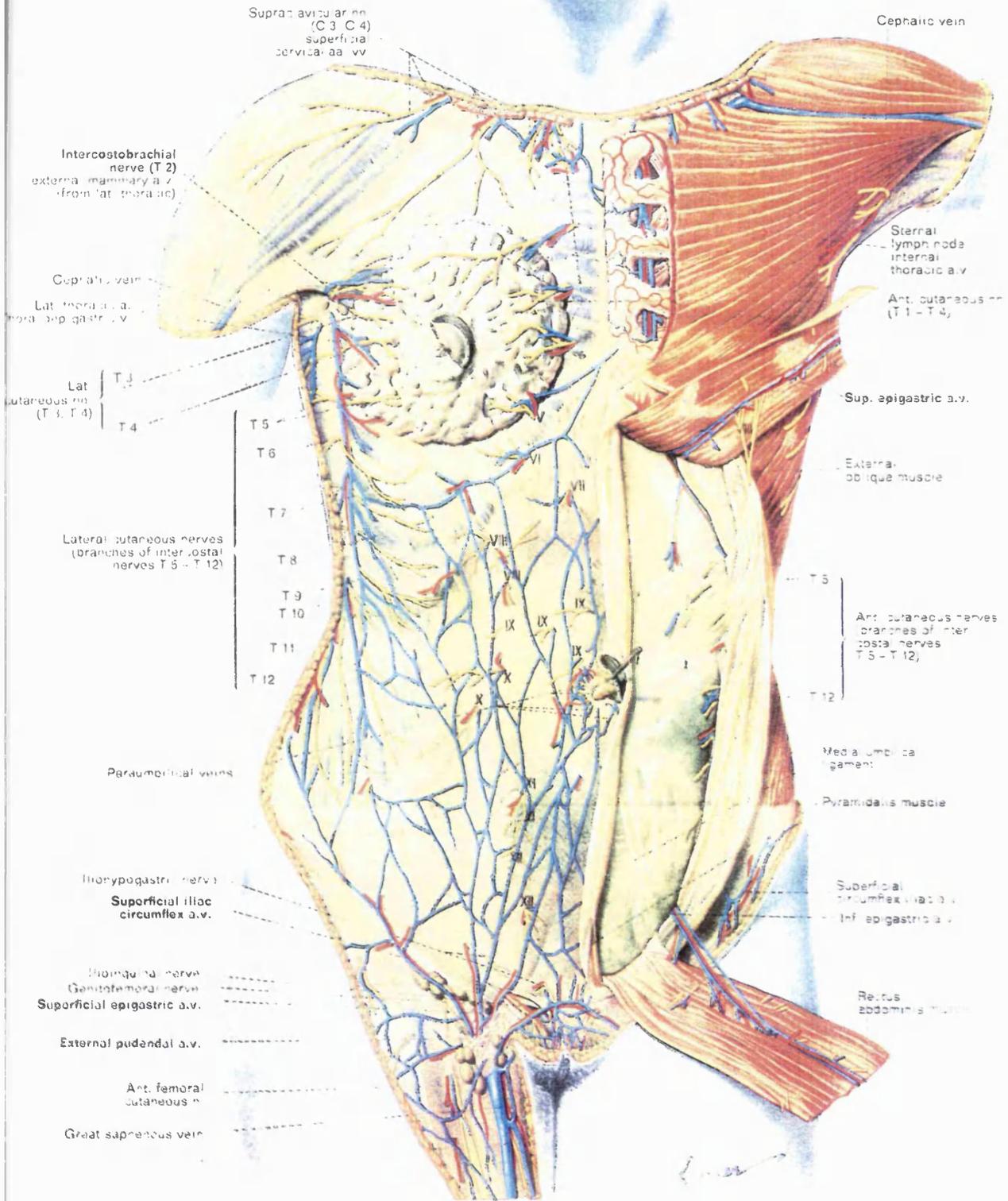


Figure 2.1

Illustration of the anatomy of the anterior abdominal wall (reproduced from Grant's Atlas of Anatomy)

Forearm venous sampling

A cannula (Abbocath 18G 51mm) was inserted retrogradely into the deep branch of the median cubital vein in the antecubital fossa. Blood samples from this vein represent drainage from forearm muscle with a minor contribution from the skin and adipose tissue of the forearm via anastomoses with the deep venous system (119). A wrist cuff was inflated above systolic pressure prior to sampling for two minutes, to isolate the forearm compartment from the hand. Paired arterial and forearm venous blood samples were taken simultaneously during the late morning (11.00-12.00hrs) for measurement of steroid hormone concentrations. In subjects with both deep venous and abdominal venous samples these were taken simultaneously.

2-6 (b) Measuring blood flow rates

Blood flow measurement in SAAT

Blood flow in the SAAT was measured by ^{133}Xe washout (120). At least forty five minutes after the injection of 5MBq of liquid ^{133}Xe (Malinkrodt, UK) into the subcutaneous fat of the anterior abdominal wall, a CsI gamma radiation detector (Mediscint, Oakfield Instruments Ltd, Witney, U.K.) was used to detect ^{133}Xe radioactivity at the injection

site. The rate of decline of radioactivity is proportional to the adipose tissue blood flow (120). For all subjects a xenon adipose/blood partition coefficient of 10 was used (121). Some authors have suggested that the partition coefficient is greater in obese subjects (122), such an assumption would increase the local production rates in the adipose tissue of obese relative to lean subjects. However not all workers have found such changes in partition coefficient (123), so we have adopted the more conservative assumption that the partition coefficient is the same in lean and obese.

Blood flow measurement in the forearm

Blood flow in the forearm from which venous samples were taken was determined by plethysmography (119) using a mercury strain gauge (Hokansen system, PMS Instruments, Maidenhead UK). Plasma flows were calculated as the blood flow multiplied by (1-haematocrit) for each subject. The haematocrit was measured in arterial, forearm and epigastric venous samples to ensure that sampling methods did not result in dilutional sampling errors. The haematocrits measured in the three sites were equal in all studies, which excluded a dilutional effect of sampling resulting in artefactual AV differences.

2-6 (c) Calculating production / clearance rates

Plasma flows were calculated as the blood flow multiplied by (1-haematocrit) for each subject. The haematocrit was measured in arterial, forearm and epigastric venous samples. The product of local plasma flow (ml/100g tissue/min) and A-V difference in hormone (nmol/l) yields the local clearance rate (pmol/100g adipose tissue/min) - according to the Fick principle (119). Hormone net clearance / production rates for total body fat (TBF) (nmol/day) or lean body mass (LBM) were estimated by multiplying local clearance rate by TBF and LBM respectively. This method of calculating TBF and LBM production rates assumes homogeneity of enzyme activity across different tissue beds. There is evidence that omental and subcutaneous fat depots vary with respect to their lipoprotein lipase (124), 11BHSD (34) and 17BHSD (62) activity. The assumptions with regard to TBF production rate calculations will be discussed in more detail in chapters 4 and 5.

2-7) Attempt to study angiotensinogen/angiotensin II release - limitations of the A-V difference technique

At one stage, early on in the research, we sought an explanation of the coexistence of hypertension and obesity. Our hypothesis, supported by *in vitro* studies carried out before and since the research was completed, was that adipose tissue secretes angiotensinogen or angiotensin II.

Angiotensinogen mRNA is expressed by adipocytes in culture and angiotensin converting enzyme is found in many tissue including adipose tissue (125;126). We looked for an A-V difference in angiotensinogen (ANG) and angiotensin II (ANG II).

Blood was collected in tubes containing inhibitors of angiotensin I-converting enzyme and angiotensinases. These tubes were supplied by the laboratory that carried out the ANG and ANGII assays (Dr J.Connell, Glasgow Western Infirmary). All necessary precautions were taken in sampling, centrifuging (4°C), storage (-70°C) and packaging (in dry ice) and posting (overnight courier). The pilot samples, including a batch of blindly labelled identical samples demonstrated that the variability in both the ANG and ANGII assays would mask all but the most extreme arteriovenous differences in concentration between artery and vein.

Power calculations demonstrated that hundreds of patients would need to

be sampled before any A-V difference would become significant. Thus, limitations of the assay (a difficult multi-step procedure with labile substrates) meant that we could not investigate the hypothesis. Our failure to find an A-V difference indicates the deficiencies of our technique to detect - whilst not precluding - a biologically significant A-V difference. "Absence of evidence is not evidence of absence". In order to reliably detect an existing A-V difference when 30 measurements are taken in a group of subjects, the assay CV (%) should be less than the percentage A-V difference of the substance being measured.

2-8) Hyperinsulinaemic euglycaemic clamp studies –Studies IV & V

The hyperinsulinaemic euglycaemic clamp technique (127) allows one to increase circulating insulin levels whilst maintaining the plasma glucose level constant. This technique can be used both to measure insulin sensitivity and to assess the metabolic consequences of hyperinsulinaemia (110). Four studies utilising this technique were discussed in Chapter 1. We carried out hyperinsulinaemic euglycaemic clamps in PCOS subjects in studies IV & V described in chapters 6 & 7 respectively.

Following completion of the baseline study, a further venous cannula was inserted in the contralateral forearm to the deep forearm venous cannula. An insulin (Actrapid, Novo Nordisk, UK) infusion was commenced at 20 $\mu\text{u}/\text{m}^2/\text{min}$ for 2 hours, with an initial loading dose of 60 $\mu\text{u}/\text{m}^2/\text{min}$ for the first 3 minutes to achieve steady state plasma levels more quickly. A 10% dextrose infusion was commenced simultaneously with the insulin infusion through the same cannula via a 3-way connector. Arterial plasma glucose samples were taken every 5 minutes during the clamp and the dextrose infusion rate was then titrated to maintain plasma glucose at the fasting level for each individual subject. Plasma glucose was measured by the glucose oxidase technique using a Beckman Glucose Analyser 2 (Beckman Instruments, Fullerton, California). At T=120 min, the insulin

infusion rate was increased to 40 $\mu\text{u}/\text{m}^2/\text{min}$, with an initial 3 minute loading dose of 120 $\mu\text{u}/\text{m}^2/\text{min}$. This infusion was continued until T=240 min.

Sampling/Blood flows

Simultaneous arterial, forearm venous and epigastric venous serum samples were taken at T= 90, 120, 210 and 240 minutes for A, T, and E2. At the same timepoints, blood (lithium heparin tube) was taken from a single vessel for plasma insulin concentration. Blood flows in SAAT and the forearm were measured (as described earlier in this chapter) at baseline and again at T=100 min and T=220 min. In the last 3 subjects, a further sampling time / blood flow measurement was introduced at T = 300 min, an hour after stopping insulin, yielding post clamp AV difference data for 3 subjects across SAAT (none of these 3 subjects had FM cannulae).

Study duration

The patients attended for baseline studies at 8.15 am. Typically, the clamp commenced at 1pm - after centrifugation and storage of baseline samples, completion of baseline blood flow readings and setting up insulin / dextrose infusions. The clamp was completed 4 hours later and

the patients were given a meal at this point, except in the last 3 subjects in which case the meal was delayed until after the post clamp sampling.

2-9) Assays

For the most part, these assays were carried out by experienced staff in other laboratories where endocrine assays are carried out on a daily basis. Because we were looking for small A-V differences, it was important that the assays were carried out by experienced laboratory personnel. I had two days personal experience in conducting radioimmunoassays for androstenedione, under the supervision of Dr H.H.G. McGarrigle, to enable me to understand the techniques involved in the assay.

These samples and the remainder of the sex steroid samples, LH, FSH and SHBG were assayed independently by Dr H.H.G. McGarrigle at his University College laboratory. The ACTH and cortisol assays in chapter 3 were carried out by Dr L. Perry's endocrine laboratory at St Bartholomew's Hospital. The cortisol and cortisone assays employed in Chapter 4 were carried out in Southampton by Dr P. Wood. Steven Goodrick in the Whittington Dept of Medicine measured Leptin and IL-6. Insulin assays were carried out by Karen Bulmer in the Whittington Dept of Medicine.

Cortisone

Cortisone was measured by radioimmunoassay (128). Serum samples were extracted with chloroform prior to cortisone radioimmunoassay (128). The cross-reactivity of cortisone antisera with cortisol was less than 0.1%. Intra-assay coefficient of variation (CV) for cortisone was 8% and 6% at cortisone concentrations of 50 and 80 nmol/l respectively. The inter-assay CV for cortisone was less than 10% over the range 15-500nmol/l.

Cortisol (Wood's Lab)

Serum was analysed by radioimmunoassay for cortisol (129). All samples from an individual subject were assayed within the same batch. The intra-assay CV for cortisol was 8.2% and 5.6% at cortisol concentrations of 156 and 410 nmol/l respectively. The inter-assay CV for cortisol was less than 10% over the range 50-2000 nmol/l.

Cortisol (CRH tests-Barts lab)

Serum cortisol was measured with the Bayer Immuno-1 automated analyser; intra-assay CV for cortisol at concentrations of 140, 400 and 950 nmol/l was 5.1%, 4.1% and 3.8%; inter-assay CV for cortisol at 140, 400 and 950 nmol/l was 5.5%, 3.6% and 3.5% respectively.

ACTH

Plasma ACTH was measured using the Nicholls immunoradiometric kit; The intra-assay CV for ACTH at 50 and 400ng/l was 6.9% and 5.8% respectively. The inter-assay CV for ACTH at 50 and 400 ng/l was 8.8% and 7.3% respectively.

Leptin

Plasma leptin was measured with an in-house radioimmunoassay, utilising a polyclonal antibody raised in a rabbit host. The intra-assay CV was 3.0% and the inter-assay CV was 5.0%.

Interleukin-6 (IL-6)

Plasma IL-6 was measured using an ELISA kit (R&D Systems, Oxon,UK). The intra-assay CV was 5.9% and the inter-assay CV was 6.7%.

Insulin

Insulin was measured by a specific 2-site ELISA (Dako Diagnostics, Cambs, UK). The assay sensitivity was 3 pmol/l, range 5-1100pmol/l. The intra-assay CV was 8.9% and the inter-assay was CV 7.5% (130).

Sex steroid assays

Radioimmunoassays for A and T were carried out following ether extraction of the plasma samples, as previously described (131). Tritiated T and A tracers (NEN Life Science Products) and specific anti-testosterone antiserum (Bioclin Services, Cardiff) and anti-androstenedione antiserum (Guildhay Ltd, Guildford) were used in the assay. Free and bound steroids were separated by the use of dextran / charcoal. Plasma oestradiol levels were assayed after ether extraction as previously described (132) using tritiated oestradiol (NEN) and specific anti-oestradiol antiserum (Bioclin Services, Cardiff) with dextran/charcoal separation of free and bound steroid.

The intra-assay CVs (most crucial for analysis of A-V differences as all assays for each patient were carried out within the same batch) were as follows: testosterone 5.7%, androstenedione 8.3%, oestradiol 4.0%. The inter-assay CVs for each of the sex steroid assays were testosterone 10.4%, androstenedione 10.4% and oestradiol 11.5%. All three sex steroid assays used were subject to monthly evaluation by the UK NEQAS scheme for Steroid Hormones.

Gonadotrophins

LH and FSH were both measured using two-site radioimmunoassays (Nichols Institute Diagnostics, San Juan Capistrano, California) supplied as kits. The intra-assay CVs for LH and FSH were 2.6% and 1.6% respectively. The inter-assay CV for LH and FSH were 5.4% and 3.8% respectively.

SHBG

SHBG was measured by an immunoradiometric assay supplied as a kit (EURO/DPC, Gwynedd, UK) . The intra-assay CV was 3.8% and the inter-assay CV was 7.9% .

2-10) Statistical Methods

It has already been noted that the recruitment of controls for all the studies was a major challenge and we did not obtain controls in sufficient numbers to define “obese” and “control” groups. In general, weight and other variables were analysed with non-parametric tests as continuous variables. Spearman’s correlations coefficient was widely used for this purpose. Where groups could be defined meaningfully (eg comparing male and female subjects in the corticosteroid studies) the Mann-Whitney test was applied. The statistical methods used in each of the five studies will be discussed in more detail in chapters 3-7.

Chapter 3

OBESITY AND THE HYPOTHALAMO-PITUITARY-ADRENAL

AXIS IN MEN AND POSTMENOPAUSAL WOMEN

3-1) Aims of Chapter3

This chapter describes a clinical study of CRH responses and urine cortisol metabolite profiles in a group of men and postmenopausal women. The aim of the study was to assess the relationship of obesity, in particular central obesity, to HPAA activity whilst controlling for the influence of depression. This has previously been studied in premenopausal women, but not in men and postmenopausal women. In addition, we sought to establish a relationship between leptin and IL-6 levels and pituitary/adrenal responses to CRH.

3-2) Study 1 : The relationship of obesity to hypothalamo-pituitary-adrenal axis activity in men and postmenopausal women

This section is based on the paper by J.R.Katz *et al* published in the International Journal of Obesity **2000:24:246-251** and is included in the appendix to the Thesis.

3-2 (a) Summary of Study 1

We examined the relationship of adiposity to pituitary-adrenal responses to CRH in 13 men and 8 postmenopausal women, controlling for the influence of depression. In men, but not in women, the ACTH and cortisol responses to CRH correlated directly with STR. This relationship was independent of depression scores. There were no significant relationships in either sex between urinary TCM or Fm/Em ratio and BMI, waist, WHR, TBF, STR or CRH responses. All subjects suppressed normally with dexamethasone. We demonstrate that central obesity in men, but not postmenopausal women, is associated with an enhanced pituitary-adrenal response to CRH and that this relationship is independent of depression scores.

3-2 (b) Study 1 : Introduction

The pituitary and adrenal response to provocation tests in obese premenopausal women has been extensively studied (22-24;26;27), but there is a lack of data on men and post-menopausal women. Centrally obese premenopausal women have been shown to have an enhanced ACTH and cortisol response to CRH (23) and some authors have extrapolated from these data to assert that a hyperactive hypothalamo-pituitary-adrenal (HPA) axis results in increased cortisol production rates, central obesity and insulin resistance (33). We have set out to clarify whether adiposity or depression influence CRH responses and cortisol metabolite levels in men and postmenopausal women. There is a growing body of *in vitro* evidence to suggest that both leptin (133-135) and IL-6 (136-138) have a regulatory role in HPA axis activity. We examine the relationship of pituitary and adrenal CRH responses to basal leptin and IL-6 levels.

3-2 (c) Methods of Study 1

Thirteen men and eight postmenopausal women were recruited from a primary health care practice and an obesity clinic. Their characteristics are outlined in Table 3.1. No subjects were being treated with topical, inhaled or oral corticosteroid preparations or had received such treatment in the year prior to the study. Four of the women were taking hormone replacement therapy. The local ethical committee approved the study and informed consent was obtained from all volunteers.

At the initial visit a full medical history was taken. Five different indices of adiposity were measured: BMI, waist circumference (waist), WHR, TBF and STR. The methods of measuring these indices of adiposity were described earlier in the Thesis: Chapter 2 , section 4. All subjects completed a 24-hour urine collection for urine cortisol metabolites immediately prior to the CRH test. The methodology for the CRH tests and overnight dexamethasone suppression tests is outlined in Methods: Chapter 2, section 5.

Table 3.1 :Subject characteristics for Study I

	MEN (n=13) Median (Interquartile Range)	WOMEN (n=8) Median (Interquartile Range)
Age (years)	62 (52 - 63)	54 (53 - 62)
BMI (kg/m²)	29.0 (26.3 - 33.1)	30.0 (23.0 - 41.0)
Waist Circumference (cm)	105 (97-111)	86 (79-117)
Waist:hip ratio (WHR)	1.03 (0.98 - 1.07)	0.94 (0.87 - 1.10)
Subscapular:Triceps Skinfold thickness Ratio (STR)	2.0 (1.2 - 2.4)	1.0 (0.85 - 1.07)
Total Body Fat (kg)	25.4 (19.8 - 28.8)	35.0 (18.7 - 48.8)
24hr Urinary TCM (µg/mmol creatinine)	440 (290-680)	540 (350-950)
Urine Fm/Em ratio	0.74* (0.66-0.83)	0.66* (0.56-0.69)
GHQ-30 Score⁵	2 (0-6)	1 (0-3)
HAD-Depression Score⁶	3 (1-4)	3 (1-12)

* Mann-Whitney – significantly higher in men (p<0.05)

⁵ By GHQ-30 criteria (score >=5) 3 men and 1 woman were depressed

⁶ By HAD criteria (score >=9) 1 man and 2 women were depressed

Depression scores

After completing the CRH test all subjects completed two questionnaires to assess depression scores. The GHQ30 and HAD questionnaires are validated methods of rating depression scores (139). The range of possible scores in the GHQ is from 0 to 30, scores greater than 5 representing significant depression. The HAD questionnaire measures depression (possible range 0-21), scores of greater than 9/21 representing significant depression.

Assays

For description of cortisol, ACTH, leptin and IL-6 assays see Methods: Chapter 2, Section 9.

Urine cortisol metabolites

In all subjects total urine cortisol metabolites and creatinine were measured in an aliquot of their 24-hour collection. Urine steroid profiles were analysed by high resolution gas chromatography (116). Urinary total cortisol metabolites (TCM) are defined as the sum of urinary cortisol (11-hydroxy) metabolites (Fm) and urinary cortisone (11-oxo) metabolites (Em). All urine cortisol metabolite levels are expressed as $\mu\text{g}/\text{mmol}$ creatinine to correct for the influence of lean body mass.

Statistical analysis

ACTH and cortisol responses to CRH were assessed by two methods: the percentage increment (%inc.) from basal to peak value; and by the area under the incremental curve (AUC). The relationships between adiposity, depression scores, urine cortisol metabolites and cortisol /ACTH response to CRH were assessed by Spearman's rank correlation coefficients. After log transformation of significantly skewed variables, linear regression was used to account for confounding variables. The Mann-Whitney test was applied to assess gender differences in adiposity, depression, urine cortisol metabolites and CRH response.

3-2 (d) Results- Study I

Adiposity and CRH responses

The basal cortisol level was 347 (277-496) nmol/l and did not vary according to gender, BMI or body composition. The ACTH and cortisol %inc. and AUC results for men and women are summarised in Table 3.2.

Table 3.2

ACTH and cortisol responses to CRH

	MEN (n=13) Median (Interquartile range)	WOMEN (n=8) Median (Interquartile range)
ACTH %inc.	122 (59 – 278)	259 (125 - 435)
Cortisol %inc.	65 (23 - 102)	69 (32 - 123)
ACTH AUC Ng/l.min	1605 (820 – 4445)	3180 (915 – 3690)
Cortisol AUC Nmol/l.min	11,280 (1610 – 19,340)	19,420 (8,570 – 28,300)

In men, there were strong positive correlations between STR and ACTH /cortisol responses to CRH (see Table 3.3). In post-menopausal women, there was an inverse correlation between STR and ACTH AUC (r= -0.81, p<0.05). There were non-significant inverse correlations between other indices of adiposity and CRH responses in women. The women on HRT (n=4) closely matched those not on HRT (n=4) with regard to age, BMI and WHR. We found no significant difference in CRH response between women on HRT and those not on HRT.

Depression Scores in relation to CRH responses and adiposity

The depression scores in men and women are summarised in Table 3.1. The correlations of depression scores with CRH responses in men are shown in Table 3.3. In men, the GHQ30 score was positively associated with all cortisol and ACTH responses to CRH. Multiple regression analysis confirmed that STR and depression scores were related to CRH responses independently of one another. In women, there was no association between depression scores and ACTH/cortisol responses to CRH.

Table 3.3 Correlations between CRH responses, adiposity and depression scores in men

	Cortisol %inc.	Cortisol AUC	ACTH %inc.	ACTH AUC
STR	+ 0.55*	+ 0.54	+ 0.70***	+ 0.64**
Waist	+ 0.32	+ 0.37	+ 0.42	+ 0.45
WHR	+ 0.36	+ 0.43	+ 0.38	+ 0.52
TBF	+ 0.21	+ 0.27	+ 0.26	+ 0.32
BMI	+ 0.23	+ 0.30	+ 0.33	+ 0.38
GHQ-30	+ 0.58**	+ 0.72***	+ 0.58**	+ 0.62**
HAD- Depression	+ 0.20	+ 0.23	+ 0.27	+ 0.43

Data are r values; *p=0.05; **p<0.05; ***p<0.01

Table 3.4.

Correlations between depression scores , adiposity and urine cortisol metabolite levels in men

	GHQ-30	HAD-Depression
STR	+ 0.29	+ 0.26
Waist	+ 0.62**	+ 0.60**
WHR	+ 0.60**	+ 0.68**
BMI	+ 0.69**	+ 0.60**
TBF	+ 0.56	+ 0.26
TCM	+ 0.57*	+ 0.51
Fm/Em	- 0.24	- 0.65**

Data are r values; *p=0.05; **p<0.05; ***p<0.01

The relationship of adiposity to depression scores in men is summarised in Table 3.4. Both GHQ-30 and HAD-Depression scores correlated positively with BMI, waist and WHR. In women, HAD-Depression scores correlated with WHR ($r=0.70$, $p<0.05$), but this was the only significant relationship between depression scores and adiposity.

Urine cortisol metabolites versus obesity and depression

Urine cortisol metabolite results, depression scores and anthropometric data are summarised in Table 3.4. Although there was no sex difference in TCM, the Fm/Em ratio was significantly higher in men than in women. There was no relationship between urinary TCM and ACTH/cortisol responses to CRH in either sex.

Depression scores correlated positively with urinary TCM and were inversely related to the urinary Fm/Em ratio (see Table 3.4). Both TCM and the Fm/Em ratio were unrelated to adiposity, as measured by BMI, WHR, TBF and STR in both sexes. This lack of a relationship persisted even when depressed patients (by either HAD or GHQ-30 criteria) were excluded from the analysis.

Dexamethasone Suppression tests

In all subjects, plasma cortisol was suppressed to <50nmol/l at 8am, the morning after a 2mg dose of dexamethasone.

Leptin

In men, basal leptin 8.5 (4.6 – 10.4) ng/ml correlated with ACTH AUC (r=0.69, p<0.05) and BMI (r=0.73, p<0.01). However, multivariate analysis showed no effect of leptin independent of STR. There was a trend towards a correlation between leptin and cortisol incremental measures, but this failed to reach significance. In women, basal leptin 16.1 (5.9 – 31.0) ng/ml correlated with BMI (r=0.79, p<0.05) but not with ACTH and cortisol responses to CRH.

Interleukin-6

In men, basal IL-6 levels of 3.8 (2.8 – 4.7) pg/ml did not relate to BMI or ACTH/cortisol responses to CRH. In women, basal IL-6 levels of 4.2 (1.6 – 10.3) pg/ml did correlate with BMI (r=0.83, p=0.01) but not with ACTH/cortisol incremental measures.

3-2 (e) Discussion – Study 1

We have demonstrated a positive correlation between central obesity, as measured by STR, and ACTH/cortisol responses to CRH in men. Our data suggests an inverse relationship between STR and the ACTH response to CRH in postmenopausal women. Cushing's disease was excluded by normal overnight dexamethasone suppression in all subjects. Skin fold thicknesses reflect subcutaneous depots rather than visceral, but can be measured in subjects with a wide range of body habitus and this represents an advantage over other methodologies. Dual energy x-ray absorptiometry (DEXA) scanning of regional fat depots is a more precise measure, but DEXA is impractical in morbidly obese subjects and was not undertaken for logistical reasons. Other studies (23;32) have demonstrated that the distribution of body fat rather than BMI predicts the pituitary and adrenal response to stimulation tests. In centrally obese premenopausal women, there is an enhanced ACTH response to CRH (23) and an enhanced cortisol response to CRH (23) and other stimulation tests (32).

In men, there were consistent positive associations between pituitary/adrenal responses to CRH and depression scores on the GHQ30

questionnaire, independent of STR. The findings with the HAD-Depression scale were in the same direction but non-significant. One possible explanation for this is that the questions in GHQ-30 were designed to elicit a change in mood (e.g. “have you been finding life a struggle *more than usual*”), whereas the HAD-Depression scale asks simply whether you feel low, whether or not this represents a change (e.g. “I feel cheerful *not at all/ not often/sometimes/ most of the time*). The HPA axis may be upregulated by short-term changes in mood rather than by chronic depression.

The positive correlations between GHQ-30 scores and CRH responses conflicts with previous data, which has shown a blunted ACTH response and a normal cortisol response in depression (30;31). There were no significant associations between depression and CRH responses in the female group.

Depression has been shown to be associated with obesity and particularly central obesity (28). This is confirmed by our finding of positive correlations between both depression scores and BMI, waist and WHR in men. We hypothesise that depression accounts for some of the inconsistencies in the literature regarding CRH responses in obesity. We

have found a direct association between central obesity and CRH responses in men, independent of depression scores.

There is limited published data on CRH responses in centrally obese men, but our data is supported by preliminary data from another group (140).

Our data, in the context of previous studies (23) on premenopausal women, suggests that the menopause may result in a diminished response to CRH. However, the lack of a relationship of central obesity to CRH responses in women in our study may reflect the narrow range of STR in the female group, as compared to the male group. There is no data as yet comparing CRH responses in large matched groups of menopausal women on and off HRT, but our preliminary data show no significant difference between these groups.

Obesity *per se*, in contrast to body fat distribution, was unrelated to CRH responses in either sex. It is conceivable that we failed to demonstrate a relationship because we only had 2 men and 4 women with a BMI of less than 25. An expanded normal weight male control group would have strengthened the analysis. However, our study is in agreement with most of the previous research in this area, which has shown normal ACTH (22;24) and cortisol (24;27) responses to CRH in obesity *per se*.

However, one study has suggested a blunted cortisol (22) response in obese subjects compared to normal weight controls. Two groups have reported an increased ACTH, but normal cortisol response to CRH in obesity (27;141). In one study, higher doses of CRH were given to the most obese patients, which may explain this finding (27). In the more recent study, CRH 100 mcg was given to obese men and a control group (141). In this study, obesity *per se* and not body fat distribution, produced an increased ACTH response to CRH.

Total urine cortisol metabolite levels (TCM) can be utilised to reflect (or indicate) cortisol production rates. Correction for creatinine controls for the influence of lean body mass (40). There is some evidence to suggest that centrally obese subjects demonstrate cortisol hypersecretion in response to transient increases in psychological stress (32;142).

Furthermore, it has been suggested that a hyperactive HPA axis in central obesity leads to increased cortisol production rates (33). However, we found no relationship of CRH responses or indices of adiposity with TCM and, in as far as urine cortisol metabolite levels reflect cortisol production rates, we were unable to confirm this hypothesis in our study. We did not, however, formally assess cortisol production rates.

Although increased TCM have been reported in obesity (41), we concur with two recent studies (42;43) which demonstrated no significant relationship between BMI and TCM in men and women. Increased urinary free cortisol (UFC) excretion has been demonstrated in centrally obese premenopausal women (23;32). However, UFC accounts for less than 1% of TCM and does not accurately reflect cortisol production rates (42).

We analysed and rejected the hypothesis that depression obscured a relationship between adiposity and urine cortisol metabolite profiles. Depression was associated directly with TCM in our study, in accordance with previous studies (116;143). However, our finding of an inverse relationship between depression and urine Fm/Em in men is at variance with one previous case-controlled study, which demonstrated no influence of depression on the Fm/Em ratio in men and an increased Fm/Em ratio in depression in women (116;143). Direct comparisons between our study and Raven's study (116;143) are difficult. The Raven study was case controlled, but there was no correction for lean body mass and they used different depression scales.

The urine Fm/Em ratio was higher in men than women in keeping with two previous studies (44;116). In our study, Fm/Em did not relate to CRH responses or adiposity in either sex. There has been debate in the literature as to the direction of the cortisol-cortisone shuttle in central obesity. Whilst Andrew *et al* found a positive correlation of the Fm/Em ratio with both hip circumference and waist circumference in men, they do not present their data on waist-hip ratio and have no other index of central obesity (42). Meanwhile, Stewart found that the Fm/Em ratio is inversely related to android (central) fat distribution, (43), which is contrary to his group's *in vitro* data on visceral adipose tissue (34). Finally, Weaver *et al* found the Fm/Em ratio to be inversely related to body weight, android fat and gynoid fat, but stepwise regression confirmed gynoid fat was the most important factor determining the Fm/Em ratio (44). Our data, in conjunction with these conflicting papers (42-44), suggests that the relationship of body fat distribution to the direction of the cortisol-cortisone shuttle is, as yet, unclear.

Regarding the role of leptin and IL-6 in regulating the HPA axis, multivariate analysis demonstrated that the positive association between leptin and ACTH AUIC was not independent of STR. This is contrary to a regulatory role for leptin at hypothalamic or adrenal levels suggested by

laboratory and animal studies (133-135). We did not find a simple relationship between basal IL-6 levels and CRH responses. Previous work has suggested a close relationship between IL-6 and ACTH circadian rhythm (144) and IL-6 administration has been shown to activate the HPA axis both in animals (137;138) and in humans (136).

Excessive alcohol consumption has been shown to blunt the ACTH and cortisol responses to CRH (145) and impair dexamethasone suppression of plasma cortisol (146). We assessed alcohol intake in all subjects as part of the medical history. There were two men who drank in excess of 20 units per week, but they did not demonstrate blunted responses to CRH and suppressed normally with dexamethasone. Excluding them from the analysis did not alter the strength of the positive relationships. We do not believe alcohol intake was a confounding factor in our study.

We have considered whether HRT use acts as a confounding variable in the assessment of CRH responses in postmenopausal women. There is *in vitro* evidence (147;148) that oestrogen increases expression of the CRH gene. However, a previous clinical study (149) found no difference in the pituitary and adrenal responses to CRH between women on HRT and those not on HRT. Our study supports the view that HRT does not

influence the ACTH and cortisol responses to CRH, although the numbers of women taking HRT (4 in our study and 4 in the previous study cited) were small. The role of oestrogen in modulating the CRH response in postmenopausal women needs to be addressed by further research.

3-3) Summary of Chapter 3

Previous studies have shown an increased pituitary-adrenal response to CRH in centrally obese premenopausal women. We have demonstrated that this effect extends to centrally obese men, but not to postmenopausal women, and is independent of depression score, basal leptin and IL-6 levels.

CHAPTER 4

**AN *IN VIVO* STUDY OF THE CORTISOL-CORTISONE
SHUTTLE IN SUBCUTANEOUS ABDOMINAL ADIPOSE TISSUE
AND FOREARM MUSCLE**

4-1) Aims of Chapter 4

Having examined abnormalities of the HPA axis in obesity in Chapter 3, in this chapter I examine the contribution of peripheral tissues to corticosteroid metabolism in obesity. I have applied the *in vivo* technique of A-V difference sampling to define the qualitative net direction of the cortisol-cortisone shuttle in SAAT and FM. In addition, I have quantified daily cortisone / cortisol production rates in the peripheral tissues of fat and muscle.

4-2) Study II : An *in vivo* study of the cortisol-cortisone shuttle in subcutaneous abdominal adipose tissue and forearm muscle

This section is based on the paper by J.R.Katz *et al* published in Clinical Endocrinology **1999:50:63-68** and is included in the appendix to the Thesis.

4-2 (a) Summary of Study II

We have conducted an *in vivo* study of the cortisol-cortisone shuttle in subcutaneous abdominal adipose tissue, measuring A-V differences in serum cortisol and cortisone across subcutaneous abdominal adipose tissue and forearm muscle in 12 men and 22 women. We found significant ($p < 0.001$) clearance of cortisone by adipose tissue, with an A-V difference of 4 (1-7) nmol/l. For cortisol there was a trend for arterial concentrations (203 (142 - 292) nmol/l) to be lower than venous (224.5 (152 - 263) nmol/l), but this was not significant. We have demonstrated net 11 β -HSD oxo-reductase activity in subcutaneous abdominal adipose tissue, which may be increased in obesity.

4-2 (b) Introduction to Study II

The nature of peripheral corticosteroid metabolism in obesity is controversial. Traditionally, it has been assumed that cortisol is converted to its inactive metabolite cortisone in adipose tissue. The *in vitro* studies on which this assumption was based (35) have been challenged by recent studies demonstrating oxo-reduction of cortisone to cortisol in visceral adipose stromal cell cultures (34). The cortisol-cortisone shuttle is controlled by the two isoforms of 11 β -Hydroxysteroid Dehydrogenase - 11 β -HSD1 and 11 β -HSD2 (36;37). Although both isoforms may dehydrogenase cortisol at the 11 β -position to form cortisone, the type 2 isoform has the higher affinity for this reaction. The type 1 isoform is a low affinity enzyme, which also promotes the reverse reaction - oxo-reduction of cortisone to cortisol. Early studies on subcutaneous abdominal fat homogenates (35) suggested dehydrogenation of cortisol to cortisone occurred in adipose tissue. However, net oxo-reduction of cortisone to cortisol has been demonstrated in breast adipose tissue (38). More recent studies detected 11 β -HSD1, but not 11 β -HSD2, mRNA in visceral and subcutaneous adipose tissue (34). Furthermore visceral adipose stromal cell cultures demonstrated predominant oxo-reductase activity (34).

There have been few studies of the cortisol-cortisone shuttle in adipose tissue *in vivo*. A previous study of cortisol metabolism utilising A-V difference techniques demonstrated no significant gradient of plasma cortisol across deep (largely muscle) and superficial (skin and adipose tissue) forearm compartments (39). However, superficial forearm veins only drain limited amounts of adipose tissue. We therefore measured the A-V differences in cortisol and cortisone across superficial abdominal adipose tissue, as well as the deep compartment of the forearm, in a heterogeneous group of men and women. This A-V difference technique has been used in the assessment of sex steroid metabolism (70) and lipolysis (150) in adipose tissue.

4-2(c) Methods of Study II

Subjects

A total of 34 subjects (male:female = 12 : 22) had superficial abdominal adipose A-V difference studies. We excluded volunteers taking topical, inhaled or oral corticosteroid medication. The volunteers' characteristics, including BMI, TBF and WHR are outlined in Table 4.1. The methodology of the A-V difference studies is described in detail in the Methods (Chapter 2, section 6) section of the Thesis. Thus, cannulae were inserted into a radial artery and a superficial epigastric vein (draining SAAT) in all 34 subjects. In addition, a deep forearm vein (draining muscle) was cannulated in 10 of these subjects. Nineteen other subjects only had A-V forearm studies without abdominal venous sampling. The characteristics of this subgroup of nineteen did not differ significantly from those shown in Table 4.1. Serum samples were taken at two timepoints (between 11.00 and 12.00hrs) simultaneously from arterial and venous lines for assay of cortisol and cortisone.

Assays

Serum was analysed by radioimmunoassay for cortisol (129) and cortisone (128) by Dr P.J.Wood's laboratory . The radioimmunoassay technique is described in Methods: Chapter 2, section 9. All samples from

an individual subject were assayed within the same batch. Intra-assay CV.s for cortisol were 8.2% and 5.6% at cortisol concentrations of 156 and 410 nmol/l respectively. Intra-assay CV.s for cortisone were 8% and 6% at cortisone concentrations of 50 and 80 nmol/l respectively.

Table 4.1

Subject Characteristics for Study II.

	Median	Range: minimum-maximum
Age (yrs)	45	19-65
BMI (kg/m ²)	32.7	20.4 – 77.1
WHR (women)	0.93	0.75 – 1.1
WHR (men)	1.01	0.85 – 1.2
Total Body Fat (kg)	34.4	5.6 – 119.1

Estimated whole-body corticosteroid interconversion by adipose tissue

The product of local plasma flow (ml/100g tissue/min) and A-V difference in cortisone (nmol/l) yields the local clearance rate (pmol/100g

adipose tissue/min) - according to the Fick principle (119). The A-V difference was taken to be the mean of the two A-V differences obtained from the two basal timepoints at which serum samples were taken. Cortisone net clearance rates for the total body fat (TBF) (nmol/day) were estimated by multiplying local clearance rate by TBF as measured by bioimpedance. Cortisol net production rates were assumed to be the same on a molar basis as cortisone clearance rates (conversion factor for cortisol nmol to mg = 3.6×10^{-4}). These calculations assume homogeneity of 11 β -HSD1 activity in all adipose tissue depots. It is likely that such activity is heterogeneous (34) and these figures serve to illustrate the potential TBF cortisol production, but should be interpreted with caution.

Statistical analysis

Non-parametric tests were used because of skewness in the data. Data are reported as median (interquartile range). Wilcoxon's paired test was used to assess whether the A-V difference in cortisol and cortisone concentrations significantly differed from zero. Spearman's rank correlation coefficients were applied to assess the relationships between the A-V difference /clearance rate of cortisol/cortisone to age, sex, blood pressure and indices of adiposity.

4-2(d) Results of Study II

A-V differences of cortisone and cortisol

In the 34 subjects who underwent superficial epigastric vein and radial artery cannulation, the arterial concentration of cortisone was 40 (32 - 58) nmol/l compared to a venous concentration of 39 (28-50) nmol/l. There was a significant ($p < 0.001$) cortisone A-V difference of 4 (1 - 7) nmol/l reflecting net cortisone clearance in abdominal adipose tissue (Figure 1). There was a trend for abdominal adipose venous cortisol concentrations [225 (152 - 263) nmol/l] to be higher than arterial [203 (142 - 292) nmol/l] but the A-V difference was not statistically significant.

In the 29 subjects who had forearm studies, there were no significant A-V differences for cortisol [arterial 255(180-293)nmol/l, deep venous 270 (176-315) nmol/l] or cortisone [arterial 54 (48-64)nmol/l, deep venous 53 (48-64) nmol/l]. In the subset of 10 subjects with samples available from artery, deep vein and abdominal vein the median A-V difference across the superficial abdominal adipose tissue bed was 4.5 (0.8 - 7.0) nmol/l compared with -1.0 (-6.5 - 7.25) nmol/l across the deep forearm compartment, but neither reached statistical significance. The haematocrits in arterial, forearm and epigastric venous blood for each subject were equal, excluding any dilutional effects of sampling.

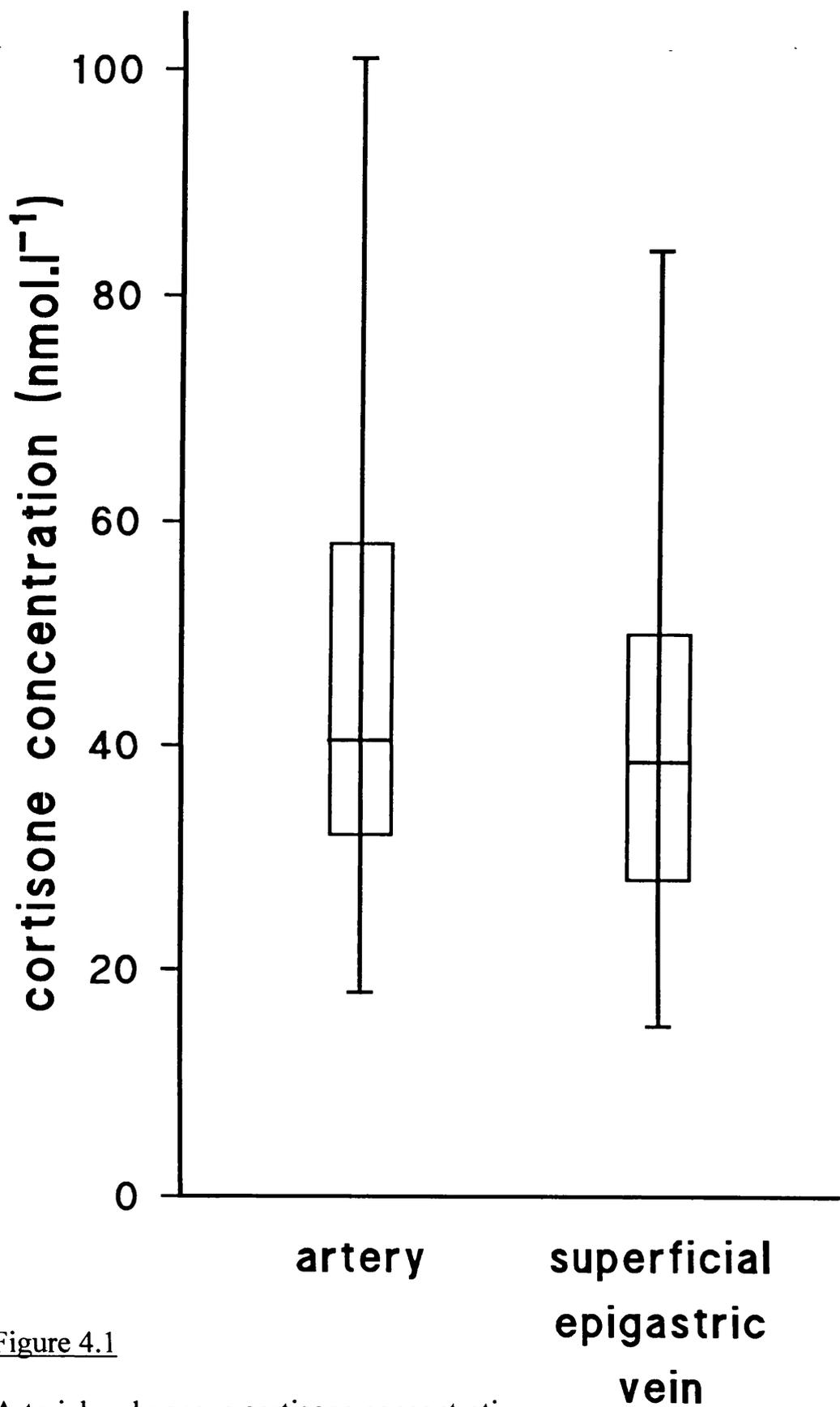


Figure 4.1

Arterial and venous cortisone concentrations in SAAT with 0th, 25th, 50th, 75th and 100th centiles shown

Cortisol:cortisone ratios

The cortisol:cortisone ratios demonstrated a significantly higher ratio ($p < 0.05$) in adipose venous blood [5.2(4.1 - 7.1)] when compared to arterial [4.8 (3.7 - 6.8)] blood. There was no significant difference between the cortisol:cortisone ratio in arterial and forearm venous blood [4.7 (3.5 - 5.5)]. In the subset of 10 subjects with samples available from both artery, deep vein and superficial epigastric vein the cortisol:cortisone ratios did not differ significantly between the different sites.

Net clearance / production rates of cortisone and cortisol

The local subcutaneous adipose tissue cortisone clearance rate was 3.62 (0.36 - 6.21) pmol/100g /min. The local adipose tissue cortisone clearance rate correlated significantly with TBF ($r = 0.35$, $p = 0.05$), but not significantly with percentage body fat ($r = 0.32$) or with BMI ($r = 0.20$). Similarly, there were no significant relationships between local cortisone clearance and age, sex or blood pressure. TBF cortisone clearance was 2000 (150 - 5400) nmol/day. There was a significant correlation between TBF cortisone clearance and BMI ($r = 0.47$, $p = 0.009$). Assuming cortisone underwent oxo-reduction in adipose tissue, the median TBF cortisol production was approximately 0.7mg /day.

4-2(e)Discussion of Study II

We have demonstrated a significant net A-V difference of cortisone across the subcutaneous abdominal adipose tissue bed. This suggests that the 11 β -HSD1 oxo-reductase enzyme is active in subcutaneous abdominal adipose tissue. Although there is no detectable A-V difference in cortisol, this is likely to be due to limitations of assay precision. Measuring a 4 nmol/l A-V difference with a median arterial cortisol level of 200nmol/l would require an assay precise enough to detect a 2% difference in cortisol concentrations between arterial and venous samples. We found no significant arteriovenous difference of cortisone or cortisol across the deep forearm tissue. Although previous studies (151) suggest there is 11 β -HSD activity in forearm skin, the deep forearm cannulae in our subjects sampled blood draining muscle rather than skin.

Previous studies indicate that the oxo-reductase activity of 11 β -HSD (34) is greater in visceral than in subcutaneous adipose tissue. However, when cortisol was added to the culture medium in these studies there was a significant increase in both subcutaneous and visceral 11 β -HSD oxo-reductase activity. Our estimate of the TBF cortisol output (median 0.7mg) assumes all adipose tissue has the same oxo-reductase activity as subcutaneous adipose tissue. However, *in vitro* studies (34) show oxo-

reductase activity in visceral fat is greater than in subcutaneous abdominal adipose tissue. Thus it is likely that our TBF cortisol output is an underestimate. *In vivo* studies of the visceral adipose tissue bed would be advantageous, but would require portal venous sampling.

Cortisol production in adipose tissue may have paracrine implications. The role of cortisol in enhancing lipolysis (150;152) is well described, but an excess of cortisol more commonly leads to central fat accumulation (124;153) as in Cushing's syndrome. Central fat accumulation is partly mediated by lipoprotein lipase action, which is increased by insulin and cortisol (124;154;155). Adipose stromal cells cultured from superficial abdominal adipose tissue show a greater response to cortisol than do femoral adipose stromal cells (153). Thus cortisol production in adipose tissue could serve a paracrine role (34) favouring central fat accumulation and contribute to the adverse metabolic consequences of central obesity. It has been postulated that cortisol-mediated vascular reactivity increases peripheral resistance and contributes to hypertension (156). But such reactivity has not been studied in the adipose tissue bed where we have identified local 11 β -HSD activity.

We have assumed that xenon partition coefficients are the same in lean and obese subjects. This assumption will reduce the local cortisone clearance rates of obese subjects relative to those of the lean subjects. Even with this assumption, we have shown that local cortisone clearance rates (pmol/100g tissue/minute) are increased in those subjects with a higher TBF, suggesting upregulation of 11 β -HSD1 oxo-reductase activity in obesity. This is compatible with a cortisol:cortisone shuttle promoting obesity through paracrine mechanisms. However, the equivalent relationships between cortisone clearance rate and BMI or percentage body fat were not significant and some caution is therefore required in interpreting the relationship between TBF and cortisone clearance rates.

It has previously been shown that basal and 24 hour mean cortisol plasma levels are normal in obesity (41). As discussed in Chapter 3, there is some controversy in the literature as to whether cortisol production rates are increased in obesity. In as far as cortisol production rates are reflected by 24 hour urine cortisol metabolite levels (TCM) there are studies suggesting TCM are increased in obesity (41) and others (42;43) including our own data (Chapter 3) which demonstrate no significant relationship between BMI and TCM in men and women. Our finding of a

modest TBF output of cortisol (median 0.7mg/day) is unlikely to contribute significantly to cortisol production rates in the presence of normal negative feedback at the hypothalamic and pituitary levels.

It has traditionally been accepted that the urine Fm/Em ratio was reduced in obesity and this was thought to reflect net increased whole body conversion of cortisol to cortisone. The explanation for these findings until recently had been that in obesity adipose tissue converts cortisol to cortisone, depleting plasma cortisol levels which are replenished by hypothalamo-pituitary-adrenal feedback mechanisms (32). Our data and Stewart's *in vitro* data suggest that adipose tissue is a site of net 11 β -HSD oxo-reductase, rather than dehydrogenase activity, which challenges the traditional view. More recently, as discussed in Chapter 3, two large studies have not confirmed the relationship of urine Fm/Em ratios with obesity (BMI or TBF) (42;43) and this is in agreement with the limited urine cortisol metabolite data, which we presented in Chapter 3.

4-3) Summary of Chapter 4

In conclusion, our data supports the conclusions of Stewart's *in vitro* studies (34) that adipose tissue is a site of 11 β -HSD oxo-reductase activity. However, we diverge on the question of oxo-reductase activity in subcutaneous adipose tissue, which is significant in our study. *In vivo* data comparing visceral and subcutaneous adipose tissue oxo-reductase activity is lacking. 11 β -HSD1 oxo-reductase is active in subcutaneous abdominal adipose tissue. This oxo-reductase activity may be upregulated in obesity.

CHAPTER 5

**SEX STEROID METABOLISM IN SUBCUTANEOUS
ABDOMINAL ADIPOSE TISSUE AND FOREARM MUSCLE IN
WOMEN**

5-1) Aims of Chapter 5

In this chapter, I examine the peripheral metabolism of sex steroids in adipose tissue and obesity. I applied the A-V difference technique to characterise and quantify the net clearance and production of androstenedione (A), testosterone (T) and Oestradiol (E2) in SAAT and FM in premenopausal women. Furthermore, I sought to establish whether peripheral sex steroid metabolism differs in those subjects with and without polycystic ovary syndrome (PCOS).

5-2) Study III : Sex steroid metabolism in SAAT and FM in premenopausal women with and without PCOS

This study was presented as an abstract (poster presentation) at the American Endocrine Society conference in Toronto in June 2000 and at the Society of Endocrinology conference in London in November 2000. I was awarded a poster prize by the Society of Endocrinology at the November meeting. A copy of the abstract is attached in the appendix and may also be found in the Journal of Endocrinology 2000: 167: Supp P66.

5-2 (a) Summary of Study III

In this study, we applied the *in vivo* method of A-V difference sampling to measure SAAT and FM sex steroid clearance and production rates. We studied 13 PCOS and 8 non-PCOS premenopausal women. We demonstrated net A clearance, and net T /E2 production in SAAT. There was no significant production or clearance of sex steroids in FM. We did not demonstrate any differences in peripheral sex steroid metabolism between women with and without PCOS.

5-2 (b) Introduction to Study III

I have outlined in the introduction (Chapter 1, Sections 4 and 5) the current spectrum of scientific opinion on androgen /oestrogen metabolism in obesity; the relationship of obesity and hyperandrogenism in PCOS; and the peripheral contribution to clearance rate of A and production rates of T and E2 in women. In most of these areas, there is considerable debate and we aim to resolve some of the controversy by the application of AV difference measurements to infer clearance / production rates of sex steroids in SAAT and muscle. In this study, we measure clearance / production rates of A, T and E2 in SAAT and forearm muscle (FM) in a group of obese women with and without PCOS.

5-2 (c) Methods of Study III

Subjects and criteria for defining PCOS

Clinically, PCOS can present with one or more of the symptoms of menstrual irregularity, hirsutism, acne or alopecia. I discussed in Chapter 1 the heterogeneity in definitions of PCOS (83;86). We have taken elements of both definitions in common use to define PCOS as the combination of clinical evidence (83) of hyperandrogenism (one of the above four symptoms) with biochemical evidence of hyperandrogenism (86) - high free testosterone index (calculated from SHBG and testosterone level, normal range 0.9-1.34 %). In addition, we have taken two key factors from the US definition (86); firstly, the presence of PCO on ultrasound is consistent with but not necessary for the definition of the syndrome. The diagnosis of PCO on ultrasound is highly operator-dependent and non-specific, limiting its usefulness as an entry criterion for a clinical study⁷. Secondly, the exclusion of conditions that could mimic PCOS was essential for the study: congenital adrenal hyperplasia and 21-hydroxylase deficiency were excluded by measurement of 9am androstenedione and, when this was > 10nmol/l, measurement of

⁷ For example, one patient, who clinically appeared to have PCOS (obese, hirsute, oligomenorrhoea and hyperandrogenism) had a “normal” ovarian ultrasound scan in clinic. A second opinion was sought from an ultrasound unit in another hospital, which described the appearances as “typical of polycystic ovaries”.

synacthen-stimulated androstenedione. Hyperprolactinaemia (prolactin >600mU/l) was excluded in all subjects. All overweight (BMI >25) patients had a 24 hour urine free cortisol to exclude Cushing's syndrome.

I recruited 13 PCOS and 8 non-PCOS premenopausal women for the study. PCOS subjects were recruited from an endocrinology clinic (supervised by Dr P. Moulton at The Whittington Hospital) between September 1996 and October 1997. All PCOS subjects were studied prior to commencing any hormonal medication. All participants were assured (as part of written informed consent) that participation in (or withdrawal from) the study would in no way influence their management in the hospital clinic. The recruitment of non-obese PCOS and non-obese non-PCOS was less successful than we anticipated and this has been discussed in chapter 2. However, there was a wide range of BMI in both PCOS and non-PCOS groups. All subjects were premenopausal women and had not taken the oral contraceptive pill, anti-androgens or any other hormonal medication for 2 months prior to the study.

The subject characteristics for PCOS and non-PCOS groups are summarised in Table 5.1.

Table 5.1

Subject characteristics for Study III:

	PCOS (n=13) Median (interquartile range)	Non-PCOS (n=8) Median (interquartile range)
Age (years)	31 (25.5 – 38.5)	39 (34.5 – 43)
Ferriman and Gallwey score *	5 (2 - 7)	0 (0)
BMI (kg/m ²)	32.8 (26.6 – 40.5)	46.2 (30.9 – 48.3)
Waist (cm)	110 (88.5 - 121)	123 (100 – 141)
WHR *	0.95 (0.91 – 1.0)	0.89 (0.87 – 0.93)
TBF * (kg)	33.6 (18.4 – 47.4)	60.7 (26.7 – 72.9)
LBM (kg)	28.7 (26.0 – 30.4)	29.0 (23.3 – 30.9)
SHBG (nmol/l)	27 (22 – 37.8)	29 (9 – 73)
Basal arterial [A] * (nmol/l)	8.2 (6.6 – 11.6)	5.8 (4.4 – 6.4)
Basal arterial [T] (nmol/l)	1.9 (1.4 – 2.6)	1.5 (1.2 – 1.9)
Basal arterial [E2] (pmol/l)	396 (252 – 616)	250 (216 – 290)

*Mann-Whitney comparisons between PCOS and non-PCOS groups revealed that the PCOS group had a higher hirsutism (Ferriman and Gallwey) score ($p<0.01$), arterial [A] ($p<0.01$), and WHR ($p<0.05$) but lower TBF ($p<0.05$) in comparison to the non-PCOS group. There were no other significant differences between the groups.

Methods

The methodology of SAAT and FM A-V difference studies has been described in Chapter 2, section 6 of the Thesis. All subjects were studied in the follicular phase of the menstrual cycle, when possible (in oligo- or amenorrhoeic subjects the follicular phase is difficult to predict for the purposes of booking a clinical study in advance). The insertion of arterial, retrograde deep forearm venous and superficial epigastric venous cannulae has already been described in Chapter 2.

As previously described in the study on corticosteroid AV difference, it was not possible to obtain both forearm venous and epigastric venous cannulae in all subjects, but all subjects had an arterial line in situ. Superficial epigastric venous lines were established in 11/13 PCOS and all 8 non-PCOS subjects. Deep forearm venous lines were in situ in 10/13 PCOS subjects and only 1/8 non-PCOS subjects. The lack of forearm venous cannulae in the non-PCOS controls relates to the fact that these patients served as controls for another study in the department. Thus control serum was obtained for SAAT AV difference, but not (bar one case) for FM AV differences.

Serum samples were taken simultaneously from all lines (as previously described in Chapter 2) for assay of A, T and E2. Two sets of basal samples (Basal 1 and Basal 2) were collected an hour apart between 9am and 11am. The results of Basal 1 and Basal 2 assays were meaned to produce (mean) basal arterial and venous values.

Clearance / production rates of sex steroids were calculated from plasma flows and significant A-V difference measures, as previously described (Chapter 2), using the following conversion factors:

Testosterone: 1 nmol = 2.8667 x 10⁻⁴ mg

Androstenedione: 1 nmol = 2.865 x 10⁻⁴ mg

Oestradiol: 1 pmol = 2.72 x 10⁻⁴ µg

Fasting plasma insulin and glucose levels were measured in the PCOS subjects. A fasting glucose : insulin ratio was calculated (plasma glucose level divided by plasma insulin level) as a measure of insulin resistance in PCOS subjects. The lower the glucose : insulin ratio, the greater is the degree of insulin resistance (157).

5-2(d) Results of Study III

SAAT AV difference studies

There was significant net clearance of A and significant net production of T and E2 across SAAT. The results of AV differences, production / clearance rates and TBF production / clearance rates for A, T and E2 are summarised in Table 5.2 below.

There was no significant difference (Mann-Whitney analysis) between PCOS and non-PCOS groups in terms of SAAT clearance rates of A and production rates of T and E2. The subjects were stratified into hirsute/non-hirsute groups. The data was reanalysed (Mann-Whitney) and production / clearance rates of A, T and E2 did not vary significantly according to the presence / absence of hirsutism.

Having concluded that the presence of PCOS did not significantly affect peripheral sex steroid clearance and production rates, PCOS and non-PCOS data were merged for further analysis (Spearman's below). The clearance of A and production of T were not equimolar. There was significantly greater clearance of A ($p < 0.01$) as compared to net production of T.

Table 5.2 : Basal SAAT sex steroid production / clearance

	Androstenedione Median (IQ range)	Testosterone Median (IQ range)	Oestradiol Median (IQ range)
AV Difference [Art] – [Ven] Production (-) Clearance (+)	+ 0.75 * (-0.18 to +1.60) nmol/l	- 0.36 ** (- 0.63 to - 0.14) nmol/l	- 54 ** (- 84 to + 1) pmol/l
SAAT production (-) / Clearance (+) rate	+ 0.67 * (- 0.18 to + 2.4) nmol/100g SAAT /day	- 0.27 * (- 0.85 to - 0.06) nmol/100g SAAT /day	- 36 ** (- 137 to + 1) pmol/100g SAAT /day
TBF production (-) / clearance (+) rate	+ 0.04 * (- 0.02 to +0.22) mg/day	- 0.04 ** (- 0.09 to - 0.01) mg/day	- 5.8 ** (- 15.8 to 0.1) µg/day

* denotes $p < 0.05$ on Wilcoxon analysis (versus zero)

** denotes $p < 0.01$ on Wilcoxon analysis (versus zero)

Spearman's-SAAT

There was a significant correlation between age of subject and basal E2 local SAAT production rate ($r=0.48$, $p<0.05$), but there was no relationship between age and TBF E2 production rate or flux of other sex steroids. There was no significant relationship between adiposity indices – BMI, waist, WHR, STR and TBF – and either AV differences or clearance / production rates of sex steroids (A, T and E2). There was no correlation between Ferriman and Gallwey score and A, T and E2 production / clearance rates (both SAAT and TBF rates).

I determined whether the concentration of substrate was related to AV differences or clearance / production rates of individual sex steroids. The arterial [A] and [T] were unrelated to AV differences or production rates of T and E2. Mean basal arterial [A] correlated with mean basal AV difference in A ($r=0.66$, $p<0.05$) but did not significantly relate to SAAT or TBF A clearance rates.

Forearm AV difference studies

The data are presented for the 11 patients who had forearm muscle (FM) AV difference studies. There were insufficient non-PCOS FM preparations to allow any meaningful comparison of PCOS and non-

PCOS FM clearance / production rates of sex steroids.

There was a significant FM AV difference of testosterone [- 0.22 (- 0.41 to + 0.03) nmol/l, $p < 0.05$] but, when converted to production rate (product of forearm plasma flow and AV difference) this just failed to reach significance ($p=0.07$). There was no significant AV difference, clearance or production rate for either A or E2 in the forearm study.

The response of SAAT and FM AV differences / production rates to hyperinsulinaemic euglycaemic clamp in PCOS subjects is the subject of Chapter 6 and will not be addressed in this chapter.

Systemic levels of sex steroids *versus* obesity

In the group of 21 subjects taken together, there was an inverse correlation between arterial [A] and obesity as measured by BMI ($r = - 0.50$, $p < 0.05$) and TBF ($r = - 0.46$, $p < 0.05$). Similarly there was an inverse relationship between arterial [E2] and both BMI ($r = - 0.54$, $p < 0.05$) and TBF ($r = - 0.51$, $p < 0.05$). These relationships were not apparent when PCOS and non-PCOS groups were analysed separately.

There was no relationship between testosterone levels and obesity, whether PCOS and non-PCOS groups analysed separately or together.

Fasting glucose:insulin ratio and SHBG

In the PCOS subjects the fasting glucose:insulin ratio was 0.11 (0.06 – 0.28). There was no relationship between insulin resistance - as measured by glucose:insulin ratio - and BMI, WHR, basal arterial sex steroid levels (A, T and E2) or flux of sex steroids in adipose tissue or muscle. SHBG levels correlated with SAAT (but not FM) AV difference in testosterone ($r=0.71$, $P<0.01$), such that subjects with a lower SHBG showed higher testosterone output from adipose tissue. However, there was no correlation of SHBG and either local SAAT or TBF production rates of testosterone. There were no relationships between SHBG and A / E2 AV differences.

5-2 (e) Discussion of Study III

This study provides evidence that the dominant action of 17-BHSD in SAAT is conversion of A to T. This suggests that the activity of type 3 17-BHSD (which converts A to T and also E1 to E2) predominates over type 2 17-BHSD (which catalyses the conversion of T to A) *in vivo*, although both enzymes are present in the SAAT of women in *in vitro* studies (64).

SAAT Clearance of A

We found net adipose tissue clearance of androstenedione. An earlier study utilising a similar technique failed to detect a significant AV difference of A across SAAT (70) in women, but they had fewer subjects (6 women) and used arterialised rather than arterial blood samples. Arterialised samples are not as accurate and can mask a significant AV difference. Our estimates of TBF clearance / production rates assume uniform activity in all adipose tissue beds.

The rate of A clearance in our study was not related to the presence of PCOS, obesity (BMI, TBF) or distribution of body fat (waist, WHR, STR). Whilst other studies (58) have suggested the MCR of A is increased in obesity, especially central obesity, we find no support for the

theory that this is due to increased sequestration in adipocytes in obese subjects (158). The net A clearance rate was greater than the net T production rate and there are two (not mutually exclusive) explanations for this. Firstly, A is aromatised to Oestrone (E1) and thence to E2 by 17BHS D activity. Secondly, although we have net production of T, there is likely to be simultaneous clearance and production of T, with the greatest net flux being production. Similarly, our results are consistent with A being converted to T by 17BHS D and thence to DHT by 5 α -reductase activity or to E2 by aromatisation of T. Infusion of radiolabelled A would have clarified the quantitative net directions of each reaction.

SAAT production of T

We found the median net TBF production of T to be 0.04 mg/day, which is very similar to the results from an earlier study using a similar technique (70). The Boulton study (from AV differences and ¹³³Xe blood flow) estimated that a woman with a fat mass of 15kg would have a TBF T production rate of 0.037mg/day (70), also suggesting that this would become proportionately greater with increasing obesity and fat mass. Although we are agreed on the quantitative estimate of the peripheral contribution to testosterone production, we find no evidence to support

the claim that there is a linear relationship between fat mass and T production rate in adipose tissue. Our data is in agreement with an earlier study by Longcope, who administered radiolabelled A/T infusions to 88 women and measured the metabolites in urine collected over the subsequent 4 days. There was no relationship between obesity and the rate of 17BHS conversion of A to T, or E1 to E2 (79). One explanation for this may be that as fat mass increases, the metabolic activity of adipocytes reduces giving the same net SAAT testosterone production rate.

In premenopausal women, testosterone is produced both by the ovaries and by peripheral conversion of androstenedione (47;48). However, the quantitative contribution of peripheral testosterone production has been debated. We discussed in chapter 1 the historical data (48;98) which consistently reports a mean daily “whole body” T production rate of 0.34 to 0.4 mg/day in premenopausal women and 4 mg/day in men.

Unfortunately, there is less data on the production rate of testosterone in women with PCOS, but one paper measured testosterone production rates in 6 women with ovarian hyperthecosis (the virilised end of the spectrum of hirsutism found in PCOS) to be 2.1 mg/day (159). There is a lack of

data on testosterone production rates in PCOS subjects with less aggressive virilisation and this may only be 1mg/day.

Based on the median (interquartile ranges) in our study, we estimate that the periphery (adipose tissue) contributes 4 (1 – 9) % to the daily total testosterone production in PCOS, increasing to 10 (3 – 23) % in non-PCOS subjects. It would appear that the ovarian output of testosterone in PCOS far outweighs the contribution from peripheral 17BHS D conversion of A to T. We believe our estimates are more accurate than earlier estimates: Horton and Tait estimated 60% of circulating T was derived from peripheral 17BHS D activity (48); Vermeulen (6;47) estimated 55% of T production in premenopausal women comes from the periphery. We would suggest that both these studies - based on indirect assessment of peripheral conversion - overestimated the relative contribution of peripheral 17BHS D activity to daily T production.

SAAT production of E2

The AV difference of E2 in our study is similar to that found in the Boulton study (35 +/- 13 pmol/l) referred to above (70). We have demonstrated net production of E2 in SAAT, estimating median TBF E2 output as 5.8 µg/day in premenopausal women. We found no relationship

of adiposity (BMI, TBF, waist circumference or WHR) to either the AV difference in E2 or the rate of TBF E2 production. Radiolabelled A and E2 infusions (2 label technique) have been used to quantify peripheral conversion of sex steroids. Using this technique, Macdonald and Sitteri demonstrated a positive correlation between body weight and the rate of aromatisation of A to E1 in postmenopausal women (78). Similarly, in 1986, Longcope studied urine steroid profiles of obese and control postmenopausal women after radiolabelled A and T infusions (79). He found obese subjects to have increased aromatase activity with increased A to E1, and T to E2 conversion (79).

The association of obesity with an increased risk of endometrial (72;73) and breast cancer (74;75) has led to a search for an endocrine basis for this relationship. It has been proposed that increased oestrogen production from aromatisation in adipose tissue explains the increased risk of breast and endometrial cancer in obese postmenopausal women. However, we studied premenopausal women and if our study were repeated with postmenopausal women, a relationship between aromatisation rate and increasing adiposity may then be confirmed. Interestingly, we did find some supportive evidence for this with increased adipose tissue E2 production in older premenopausal subjects.

Alternatively, increased aromatisation in obese subjects may be taking place at a site other than adipose tissue (e.g. liver or ovary). Interestingly, when Sitteri studied the effects of weight loss in obese subjects (160), he found that weight reduction of 45kg did not decrease the aromatisation rate of A to E1 *in vivo*, suggesting that mechanisms other than fat mass dictate rates of aromatisation. Indeed, after 20 years of research in the field, Sitteri concluded that the exact site of peripheral aromatisation and the factors controlling the process had not yet been defined clearly (80;81). The advantage of the AV difference technique is that it defines the contribution of individual tissue beds. Thus we have provided a quantitative estimate of adipose tissue E2 production and concluded that muscle is not responsible for peripheral aromatisation.

PCOS versus non-PCOS and choice of definition

Obesity is strongly associated with PCOS, with 35% of PCOS women being overweight (BMI>25) in one series (83). Obese PCOS subjects in addition to greater insulin resistance (86), also have higher levels of total T and increased hirsutism (83) when compared to lean PCOS females. However, we found no evidence to support our hypothesis that peripheral testosterone production rates are increased in obese women with PCOS.

We considered whether our definition of PCOS biased the results. The database (SPSS for Windows version 6.0) was set up to stratify the subjects according to 3 different definitions of PCOS (set up as 3 different variables), according to the US criteria (86), UK criteria (83) or our combination of the two. No matter which definition was selected, no significant differences in peripheral sex steroid metabolism were detected between PCOS and control groups.

The Forearm Studies

There was a significant AV difference in testosterone across forearm muscle, suggesting 17BHS activity, but when blood flows were taken into account this did not translate into a significant production rate. We found no evidence for significant clearance or production of sex steroids in muscle. This is in agreement with an earlier AV difference study by Chang in 1975, who found no forearm (deep compartment) AV difference in DHEA, DHEAS, A, T and DHT (68).

Dihydrotestosterone (DHT) is produced entirely in the periphery, by 5- α reductase action on testosterone (47). In 1985, Longcope combined the A-V difference technique with infusions of radiolabelled A and T to measure DHT production in the forearm (69). He found that 14% of

radiolabelled A and 6% of T were converted to DHT in the superficial compartment (adipose tissue) of the forearm. We considered the question of measuring peripheral DHT production, but DHT measurements were not carried out due to limitations of the assay. For DHT, the assay method involves a 2-stage extraction technique and the intra-assay CV exceeds 15%. This is unlikely to allow us to detect small AV differences (without the benefit of radiolabelled hormone) as discussed in chapter 2, so time and research funds were not invested in DHT analysis.

Systemic levels of sex steroids

In Chapter 1, we outlined the debate as to whether systemic sex steroid levels were increased, normal or reduced in obesity. Most studies have found no difference in E2 levels between obese and non-obese premenopausal women (50). Meanwhile, total androgen levels in obese eumennorrhoeic women have previously been shown to be normal or low (6;50;51). We found no evidence to support a relationship between systemic (arterial) T levels and BMI or TBF in our cohort. Although we found an inverse relationship between BMI, TBF and both arterial [A] and [E2], this disappeared when PCOS and non-PCOS groups were analysed separately. The fact that E2 and A levels were higher in the PCOS group, which had a significantly lower TBF than the non-PCOS

group, skewed the results, giving rise to an apparent relationship between sex steroid levels and obesity when our 21 subjects were analysed as a single group.

The lack of a relationship between SHBG and obesity in our study reflects the lack of normal weight controls (only 3 out of 21) in our cohort. Our study was not designed to specifically address the issue of sex steroid levels in obesity, which would require a study of larger numbers of subjects with an appropriate non-obese control group.

5-3) Chapter Summary

We have established that adipose tissue contributes a median of 4 % to the daily whole body testosterone production in women with PCOS, increasing to 10 % in non-PCOS subjects. Previous studies had overestimated the contribution of peripheral 17BHS D activity to daily T production in premenopausal women. Our study suggests that type-3 17-BHSD enzyme activity predominates in SAAT, providing net clearance of A and net production of T and E2 in premenopausal women. Peripheral sex steroid metabolism is similar qualitatively and quantitatively in PCOS and non-PCOS women.

Chapter 6

The effect of acute hyperinsulinaemia on SAAT and FM sex steroid metabolism in PCOS

6-1) Aims of Chapter 6

Having established the baseline characteristics of peripheral sex steroid metabolism in women with and without PCOS in chapter 5, in this chapter I go on to assess the influence of insulin on peripheral sex steroid metabolism in women with PCOS. I applied the hyperinsulinaemic euglycaemic clamp technique to study the effect of acute hyperinsulinamia on peripheral sex steroid metabolism.

6 –2) Study IV: The effect of acute hyperinsulinaemia on production / clearance rates of sex steroids in SAAT and FM in PCOS

This study was presented by Dr J.R.Katz as an abstract at the American Endocrine Society (ENDO 2000) in Toronto in June 2000. This work was completed in collaboration with Dr J.Patel, Dr H.McGarrigle, Prof.J.S.Yudkin and Dr S.W.Coppack.

6-2 (a) Summary of Study IV

The PCOS subjects in Study III, having completed the baseline study described in chapter 5, proceeded to a 4 hour insulin infusion, with a euglycaemic clamp technique to assess the impact of insulin on SAAT and FM sex steroid metabolism. Insulin was infused at low dose (20 μ m²/min) for 2 hours and then at high dose (40 μ m²/min) for a further 2 hours. In SAAT, in response to insulin there was a small but significant increase in T production, with no significant increase in A clearance. E2 output was not detected during the insulin infusion in SAAT or FM. In FM, A clearance occurred during both low and high dose clamps with significant T production during the low-dose insulin infusion.

6-2 (b) Introduction of Study IV

I outlined in chapter 5 the baseline adipose tissue and muscle production / clearance rates of A, T and E2. The question remains as to the mechanism of regulation of peripheral production. Insulin is known to regulate glucose and free fatty acid metabolism in adipose tissue and muscle (161;162), but it has not been established whether insulin influences peripheral sex steroid metabolism. I tested the hypothesis that insulin up-regulates 17-BHSD and aromatase activity in adipose tissue and muscle.

6-2 (c) Methods of Study IV

Subjects

Twelve of the thirteen PCOS subjects who completed the baseline studies described in chapter 5 proceeded immediately to a hyperinsulinaemic euglycaemic clamp. In one subject (JKPC12), the arterial line failed after completion of the baseline sample collection and she could not be included in this wing of the study.

The characteristics of the 12 PCOS subjects who commenced euglycaemic clamps as part of this AV difference study are as follows:

median [interquartile range] age 30 [25 - 38] years, FGS 5 [2 – 7.5], BMI 33.6 [26.6 –40.9] kg/m², TBF 33.8 [17.3 – 48.7] kg, WHR 0.94 [0.91 - 1.00], fasting plasma glucose (FPG) 5.5 [4.9 – 6.7] mmol/l, fasting insulin 57.8 [32.7 – 77.3] pmol/l.

Basal hormone levels were as follows: LH 4.0 [1.3 – 10.5] IU/l, FSH 4.4 [2.2 – 8.0] IU/l, SHBG 26.5 [19.0 – 41.2] nmol/l, androstenedione (A) 8.2 [6.8 - 11.2] nmol/l, testosterone (T) 2.1 [1.4 – 2.6] nmol/l, oestradiol (E2) 424 [243 - 659] pmol/l.

Methods

All twelve subjects were studied after an overnight fast and were studied in the follicular phase of the menstrual cycle when possible. Basal samples were taken as described in Chapter 5. All subjects had radial arterial lines in situ. Superficial epigastric venous cannulae were in tact during the clamp in 9/12 subjects. Forearm venous cannulae were in situ in 8/12 subjects at the beginning of the clamp study.

Insulin/dextrose infusion

For hyperinsulinaemic euglycaemic clamp method, see Methods: chapter 2, section 8.

Sampling

Simultaneous arterial, forearm venous and epigastric venous serum samples were taken at T= 90, 120, 210 and 240 minutes for A, T, and E2. At the same timepoints, blood (lithium heparin tube) was taken from a single vessel for plasma insulin concentration. Blood flows in SAAT and the forearm were measured as previously outlined in chapters 2 and 5 at baseline and again at T=100 min and T=220 min. In the latest 3 studies, a further sampling time / blood flow measurement was introduced at T = 300 min, an hour after stopping insulin, yielding post clamp AV difference data for 3 subjects across SAAT and none across FM.

Assays

The concentration of each hormone at each timepoint for each site (artery, forearm vein, epigastric vein) was assayed. Each assay was carried out on duplicate aliquots. The basal values for each hormone were calculated from the mean of two basal timepoints. The “low dose clamp” values

were the mean of T=90 and T=120 min values. The “high-dose clamp” values were the mean of T=210 and T=240 min samples.

Assay techniques for A, T, E2 and insulin are described in Methods: Chapter 2, section 9. Glucose was assayed in a Beckman analyser after microcentrifugation to separate plasma from whole blood.

Glucose disposal rate and metabolic clearance rate

I calculated the glucose disposal rate (M-value) and metabolic clearance rate (MCR) for both low-dose (M1, MCR1) and high-dose (M2, MCR2) clamps. The calculations were based on the following formulae:

1 ml 10% Dextrose = 100 mg glucose

Infusing 10% dextrose, 1 ml/hr = 1.6666mg/min

Glucose concentration of 1 mmol/l equates to 18 mg/dl

$$\text{MCR (dl/min)} = \frac{\text{Mean infusion rate at steady state (mg/min)}}{\text{Steady state plasma [glucose] (mg/dl)}}$$

$$\text{M-value (mg/kg/min)} = \frac{\text{Mean infusion rate at steady state (mg/min)}}{\text{Weight (kg)}}$$

6-2(d) Results of Study IV

Insulin levels /M-values/MCR glucose

The insulin levels, glucose disposal rate (M-values) and metabolic clearance rate (MCR) at each stage of the clamp are summarised in Table 6.1.

Table 6.1

Insulin , M-Values and MCRs for glucose in Study IV

	Plasma [Insulin] (pmol/l)	M-value (mg/kg/min)	MCR Glucose (dl/min)
Basal	51 (30 – 74)	N/A	N/A
Low Dose Clamp	430 (367 – 458)	2.42 (1.51 – 3.89)	2.08 (1.46 – 3.15)
High dose clamp	1096 (976 – 1127)	4.97 (3.94 – 7.47)	5.21 (3.92 – 6.33)
Post clamp	71 (76 – 81)	N/A	N/A

Plasma insulin level increased significantly ($p<0.05$) from baseline to low dose clamp, and then again from low dose to high dose clamp. The glucose disposal rate (M-value) and metabolic clearance rate (MCR) both increased ($p<0.01$) significantly from the low dose to high dose clamp.

Age, BMI, waist circumference and WHR did not correlate with (a) basal insulin, (b)MCR at low and high dose clamp (MCR 1 and MCR2 respectively) and (c) M-value at low and high dose clamps (M1 and M2 respectively).

Basal [insulin] was directly related to LBM ($r=0.86$, $p<0.05$) and inversely related to M1 ($r= - 0.81$, $p<0.05$) and M2 ($r = - 0.89$, $p < 0.01$). M1 was inversely related to LBM ($r=- 0.70$, $p<0.05$) and TBF ($r= - 0.65$, $p<0.05$) and directly related to MCR1 ($r= 0.83$, $p<0.01$). M2 was directly related to MCR2 ($r=0.75$, $p<0.05$).

SAAT- Testosterone

There was significant output of testosterone (as measured by AV difference, local SAAT and TBF production rates) at all stages of the clamp. The testosterone production rate was significantly increased – relative to baseline –during both low dose and high dose clamps. There was no significant difference between low and high dose clamp testosterone production rates. The numerical data are summarised in Table 6.2 below and in Figure 6.1.

Table 6.2 : The effect of insulin infusion on SAAT testosterone production in PCOS subjects

	AV-difference (nmol/l)	SAAT production rate (nmol/100g/day)	TBF production rate (mg/day)
Basal	- 0.32 * (- 0.48 to - 0.08)	- 0.44 * (- 0.85 to 0.00)	- 0.03 * (- 0.08 to 0.00)
Low-Dose Clamp	- 0.40 **/ns (- 0.57 to - 0.20)	- 0.57 **/* (- 2.44 to - 0.22)	- 0.05 **/* (- 0.02 to - 0.24)
High Dose Clamp	-0.48 **/* (- 0.69 to - 0.29)	- 0.56 **/* (- 1.82 to - 0.29)	- 0.08 **/* (- 0.03 to - 0.18)

All values quoted as median (interquartile range)

The first p value is Wilcoxon versus zero

The second p value (after /) is Wilcoxon versus basal value

*p<0.05, **p<0.01

Testosterone Production Rates before and after Insulin Infusion in PCOS Subjects

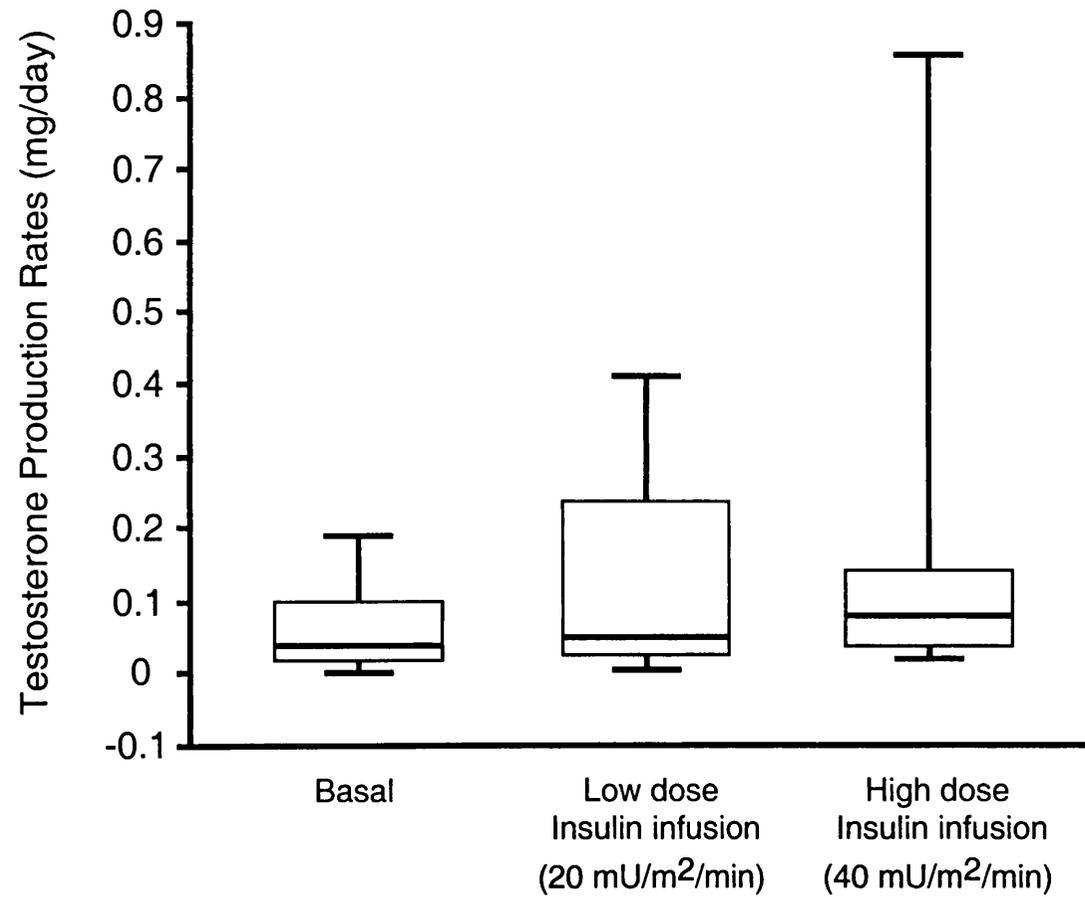


Figure 6.1

Total body fat testosterone production rates in PCOS subjects before and after insulin infusion

The post-clamp data showed a local SAAT output of -0.67 (-0.20 to -16.2) nmol/100g adipose tissue/day, suggesting a continuing increase in testosterone output, but the numbers were too small ($n=3$) for statistical analysis.

There was no relationship (Spearman's) between clamp T output (AV difference or production rate) and (a) indices of adiposity, as measured by BMI, waist, WHR, TBF, (b) the hirsutism score (FGS), (c) prevailing arterial concentration of A or T at each timepoint or (d) fasting glucose:insulin ratio, M1,M2,MCR1 or MCR2.

SAAT - Androstenedione

There was significant clearance of A across SAAT at baseline (as measured by AV-difference and local SAAT clearance rate), low dose clamp and high dose clamp (as measured by SAAT and TBF clearance rates). The clamp did not alter the clearance rate of A in SAAT at either low or high dose. The numerical data are summarised in Table 6.3 below.

Table 6.3

The effect of insulin infusion on SAAT androstenedione clearance in PCOS subjects

	AV-difference (nmol/l)	SAAT production rate (nmol/100g/day)	TBF production rate (mg/day)
Basal	+ 1.26 * (+0.29 to +1.79)	+ 1.53 * (-0.06 to +3.37)	+0.10 ns (0.00 to +0.46)
Low dose clamp	+ 0.52 **/ns (+0.33 to +1.80)	+ 1.19 **/ns (+0.45 to +3.81)	+0.07 **/ns (+0.04 to +0.61)
High dose Clamp	+ 0.97 ns/ns (-0.34 to +2.13)	+1.99 */ns (+0.49 to +7.0)	+0.16 */ns (+0.06 to +0.46)

All values quoted as median (interquartile range)

The first p value is Wilcoxon versus zero

The second p value (after / in low and high dose clamps) denotes Wilcoxon of clamp value versus basal value

*p<0.05, **p<0.01

There was no relationship (Spearman's) between clamp A output (AV difference or production rate) and (a) indices of adiposity, as measured by BMI, waist, WHR, TBF, (b) the hirsutism score (FGS) or (c) prevailing arterial concentration of A or T at each timepoint or (d) fasting glucose:insulin ratio, M1,M2,MCR1 or MCR2.

SAAT - Oestradiol

There was significant output of E2 at baseline, but there was no significant AV difference or production of E2 at the end of both low dose and high dose clamps. The numerical data are summarised in Table 6.4 below. Non-significant AV differences are shown for completion, but the non-significant production rates are not shown.

Table 6.4

The effect of insulin infusion on SAAT oestradiol production in PCOS subjects

	AV-difference (pmol/l)	SAAT production rate (pmol/100g/day)	TBF production rate (µg/day)
Basal	- 68 ns (- 84 to + 18)	- 94 * (- 177 to + 3)	- 9 * (- 16 to + 0.2)
Low dose clamp	-20 ns (- 43 to + 40)	ns	Ns
High dose Clamp	-36 ns (- 64 to + 34)	ns	Ns

All values quoted as median (interquartile range)

*p<0.05

There was no relationship (Spearman's) between basal / clamp E2 production rates and (a) indices of adiposity, as measured by BMI, waist, WHR, TBF, (b) the hirsutism score (FGS), (c) prevailing arterial concentration of A or T at each timepoint or (d) fasting glucose:insulin ratio, M1,M2,MCR1 or MCR2.

Forearm Muscle – Testosterone

There were significant AV differences of T, indicating T output, at all stages of the clamp (see Table 6.5). However, on correction for blood flow, there was only significant production of T at the end of the low dose clamp. On further analysis (Wilcoxon) there were no significant alterations in T AV difference /production rates from baseline to either low or high dose clamp, or from low dose to high dose clamp.

Table 6.5

Summary of forearm testosterone clamp data

	AV difference (nmol/l)	FM production rate (nmol/100g/day)	LBM production rate (mg/day)
Basal	- 0.30 * (- 0.42 to +0.05)	- 0.34 ns (- 0.47 to +0.11)	ns
Low Dose Clamp	- 0.16 */ns (- 0.40 to - 0.03)	- 0.24 */ns (- 0.60 to - 0.03)	- 0.02 */ns (- 0.06 to - 0.01)
High dose clamp	- 0.22 */ns (- 0.25 to - 0.06)	- 0.25 ns/ns (- 0.50 to - 0.03)	ns/ns

All values quoted are median (interquartile range)

The first p value is Wilcoxon versus zero

The second p value (after / in low and high dose clamps) denotes Wilcoxon of clamp value versus basal value

*p<0.05

There was no significant correlations (Spearman's) between low dose clamp T production rates and (a) indices of adiposity, as measured by BMI, waist, WHR, TBF, (b) hirsutism (FGS) score (c) prevailing arterial concentration of A or T at each timepoint or (d) glucose : insulin ratio, M1 or MCR1.

Forearm – Androstenedione

There was no significant basal A-V difference or clearance of A.

However, at the end of both low and high dose clamps there was significant clearance of A across the deep forearm compartment. There was a significant increase in A clearance from baseline to high dose clamp. The results are summarised in Table 6.6.

Table 6.6

Summary of FM androstenedione clamp data

	AV difference (nmol/l)	FM clearance rate (nmol/100g/day)	LBM clearance rate (mg/day)
Basal	+ 0.24 ns (-0.75 to +1.49)	ns	ns
Low Dose Clamp	+ 1.07 */ns (+0.16 to +2.19)	+ 1.48 */ns (+0.31 to + 2.39)	+ 0.11 */ns (+0.01 to + 0.18)
High dose clamp	+ 0.88 */* (+0.55 to +2.03)	+ 2.05 */* (+0.70 to + 3.14)	+ 0.19 */* (+0.06 to + 0.32)

All values quoted are median (interquartile range)

The first p value is Wilcoxon versus zero

The second p value (after / in low and high dose clamps) denotes Wilcoxon of clamp value versus basal value

*p<0.05

There was no relationship (Spearman's) between basal /clamp A clearance rates and (a) indices of adiposity, as measured by BMI, waist, WHR, TBF, (b) the hirsutism score (FGS) or (c) prevailing arterial concentration of A at each timepoint. The A clearance rate in the high dose clamp was inversely related to the fasting glucose : insulin ratio ($r = - 0.90$, $p < 0.05$), suggesting increasing A clearance with greater insulin resistance. However, low/high dose clamp A clearance was not related to M1/M2 or MCR1/MCR2 respectively.

Forearm - Oestradiol

There was no significant AV difference, production or clearance of E2 across the forearm, either basally or in response to low / high dose insulin infusion.

6-2 (e) Discussion of Study IV

Effects of insulin on SAAT sex steroid metabolism

Acute hyperinsulinaemia increases SAAT output of testosterone, with median net TBF output increasing from 0.03mg/day at baseline to 0.08mg/day at the end of the high dose clamp. This effect was apparent within 2 hours of the onset of the clamp and appeared to continue even when insulin levels returned to normal. This is may be a result of insulin upregulating 17BHSD activity within adipose tissue. There is evidence that insulin increases the metabolism of steroid hormones in rat hepatocytes (163), but there is no data to my knowledge⁸ on the effect of insulin on 17-BHSD activity in adipose tissue.

There was no clear effect of insulin on A clearance in SAAT, with a non-significant trend towards increasing A clearance with increasing insulin infusion rates. Interestingly, E2 output became undetectable during the clamp. Although there was a trend towards decreasing E2 output on insulin, this was not confirmed on Wilcoxon comparison of basal and clamp data. A study with larger numbers would be required to confirm this observation.

⁸ Complete medline literature search on “17 beta-hydroxysteroid dehydrogenase” and “insulin” as keywords

Effects of insulin on forearm sex steroid metabolism

As in chapter 5, in this cohort there was no significant basal production (as measured by production/clearance rates) of T. The low dose clamp resulted in significant T output, although this was not the case on the high-dose clamp. Direct comparisons of basal and low dose clamp testosterone production rates did not reveal significant differences. It is possible that larger numbers of patients would reveal a clearer trend, but our data suggests upregulation of FM 17BHS D activity by insulin.

Perhaps the most striking effect of the insulin infusion was the significant increase in FM A clearance from baseline to high dose clamp. As in Chapter 5, there was no detectable basal A clearance, but sampling at the end of both low and high dose clamps revealed significant A clearance. We cannot determine whether the increasing A clearance from low to high dose clamp reflects a dose response to insulin or the effect of a continuing insulin infusion.

The inverse correlation of fasting glucose : insulin ratio (FGIR) with high dose FA A clearance rate is difficult to explain as it suggests that increasing FGIR, which reflects increased insulin sensitivity, corresponds with reduced A clearance. The correlation matrix from which this result

arose included over 20 combinations of M1,M2,MCR1,MCR2,FGIR and SAAT/FM production/clearance rates of A,T and E2. It is possible that this association arose by chance and this is a common problem in correlation matrixes. Some would suggest only taking not of p-values<0.01 in this context, which would make this finding non-significant.

The lack of any E2 output basally and during the clamp suggests that there is no significant aromatase activity in FM. This data indicates that insulin upregulates 17BHSB activity in FM, resulting in clearance of A and production of T in FM during the clamp. The alternative explanation for increasing A clearance would be aromatase conversion to E1, but we have no evidence to support the presence of aromatase activity in FM.

6-3) Summary of Chapter 6

Acute hyperinsulinaemia increases 17 β HSD activity in adipose tissue and muscle, resulting in increased T production in SAAT and increased T production / A clearance in FM. Insulin also appears to inhibit aromatase activity in SAAT, but this observation requires confirmation with larger numbers of subjects.

Chapter 7

The effect of acute hyperinsulinaemia on systemic levels of sex steroids, gonadotrophins and SHBG in PCOS

7-1) Aims of Chapter 7

Having assessed the impact of acute hyperinsulinaemia on peripheral sex steroid metabolism in Chapter 6, in this chapter I assess the impact of insulin infusion on systemic circulating levels of sex steroids, gonadotrophins and SHBG in women with PCOS. Levels of A, T, E2, LH, FSH and SHBG were monitored during a hyperinsulinaemic euglycaemic clamp in our PCOS cohort.

7-2) Study V: The effect of acute hyperinsulinaemia on systemic levels of SHBG, gonadotrophins and sex steroids in PCOS

This study was carried out in collaboration with Dr H.McGarrigle, Prof J.S.Yudkin and Dr S.W.Coppack.

7-2 (a) Summary of Study V

Hyperinsulinaemic euglycaemic clamps were carried out in 15 PCOS women. In addition 3 time control subjects were studied. Contrary to our hypothesis, there was no rise in systemic A, T or LH levels and there was no fall in SHBG. In fact, testosterone levels fell in both insulin infusion and control groups. This fall in testosterone levels is thought to represent underlying circadian rhythm rather than a direct effect of insulin.

7-2 (b) Introduction to Study V

The effect of insulin on sex steroid, gonadotrophin and SHBG levels in PCOS subjects has been examined previously, with conflicting results, as outlined in the Chapter 1, section 5(f). Whilst two studies demonstrated no effect of a 2 hour insulin infusion ($40\mu\text{u}/\text{m}^2/\text{min}$) on A or T levels (110;112), another study demonstrated a rise in testosterone levels after a 4.5 hour insulin infusion (111). LH levels did not alter during a 16-hour clamp study (112). Another study demonstrated a fall in SHBG levels after a 6-hour insulin infusion (113).

The aim of this study is to assess the response of sex steroids, gonadotrophins and SHBG to acute hyperinsulinaemia – a question which has not previously been addressed in a single study.

7-2 (c) Methods of Study V

Subjects

Sixteen PCOS subjects were allocated to receive insulin infusion and eleven of these had formed part of the AV difference study outlined in the previous section. In addition, 4 time controls were studied. Two patients were excluded (JKPC06-insulin infusion, and JKPC22- a time control) as they were found to have LH levels above 20 IU/l and were entering the luteal phase of the menstrual cycle. The effect of their high LH/FSH levels on sex steroid levels could not be predicted and they were excluded on this basis. This left 15 PCOS insulin infusion subjects and 3 time controls, two of whom had PCOS and one of whom was non-PCOS.

The characteristics of the 15 PCOS subjects did not differ significantly from the PCOS group described in the clamp AV difference studies (described in the previous section 6.1). Time controls are described individually in Table 7.1.

Table 7.1

Characteristics of time controls for Study V

	Study code PC18	Study code PC20	Study code PC21
PCOS?	Yes	Yes	No
Age (yrs)	36	23	33
FGS	6	0	0
BMI (kg/m ²)	41.1	28.1	45.4
WHR	0.94	0.90	0.87
TBF (kg)	45.5	20.3	48.0
Basal [A] (nmol/l)	5.1	12.4	6.4
Basal [T] (nmol/l)	1.8	2.3	1.2
Basal [E2] (pmol/l)	317	149	202
Basal [LH] IU/l	2.6	6.5	1.4
Basal [FSH] IU/l	7.3	7.9	9.6
Basal [SHBG] (mmol/l)	27.5	15.7	24.9

Study protocol

All subjects were studied in the follicular phase of the menstrual cycle, where possible and those with a LH >20 IU/l (suggesting mid-cycle LH surge) were excluded as described in the previous section. The studies commenced in the morning after an overnight fast. The insulin infusion protocol /sampling methods were as previously described in section 6.1 for the AV difference studies, except that all blood samples at each timepoint were taken from a single vessel in each patient. The resulting values for all assays shall be described as “systemic levels”.

The time control patients were given IV fluids (0.9% saline) to maintain hydration and match the volumes of IV fluid given to clamp patients. The plasma glucose was checked hourly on time control patients and IV dextrose was not required to maintain plasma glucose at the fasting level. The same restrictions with respect to fasting applied to the time controls. The timing of blood sampling for hormones was matched in infusion subjects and time controls.

Wilcoxon's test was used to analyse differences between basal hormone values (sex steroid, LH,FSH and SHBG) and both low and high dose clamp values. Spearman's correlation coefficients were applied to analyse relationships of adiposity, hormone levels, FGIR, MCR glucose and M-values.

7-2 (d) Results of Study V

Insulin levels/M-values/MCR glucose in clamp subjects

The basal and clamp insulin levels, M- values and MCR for glucose during low and high dose clamps did not differ significantly from those described in section 6.1 (clamp AV difference studies). Please refer to section 6.1 for these levels. The peak insulin level at the end of the high dose clamp was 1100 (980 –1130) pmol/l.

The fasting glucose: insulin ratio (FGIR) correlated directly with M-values of low-dose ($r= 0.81, p<0.01$) and high-dose ($r= 0.82, p<0.01$) clamps. Basal systemic [A] correlated inversely with FGIR ($r= - 0.67, p<0.05$). The low dose clamp M value correlated inversely with WHR ($r= - 0.57, p =0.05$).

Relationship of basal hormone levels to adiposity / hirsutism in clamp subjects

Basal systemic [A] correlated with basal LH ($r=0.61, p<0.05$). There were no relationships between basal sex steroid levels and indices of adiposity, as measured by BMI, waist, WHR, STR and TBF). Basal [T] was directly related to hirsutism, as measured by FGS ($r= 0.51, p=0.05$). Basal [A] , [E2], LH, FSH and SHBG were not related to FGS.

Basal LH was directly related to WHR ($r=0.58$, $p<0.05$). Basal FSH was directly related to BMI ($r=0.57$, $p<0.05$), TBF ($r=0.57$, $p<0.05$), WHR ($r=0.88$, $p<0.001$) and waist ($r=0.74$, $p<0.01$). SHBG was inversely related to BMI ($r= - 0.55$, $p<0.05$).

Sex steroid levels during clamp

There was a significant fall in T levels from basal levels of 2.2 (1.5 – 2.7) nmol/l to high dose clamp levels of 1.9 (1.3 – 2.3) nmol/l ($p<0.05$). There were no changes in T levels from basal to low dose clamp. There were no significant changes during the clamp of systemic A or E2 levels.

Gonadotrophin and SHBG levels

There was no significant change in LH, FSH or SHBG levels from baseline to either low-dose or high-dose clamp.

Time controls

The main aim of the time controls was to control for the influence of circadian rhythm and associated changes in hormone levels. The only significant finding in the clamp was a fall in T levels from baseline to high dose clamp. $\Delta[T]$ was defined as the high dose clamp [T] minus low

dose clamp[T]. Mann-Whitney analysis did not demonstrate a significant difference in $\Delta[T]$ between time controls and clamp subjects.

Similarly, $\Delta[A]$, $\Delta[E2]$, $\Delta[SHBG]$, $\Delta[LH]$ and $\Delta[FSH]$ were defined as the difference between high dose and basal concentrations of the respective hormone. Mann-Whitney analysis excluded a significant difference in these values between time controls and clamp subjects.

7-2 (e) Discussion of Study V

We found a significant fall in T levels during the clamp, but the comparison with time controls suggests this may be an effect of diurnal rhythm in T levels. There is evidence that both T and A exhibit diurnal rhythm with levels falling in the afternoon (164;165). This diurnal rhythm is seen in normal women and women with PCOS. Thus, I would have expected a fall in [A] during the afternoon in normal circumstances, but the failure of A to fall may represent stimulation of ovarian or adrenal A secretion. Ideally, an equal number of PCOS subjects would have been studied as time controls (15) and this represents a problem with the study

design. The failure to detect differences between control and insulin infusion groups may well relate to the small size of the control group.

Another criticism of this study might be the insulin dose / duration of insulin infusion. We used an insulin dose at a near physiological post-prandial rate of 40 $\mu\text{u}/\text{m}^2/\text{min}$ for our high dose clamp, which had previously been shown not to alter A or T levels in PCOS subjects in another study (110). Similar doses of insulin in euglycaemic clamps have been shown to influence both glucose uptake in muscle and lipolysis in adipose tissue (162).

Insulin infusions have been employed at much higher rates in other studies of PCOS women: Micic *et al* increased insulin levels up to 4,300 pmol/l for 4.5 hours resulting in increased [T] at the end of the clamp (111); Nestler *et al* achieved insulin levels of 12,900 pmol/l for 16 hours, with no effect on hourly measured LH or testosterone (112). Fendri *et al* found that SHBG levels fell after a 6-hour insulin infusion, plasma insulin concentrations peaking at 7,900 pmol/l (113).

Thus, the length of our clamp may have been insufficient to allow an effect to take place. If insulin acts at a nuclear level to influence gene

transcription, it may take several hours before mRNA is produced and translated (to the relevant hormone) and hormone release into the circulation takes place. If however, insulin is affecting release of stored hormone, the response would be quicker. It is certainly plausible that our experiment was too short to see an effect on systemic hormone levels, despite the notable effects on peripheral sex steroid conversions referred to in chapter 6.

Our reasons for choosing a relatively short insulin infusion were based mainly on practicality. Because the clamp protocol was added on to the baseline study described in Chapter 5, the length of the infusion had to be limited in the interests of a) patient comfort..most subjects had 4 cannulae in situ for 10 hours, with limited mobility during this time and b) limiting the volume of blood taken, which did not exceed 500ml in any study. The baseline study of multiple site sampling used significant volumes of blood.

7-3) Summary of Chapter 7

We demonstrated a reduction in testosterone levels after a 4-hour insulin infusion in PCOS subjects. This effect is likely to be a result of circadian rhythm rather than a direct effect of insulin. The lack of a fall in [A] during the day may be a result of hyperinsulinaemia increasing ovarian or adrenal A secretion. The effects of acute hyperinsulinaemia (at post-prandial physiological levels) on systemic hormone levels is much less striking than the effects of insulin on peripheral sex steroid metabolism.

Chapter 8

Conclusions

Successes and limitations of the Thesis **and directions for future research**

8-1) Aims of Chapter 8

The aim of this chapter is to review the novel clinical scientific findings presented in this Thesis. Here, the contribution of this Thesis to the current understanding of the production and interconversion of steroid hormones in obesity and PCOS is summarised. The limitations of this research are discussed and directions for future research are outlined.

8-2) Contributions of this Thesis to clinical endocrinology

This Thesis examines the production and interconversion of steroid hormones in obesity and PCOS. Corticosteroid metabolism has been examined by studying the influence of adiposity on the HPAA and the endocrine function of adipose tissue with respect to the cortisol-cortisone shuttle. The studies on sex steroid metabolism have assessed the contribution of the periphery to sex steroid production in obesity and PCOS. This thesis demonstrates how insulin may regulate peripheral sex steroid production in PCOS. The findings are now discussed in detail, firstly with respect to corticosteroid metabolism and then with respect to sex steroid metabolism in obesity and PCOS.

8-2 (a) Corticosteroid metabolism in obesity

In Chapter 3 it was established that the pituitary-adrenal axis is hyper-responsive to CRH in centrally obese men, but not in postmenopausal women. No evidence was found to support a role for leptin in determining the response of the pituitary or adrenal to CRH in humans *in vivo*. This study has been published (see appendix) and is part of an evolving literature in this area and other groups have now also

demonstrated abnormal HPAA activity in obese men, as discussed in chapter 3, section 2(e).

The main conclusion from Chapter 3, putting to one side the limitations and methodological problems that will be discussed later in this chapter, is that central obesity is associated with a hyperactive HPAA axis in men. It is suggested that a hyperactive HPAA encourages the central accumulation of adipose tissue, giving rise to a subclinical form of Cushing's syndrome. The alternative though not mutually exclusive hypothesis is that centrally obese subjects produce hormones and cytokines from central adipose tissue sites, feeding back to the HPAA to enhance its activity.

No relationship was found between basal leptin and IL-6 levels and pituitary-adrenal responses to CRH in men and postmenopausal women with a range of body fat distribution. The negative findings with regard to leptin and IL-6 do not support the theory that adipose tissue secretes hormones/cytokines which then signal at the hypothalamo-pituitary or adrenal level to create a hyperactive HPAA. However, detailed studies of the influence of leptin and IL-6 on the HPAA would require infusions of leptin and IL-6 and direct study of their influence on ACTH/cortisol

levels in human subjects. Our methodology was not robust enough to study the influence of leptin particularly, which has a circadian rhythm (with a trough between 8 and 9am, the time at which we measured leptin). Leptin has been shown to inhibit the activity of the HPAA (134,135) in animal studies, so it is unlikely that central adipose tissue leptin secretion serves to enhance HPAA activity.

In chapter 4, data was presented which has now been published (see appendix) supporting the presence of net 11-BHSD oxo-reductase activity in adipose tissue. This was important for two reasons: firstly, it demonstrated to me the importance of questioning the previous literature in the area - cortisol has always been assumed to undergo oxidation to cortisone in adipose tissue; secondly this suggests that adipose tissue generates its own growth factor (cortisol) which may serve more important paracrine than endocrine roles.

Thus, this thesis provides evidence that a hyperactive HPAA gives rise to central obesity and that adipose tissue produces its own growth factor (cortisol). These findings combined suggest that obesity and, in particular central obesity, once it has developed, may be a self-sustaining condition.

Furthermore, this Thesis may provide some explanation for the great difficulties faced by those patients trying to lose weight and despite a calorie-controlled diet are unable to do so. It may be that a subset of obese patients are faced with a hyperactive HPAA and an unfavourable cortisol-cortisone shuttle, whilst fighting “the battle of the bulge”.

8-2 (b) Sex steroid metabolism in obesity and PCOS

In chapter 5, the contribution of peripheral tissues to androstenedione clearance, testosterone production and oestradiol production was quantified. The figures in this thesis do not concur with previous metabolic studies that suggested 50% of testosterone is derived from peripheral conversion of androstenedione in premenopausal women.

In chapter 5, it was shown that the absolute contribution of the periphery to testosterone production is the same in simple obesity and PCOS (0.04mg/day). When this is viewed as a proportion of total daily testosterone production (which is 1-2 mg/day in PCOS and 0.4 mg/day in non-PCOS subjects) the contribution of the periphery lies closer to 10% in normal women and 4% in PCOS.

This thesis suggests that the ovaries are the main source of testosterone production in PCOS and that the contribution of peripheral testosterone production (from 17-BHSD conversion of androstenedione produced in adrenals or ovaries) has been overestimated in the past. Insulin infusion doubles the production rate of testosterone in adipose tissue in obese PCOS subjects, without increasing circulating testosterone levels. Thus, whilst the net contribution of the periphery to testosterone production is small in PCOS, this may be enhanced in hyperinsulinaemic PCOS subjects – that is anovulatory PCOS and, in particular obese anovulatory PCOS subjects.

8-3) Limitations of the Thesis

The over-riding problem encountered by most of the studies was a lack of a normal weight control group. The main reason for this was the invasive nature of the A-V difference studies which achieved some level of meaning for “patients” from reproductive endocrine and obesity clinics, but perhaps not for the general public. The consequences of a small control group have been discussed, particularly in Chapters 2 and 3.

Another logistical problem lay in recruiting for and conducting 3 broadly separate clinical studies (classifying studies III to V here as one study).

Thus, instead of one large study with 60 subjects, this thesis contains three smaller studies with 21-34 subjects each. In part, this was pragmatic as it was not certain that any individual study would succeed and it seemed sensible to keep the research options open. In retrospect, resources might have been better applied spread across one or two studies, such that the findings of individual studies would have greater statistical power. Nevertheless, it has been gratifying to see that each study has in its own way succeeded in contributing to the science of clinical endocrinology.

The CRH study (Study II) would have benefited from larger groups better characterised for distribution of body fat. DEXA scanning was not available locally and there were resource issues in obtaining DEXA scans on those individuals where DEXA was a viable option. It would have been particularly interesting to assess a larger group of postmenopausal women, half of whom were taking HRT, to assess the impact of oestrogen on the pituitary-adrenal response to CRH.

The sex steroid studies would have been strengthened by infusing radiolabelled androstenedione and measuring peripheral testosterone production by the A-V difference method. Accurate measures of systemic

sex steroid production/metabolic clearance rates in each subject would have been highly informative. The proportional contribution of TBF or LBM to sex steroid production could then have been calculated in each individual, rather than relying on the more generalised literature available on testosterone production rates in women with and without PCOS.

Regarding the hyperinsulinaemic euglycaemic clamp studies, a control group would have added to Study IV – do women without PCOS also exhibit enhanced type 3 17-BHSD activity in response to insulin?

Comparing our own study with the existing literature, one could perhaps have predicted that insulin should have been given in higher doses for a longer period to see an increase in androgen levels. However, it is questionable whether such supraphysiological studies have a great deal of clinical relevance.

8-4) Directions for future research

Thus far, corticosteroid and sex steroid metabolism have been examined as two almost distinct entities. There is literature suggesting that the cortisol-cortisone shuttle may be affected by the presence of PCOS.

Examining the direction of the cortisol-cortisone shuttle in SAAT in our PCOS cohort is a matter of ongoing research – preliminary data suggesting that the direction remains oxo-reductase in PCOS.

The data in this thesis on peripheral oestradiol production in premenopausal women suggests modest production of oestradiol by aromatase or 17-BHSD action in adipose tissue. It is a widely held belief that obese menopausal women are protected from osteoporosis - and have increased risk of breast and endometrial cancer – due to oestrogen production in peripheral tissues. However, it would be interesting to quantify this by our A-V difference technique to see if this is in fact the case. In premenopausal women, peripheral E2 production did not vary according to adiposity.

In terms of the PCOS studies, it would be interesting to see the effect of metformin therapy on peripheral testosterone production. The protocol for studies III and IV would have to be conducted before and after 8 weeks of metformin therapy. This would be an ambitious undertaking and not necessarily warranted given the relatively modest contribution of adipose tissue to testosterone production that we have demonstrated.

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Our Ref: **EC96/28**

16 September, 1996

Dr Katz
Dept of Medicine
G Block
Archway Wing
Whittington Hospital

Dear Dr Katz,

Re: EC96/28 - Study on the endocrine function of adipose tissue and muscle in health, simple obesity, hypertension and polycystic ovarian syndrome

I am writing to inform you that your submission has been considered by the Ethics Committee and it has been approved.

I should remind you of your undertaking to inform the Ethics Committee of any adverse or unforeseen circumstances arising out of the research, or of any changes during the research which might affect ethical approval, and to provide the Committee with re-prints of any publication resulting from the project.

All correspondence relating to your submission should be sent to the Secretary, Janice Wright, at the above address. If we do not hear from you further, you may be asked to provide a brief report in approximately six months after your stated completion date.

Yours sincerely,



John Farrell
Chairman - Ethics Committee

Central obesity, depression and the hypothalamo – pituitary – adrenal axis in men and postmenopausal women

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OBJECTIVE: We examined the relationship of adiposity to pituitary – adrenal responses to corticotrophin-releasing hormone (CRH) in men and postmenopausal women, controlling for the influence of depression.

DESIGN: Studies of CRH responses, cortisol metabolite levels and depression scores in relation to adiposity in men and postmenopausal women.

SUBJECTS: Thirteen men: age (median, interquartile range) 62 y (52–63), body mass index (BMI) 29.0 kg/m² (26.3–33.1), waist circumference (waist) 105 cm (97–111), waist:hip ratio (WHR) 1.03 (0.98–1.07), subscapular to triceps skinfold thickness ratio (STR) 2.0 (1.2–2.4), total body fat (TBF) 25.4 kg (19.8–28.8); and eight women: age 54 y (53–62), BMI 30 kg/m² (23–41), waist 86 cm (79–117), WHR 0.94 (0.87–1.10), STR 1.0 (0.85–1.07), TBF 35.0 kg (18.7–48.8).

MEASUREMENTS: A standard CRH test was conducted with additional basal samples taken for leptin and interleukin 6 (IL-6). Total urine cortisol metabolites (TCM) and the ratio of urinary cortisol:cortisone (Fm/Em) metabolites were measured. Depression scores were measured by the General Health Questionnaire (GHQ-30) and Hospital Anxiety and Depression Scale (HAD) questionnaire. All subjects completed an overnight dexamethasone suppression test.

RESULTS: The basal to peak percentage increments (%inc.) in adrenocorticotrophic hormone (ACTH) and cortisol in men correlated directly with STR (ACTH %inc. $r=0.70$, $P<0.01$; cortisol %inc. $r=0.55$, $P=0.05$); this relationship was independent of depression scores. In women, the ACTH area under incremental curve (AUC) correlated negatively with STR ($r=-0.81$, $P<0.05$). In men, but not in women, there was a significant correlation between GHQ-30 score and ACTH AUC ($r=0.62$, $P<0.05$) and cortisol AUC ($r=0.72$, $P<0.01$). Depression scores were consistently and directly related to indices of obesity and central obesity. There were no significant relationships in either sex between urinary TCM or Fm/Em ratio and BMI, waist, WHR, TBF, STR or CRH responses. The urinary Fm/Em ratio was higher in men than in women (median 0.74 vs 0.66, $P<0.05$). In men, but not in women, GHQ-30 scores correlated positively with urinary TCM ($r=0.57$, $P=0.05$) and HAD-depression scores were inversely related to the urine Fm/Em ratio ($r=-0.65$, $P<0.05$). All subjects suppressed normally with dexamethasone.

CONCLUSIONS: Cortisol metabolite levels were increased in depression in men, but were not related to adiposity in either sex. We demonstrate that central obesity in men, but not postmenopausal women, is associated with an enhanced pituitary – adrenal response to CRH and that this relationship is independent of depression score.

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Keywords: CRH; corticotrophin; central obesity; obesity; leptin; depression

Introduction

The pituitary and adrenal response to provocation tests in obese premenopausal women has been extensively studied,^{1–5} but there is a lack of data on men and post-menopausal women. Centrally obese premenopausal women have been shown to have an enhanced ACTH and cortisol response to CRH² and some authors have extrapolated from these data to assert that a hyperactive hypothalamo – pituitary – adrenal (HPA) axis results in increased cortisol production rates, central obesity and insulin resistance.⁶

We have set out to clarify whether adiposity or depression influence CRH responses and cortisol metabolite levels in men and postmenopausal women. There is a growing body of *in-vitro* evidence to suggest that both leptin^{7–9} and IL-6^{10–12} have a regulatory role in HPA axis activity. We examine the relationship of pituitary and adrenal CRH responses to basal leptin and IL-6 levels.

Methods

Thirteen men and eight postmenopausal women were recruited from a primary health care practice and an obesity clinic. Their characteristics are outlined in Table 1. No subjects were being treated with topical, inhaled or oral corticosteroid preparations or had received such treatment in the year prior to the

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Table 1 Subject characteristics

	Men (n=13) Median (interquartile range)	Women (n=8) Median (interquartile range)
Age (y)	62 (52–63)	54 (53–62)
BMI (kg/m ²)	29.0 (26.3–33.1)	30.0 (23.0–41.0)
Waist-circumference (cm)	105 (97–111)	86 (79–117)
Waist:hip ratio (WHR)	1.03 (0.98–1.07)	0.94 (0.87–1.10)
Subscapular:triceps skinfold thickness ratio (STR)	2.0 (1.2–2.4)	1.0 (0.85–1.07)
Total body fat (kg)	25.4 (19.8–28.8)	35.0 (18.7–48.8)
24-h urinary TCM (µg/mmol creatinine)	440 (290–680)	540 (350–950)
Urine Fm/Em ratio	0.74 ^c (0.66–0.83)	0.66 ^c (0.56–0.69)
GHQ-30 score ^a	2 (0–6)	1 (0–3)
HAD-depression score ^b	3 (1–4)	3 (1–12)

^a By GHQ-30 criteria (score ≥ 5) three men and one woman were depressed.

^b By HAD criteria (score ≥ 9) one man and two women were depressed.

^c Mann-Whitney: significantly higher in men ($P < 0.05$).

study. Four of the women were taking hormone replacement therapy. The local ethical committee approved the study and informed consent was obtained from all volunteers.

At the initial visit a full medical history was taken. Five different indices of adiposity were measured: body mass index (BMI), waist circumference (waist), waist:hip ratio (WHR), total body fat (TBF) and subscapular:triceps skinfold thickness ratio (STR). Weight was measured using a digital weighing machine (Seca, Marsden, UK) to the nearest 0.1 kg. Height was measured using a Harpenden stadiometer (Holtain Ltd., Crymych, UK). TBF was measured by bioimpedance with the subjects recumbent and electrodes (Bodystat 1500, Bodystat Ltd, Douglas, UK) attached to the dominant side. Skinfold thickness was measured with a pair of calipers (Holtain Ltd., Crymych, UK). Subscapular skinfold thickness was measured immediately below the tip of the scapula with the skinfold at an angle of 45° to the vertical. The triceps skinfold was measured in a vertical plane at the mid-point between the acromion process of the scapula and the tip of the olecranon process. Waist and hip circumference were both measured in a transverse plane, at the level of the umbilicus and greater trochanters respectively.

All subjects completed a 24-h urine collection for urine cortisol metabolites immediately prior to the corticotrophin-releasing hormone (CRH) test. Subjects fasted overnight and a cannula was inserted in the antecubital fossa at 8 am. They rested supine for at least 30 minutes before the first blood sample was taken. They remained supine for the duration of the test. At 9 am CRH (100 µg human CRH, Ferring

Pharmaceuticals Ltd.) was injected intravenously. Serum and ethylenediaminetetra-acetic acid (EDTA) samples were collected at -15, 0, 15, 30, 45, 60, 90 and 120 minutes after CRH administration. EDTA samples for ACTH, leptin and IL-6 analysis were stored on ice, centrifuged (4°C) and frozen (-20°C) within an hour. Serum samples were centrifuged at room temperature and stored (-20°C).

Overnight dexamethasone suppression tests

At least two weeks after the CRH test, all subjects took 2 mg of dexamethasone at 11 pm. The following morning at 8 am, a serum sample was taken from each subject for cortisol assay.

Depression scores

After completing the CRH test all subjects completed two questionnaires to assess depression scores. The GHQ-30 and HAD questionnaires are validated methods of rating depression scores.¹³ The range of possible scores in the GHQ is from 0–30, scores > 5 representing significant depression. The HAD questionnaire measures depression (possible range 0–21), scores of greater than 9/21 representing significant depression.

Assays

Serum cortisol was measured with the Bayer Immuno-1 automated analyser; inter-assay imprecision for cortisol at 140, 417 and 948 nmol/l was 5.5, 3.6 and 3.5% respectively. Plasma ACTH was measured using the Nicholls immunoradiometric kit; inter-assay imprecision for ACTH at 50 and 400 ng/l was 8.8% and 7.3% respectively. Plasma leptin was measured with an in-house radioimmunoassay (utilising a polyclonal antibody raised in a rabbit host) with an inter-assay imprecision of 5.0%. Plasma IL-6 was measured using an ELISA kit (R&D Systems, Oxon, UK) with an inter-assay imprecision of 6.7%.

Urine cortisol metabolites

In all subjects total urine cortisol metabolites and creatinine were measured in an aliquot of their 24-h collection. Urine steroid profiles were analysed by high resolution gas chromatography.¹⁴ Urinary total cortisol metabolites (TCM) are defined as the sum of urinary cortisol (11-hydroxy) metabolites (Fm) and urinary cortisone (11-oxo) metabolites (Em). All urine cortisol metabolite levels are expressed as µg/mmol creatinine to correct for the influence of lean body mass.

Statistical analysis

ACTH and cortisol responses to CRH were assessed by two methods: the percentage increment (%inc.) from basal to peak value; and by the area under the incremental curve (AUC). The relationships between

adiposity, depression scores, urine cortisol metabolites and cortisol/ACTH response to CRH were assessed by Spearman's rank correlation coefficients. After log transformation of significantly skewed variables, linear regression was used to account for confounding variables. The Mann–Whitney test was applied to assess gender differences in adiposity, depression, urine cortisol metabolites and CRH response.

Results

Adiposity and CRH responses

The basal serum cortisol concentration was 347 (277–496) nmol/l and did not vary according to gender, BMI or body composition. The ACTH and cortisol %inc. and AUC results for men and women are summarised in Table 2. In men, there were strong positive correlations between STR and ACTH/cortisol responses to CRH (Table 3). In postmenopausal women, there was an inverse correlation between STR and ACTH AUC ($r = -0.81$, $P < 0.05$). There were non-significant inverse correlations between other indices of adiposity and CRH responses in women. The women on HRT ($n = 4$) closely matched those not on HRT ($n = 4$) with regard to age, BMI and WHR. We found no significant difference in CRH response between women on HRT and those not on HRT.

Table 2 ACTH and cortisol responses to injection of CRH (100 µg)

	Men ($n = 13$) Median (interquartile range)	Women ($n = 8$) Median (interquartile range)
ACTH %inc.	122 (59–278)	259 (125–435)
Cortisol %inc.	65 (23–102)	69 (32–123)
ACTH AUC ng/l.min	1605 (820–4445)	3180 (915–3690)
Cortisol AUC nmol/l.min	11 280 (1610–19 340)	19 420 (8570–28 300)

Table 3 Correlations between CRH responses, adiposity and depression scores in men

	Cortisol %inc.	Cortisol AUC	ACTH %inc.	ACTH AUC
STR	+0.55 ^a	+0.54	+0.70 ^c	+0.64 ^b
Waist	+0.32	+0.37	+0.42	+0.45
WHR	+0.36	+0.43	+0.38	+0.52
TBF	+0.21	+0.27	+0.26	+0.32
BMI	+0.23	+0.30	+0.33	+0.38
GHQ-30	+0.58 ^b	+0.72 ^c	+0.58 ^b	+0.62 ^b
HAD-depression	+0.20	+0.23	+0.27	+0.43

Data are r values: ^a $P = 0.05$; ^b $P < 0.05$; ^c $P < 0.01$.

Depression scores in relation to CRH responses and adiposity

The depression scores in men and women are summarised in Table 1. The correlations of depression scores with CRH responses in men are shown in Table 3. In men, the GHQ-30 score was positively associated with all cortisol and ACTH responses to CRH. Multiple regression analysis confirmed that STR and depression scores were related to CRH responses independently of one another. In women, there was no association between depression scores and ACTH/cortisol responses to CRH.

The relationship of adiposity to depression scores in men is summarised in Table 4. Both GHQ-30 and HAD-depression scores correlated positively with BMI, waist and WHR. In women, HAD-depression scores correlated with WHR ($r = 0.70$, $P < 0.05$), but this was the only significant relationship between depression scores and adiposity.

Urine cortisol metabolites vs obesity and depression

Urine cortisol metabolite results, depression scores and anthropometric data are summarised in Table 1. Although there was no sex difference in TCM, the Fm/Em ratio was significantly higher in men than in women. There was no relationship between urinary TCM and ACTH/cortisol responses to CRH in either sex. Depression scores in men, but not in women, correlated positively with urinary TCM and were inversely related to the urinary Fm/Em ratio (see Table 4). Both TCM and the Fm/Em ratio were unrelated to adiposity, as measured by BMI, WHR, TBF and STR in both sexes. This lack of a relationship persisted even when depressed patients (by either HAD or GHQ-30 criteria) were excluded from the analysis.

Dexamethasone suppression tests

In all subjects, plasma cortisol was suppressed to < 50 nmol/l at 8 am, the morning after a 2 mg dose of dexamethasone had been administered.

Leptin

In men, basal leptin 8.5 (4.6–10.4) ng/ml correlated with ACTH AUC ($r = 0.69$, $P < 0.05$) and BMI ($r = 0.73$, $P < 0.01$). However, multivariate analysis

Table 4 Correlations between depression scores, adiposity and urine cortisol metabolite levels in men

	GHQ-30	HAD- depression
STR	+0.29	+0.26
Waist	+0.62 ^b	+0.60 ^b
WHR	+0.60 ^b	+0.68 ^b
BMI	+0.69 ^b	+0.60 ^b
TBF	+0.56	+0.26
TCM	+0.57 ^a	+0.51
Fm/Em	-0.24	-0.65 ^b

Data are r values: ^a $P = 0.05$; ^b $P < 0.05$.

showed no effect of leptin independent of STR. There was a trend towards a correlation between leptin and cortisol incremental measures, but this failed to reach significance. In women, basal leptin 16.1 (5.9–31.0) ng/ml correlated with BMI ($r=0.79$, $P<0.05$) but not with ACTH and cortisol responses to CRH.

Interleukin-6

In men, basal IL-6 concentrations of 3.8 (2.8–4.7) pg/ml did not relate to BMI or ACTH/cortisol responses to CRH. In women, basal IL-6 values of 4.2 (1.6–10.3) pg/ml did correlate with BMI ($r=0.83$, $P=0.01$) but not with ACTH/cortisol incremental measures.

Discussion

We have demonstrated a positive correlation between central obesity, as measured by STR, and ACTH/cortisol responses to CRH in men. Our data are consistent with an inverse relationship between STR and the ACTH response to CRH in postmenopausal women. Cushing's disease was excluded by normal overnight dexamethasone suppression in all subjects. Studies in premenopausal women have demonstrated that the distribution of body fat rather than BMI predicts the pituitary and adrenal response to stimulation tests.^{2,15} Centrally obese premenopausal women demonstrate an enhanced ACTH response to CRH² and an enhanced cortisol response to CRH, synthetic corticotropin and psychological stress.¹⁵

Skinfold thickness reflects subcutaneous rather than visceral fat depots, but can be measured in subjects with a wide range of body habitus and this represents an advantage over other methodologies. Dual energy X-ray absorptiometry (DEXA) scanning of regional fat depots was not undertaken in this study as DEXA is less accurate in the very obese and morbidly obese subjects do not fit on the table. There are similar methodological problems with computed tomographs (CT) and magnetic resonance imaging (MRI) (except where large diameter machines are available).

In men, there were consistent positive associations between pituitary/adrenal responses to CRH and depression scores on the GHQ-30 questionnaire, independent of STR. This conflicts with previous data, which has shown a blunted ACTH response and a normal cortisol response in depression.^{16,17} There were no significant associations between depression and CRH responses in the female group. Depression has been shown to be associated with obesity and particularly central obesity¹⁸ and this is confirmed by our finding of positive correlations between depression scores and BMI, waist and WHR in men. We hypothesise that depression accounts for some of the inconsistencies in the literature regarding CRH responses in obesity. We have found a direct

association between central obesity and CRH responses in men, independent of depression scores.

There are limited published data on CRH responses in centrally obese men, but our data are supported by preliminary data from another group.¹⁹ Our data, in the context of previous studies² on premenopausal women, are consistent with its suggestion that the menopause may result in a diminished response to CRH. However, the lack of a relationship of central obesity to CRH responses in women may reflect the narrow range of STR in the female group, as compared to the male group in our study. There is no data as yet comparing CRH responses in large matched groups of menopausal women on and off hormone replacement therapy (HRT), but our preliminary data show no significant difference between these groups.

Obesity *per se*, in contrast to body fat distribution, was unrelated to CRH responses in either sex. Although only 2/13 men and 4/8 women had a BMI <25 kg/m², both sexes had a wide range of BMI and this should be sufficient to establish a correlation. Expanded normal weight control groups would have strengthened the analysis. However, our study is in agreement with most of the previous research in this area, which has shown normal ACTH^{1,3} and cortisol^{3,5} responses to CRH in obesity *per se*. Only one study has suggested a blunted cortisol¹ response to CRH in obese subjects compared to normal weight controls. Another group reported an increased ACTH, but normal cortisol response to CRH in obesity,⁵ but higher doses of CRH were given to the most obese patients, which may explain this finding.

Total urine cortisol metabolite levels (TCM) can be utilised to reflect (or indicate) cortisol production rates. Correction for creatinine controls for the influence of lean body mass.²⁰ There is some evidence to suggest that centrally obese subjects, when compared to subjects with generalised obesity, demonstrate an exaggerated increase in cortisol secretion in response to transient psychological stress.^{15,21} Furthermore, it has been suggested that a hyperactive HPA axis in central obesity leads to increased cortisol production rates.⁶ However, we found no relationship of CRH responses or indices of adiposity with TCM and, in as far as urine cortisol metabolite levels reflect cortisol production rates, we were unable to confirm this hypothesis in our study. We did not, however, formally assess cortisol production rates.

Although increased TCM have been reported in obesity,²² we concur with two recent studies^{23,24} which demonstrated no significant relationship between BMI and TCM in men and women. Increased urinary free cortisol (UFC) excretion has been demonstrated in centrally obese premenopausal women.^{2,15} However, UFC accounts for less than 1% of TCM and does not accurately reflect cortisol production rates.²³

We analysed and rejected the hypothesis that depression obscured a relationship between adiposity and urine cortisol metabolite profiles. Whilst we found

depression was associated directly with TCM in men only, previous studies have found a similar relationship in both men and women.^{14,25} Our finding of an inverse relationship between depression and urine Fm/Em in men is at variance with one previous case-controlled study, which demonstrated no influence of depression on the Fm/Em ratio in men and an increased Fm/Em ratio in depression in women.^{14,25} Direct comparisons between our study and Raven's study^{14,25} are difficult. The Raven study was case-controlled, but there was no correction for lean body mass and they used a different depression scale.

The urine Fm/Em ratio was higher in men than women in keeping with two previous studies.^{14,26} In our study, Fm/Em did not relate to CRH responses or adiposity in either sex. There has been debate in the literature as to the direction of the cortisol–cortisone shuttle in central obesity. Whilst Andrew *et al*²³ found a positive correlation of the Fm/Em ratio with both hip circumference and waist circumference in men, they do not present their data on waist-hip ratio and have no other index of central obesity. Meanwhile, Stewart found that the Fm/Em ratio is inversely related to android (central) fat distribution,²⁴ which is contrary to his group's *in-vitro* data on visceral adipose tissue.²⁷ Finally, Weaver *et al*²⁶ found the Fm/Em ratio to be inversely related to body weight, android fat and gynoid fat, but stepwise regression confirmed gynoid fat was the most important factor determining the Fm/Em ratio.²⁶ Our data, in conjunction with these conflicting papers,^{23,24,26} suggests that the relationship of body fat distribution to the urine Fm/Em ratio is, as yet, unclear.

Regarding the role of leptin and IL-6 in regulating the HPA axis, multivariate analysis demonstrated that the positive association between leptin and ACTH AUC was not independent of STR. This is contrary to a regulatory role for leptin at hypothalamic or adrenal levels suggested by laboratory and animal studies.^{7–9} We did not find a simple relationship between basal IL-6 levels and CRH responses. Previous work has suggested a close relationship between IL-6 and ACTH circadian rhythm²⁸ and IL-6 administration has been shown to activate the HPA axis both in animals^{11,12} and in humans.¹⁰

Excessive alcohol consumption has been shown to blunt the ACTH and cortisol responses to CRH²⁹ and impair dexamethasone suppression of plasma cortisol.³⁰ We assessed alcohol intake in all subjects as part of the medical history. There were two men who drank in excess of 20 units per week, but they did not demonstrate blunted responses to CRH and suppressed normally with dexamethasone. Excluding them from the analysis did not alter the strength of the positive relationships. We do not believe alcohol intake was a confounding factor in our study.

We have considered whether HRT use acts as a confounding variable in the assessment of CRH responses in postmenopausal women. There is *in-vitro* evidence^{31,32} that oestrogen increases expression

of the CRH gene. However, a previous clinical study³³ found no difference in the pituitary and adrenal responses to CRH between women on HRT and those not on HRT. Our study supports the view that HRT does not influence the ACTH and cortisol responses to CRH, although the numbers of women taking HRT (four in our study and four in the previous study cited) were small. The role of oestrogen in modulating the CRH response in postmenopausal women needs to be addressed by further research.

Conclusions

Previous studies have shown an increased pituitary–adrenal response to CRH in centrally obese premenopausal women. We have demonstrated that this effect extends to centrally obese men, but not to postmenopausal women, and is independent of depression score, basal leptin and IL-6 levels.

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An *in vivo* study of the cortisol–cortisone shuttle in subcutaneous abdominal adipose tissue

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Summary

OBJECTIVE Previous *in vitro* studies have demonstrated significant 11-beta hydroxysteroid dehydrogenase (11 β -HSD) oxo-reductase activity in visceral, but not subcutaneous adipose stromal cells. We have conducted an *in vivo* study of the cortisol–cortisone shuttle in subcutaneous abdominal adipose tissue.

DESIGN We measured arteriovenous (A-V) differences in serum cortisol and cortisone across subcutaneous abdominal adipose tissue and forearm muscle in a heterogeneous group of subjects.

PATIENTS We studied 34 subjects (male:female = 12:22), age median (interquartile range) 45 (19–65) years, body mass index 32.7 (20.4–77.1) kg m⁻², total body fat 34.4 (5.6–119.1) kg.

MEASUREMENTS Serum cortisol and cortisone were measured in serum samples from a radial artery, superficial epigastric vein and deep forearm vein. Abdominal adipose and forearm blood flow rates were measured by ¹³³Xenon washout and plethysmography, respectively.

RESULTS For cortisone, there was significant ($P < 0.001$) clearance by adipose tissue, with an A-V difference of 4 (1–7) nmol/l. For cortisol there was a trend for arterial concentrations (203 (142–292) nmol/l) to be lower than venous (225 (152–263) nmol/l), but this was not significant. The adipose tissue cortisone clearance rate correlated with total body fat ($r = 0.35$, $P = 0.05$).

CONCLUSIONS We have demonstrated 11 β -HSD oxo-reductase activity in subcutaneous abdominal adipose tissue, which may be increased in obesity.

Obesity is associated with a variety of endocrine abnormalities (Kopelman, 1998). The nature of peripheral corticosteroid metabolism in obesity is controversial. Traditionally, it has been assumed that cortisol is converted to its inactive metabolite cortisone in adipose tissue. The *in vitro* studies on which this assumption was based (Weidenfeld *et al.*, 1982) have been challenged by recent studies demonstrating oxo-reduction of cortisone to cortisol in visceral adipose stromal cell cultures (Bujalska *et al.*, 1997).

The cortisol–cortisone shuttle is controlled by the two isoforms of 11- β hydroxysteroid dehydrogenase, 11 β -HSD1 and 11 β -HSD2 (Whitworth *et al.*, 1989; Stewart, 1996). Although both isoforms may dehydrogenize cortisol at the 11 β -position to form cortisone, the type 2 isoform has the higher affinity for this reaction. The type 1 isoform is a low affinity enzyme, which also promotes the reverse reaction—oxo-reduction of cortisone to cortisol.

Early studies on subcutaneous abdominal fat homogenates (Weidenfeld *et al.*, 1982) suggested dehydrogenation of cortisol to cortisone occurred in adipose tissue. However, net oxo-reduction of cortisone to cortisol has been demonstrated in breast adipose tissue (Yang *et al.*, 1997). More recent studies detected 11 β -HSD1, but not 11 β -HSD2, mRNA in visceral and subcutaneous adipose tissue (Bujalska *et al.*, 1997). Furthermore, visceral adipose stromal cell cultures demonstrated predominant oxo-reductase activity (Bujalska *et al.*, 1997).

There have been few studies of the cortisol–cortisone shuttle in adipose tissue *in vivo*. A previous study of cortisol metabolism utilizing A-V difference techniques demonstrated no significant gradient of plasma cortisol across deep (largely muscle) and superficial (skin and adipose tissue) forearm compartments (Asmal *et al.*, 1974). However, superficial forearm veins drain only limited amounts of adipose tissue. We therefore measured the A-V differences in cortisol and cortisone across superficial abdominal adipose tissue, as well as the deep compartment of the forearm, in a heterogeneous group of men and women. This A-V difference technique has been used in the assessment of sex steroid metabolism (Boulton *et al.*, 1992) and lipolysis (Samra *et al.*, 1996) in adipose tissue.

Subjects and methods

Subjects

A total of 34 subjects (male:female = 12:22) had superficial

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abdominal adipose A-V difference studies. We excluded volunteers taking topical, inhaled or oral corticosteroid medication. The volunteers' characteristics are outlined in Table 1. In order to examine the metabolism of forearm muscle, 10 of these subjects also had forearm A-V studies performed using a retrograde forearm cannula. Nineteen other subjects had only A-V forearm studies without abdominal venous sampling. The characteristics of this subgroup of 19 did not differ significantly from those shown in Table 1. The Whittington Hospital Ethics Committee approved the protocol and informed consent was obtained.

Study design

All subjects were studied in the morning after an overnight fast. Only water was permitted during the study to avoid any influence of caloric or nicotine intake on peripheral corticosteroid metabolism. Three different indices of adiposity were measured: body mass index (BMI), and percentage body fat (bioimpedance: Bodystat 1500, Bodystat Ltd, Douglas, UK). Blood pressure was recorded with the subjects resting supine, using cuffs appropriate to the individual.

Arterial and abdominal venous sampling

Lignocaine 1% was used for local anaesthesia prior to cannulation. First, a radial artery cannula (Insite-20g, 30 mm) was inserted. Then, in 34 subjects, a polyurethane catheter (Ohmeda Hydrocath 20G 20 cm) was passed into the superficial inferior epigastric vein, draining the subcutaneous adipose tissue of the anterior abdominal wall (Frays *et al.*, 1989). The blood collected from this abdominal vein represents the drainage from subcutaneous abdominal adipose tissue with a minor contribution from skin and none from muscle (Frays *et al.*, 1989). Pairs of arterial and adipose venous serum samples were taken simultaneously during the late morning (1100–1200 h) for measurement of serum cortisol and cortisone.

Forearm venous sampling

In 29 subjects, a cannula (Abbocath 18G 51 mm) was inserted retrogradely into the deep branch of the median cubital vein in the antecubital fossa. Blood samples from this vein represent drainage from forearm muscle with a minor contribution from the skin and adipose tissue of the forearm via anastomoses with the deep venous system (Butler & Home, 1987). A wrist cuff was inflated above systolic pressure prior to sampling for 2 min to isolate the forearm compartment from the hand. Paired arterial and forearm venous blood samples were taken simultaneously during the late morning (1100–1200 h) for measurement of serum cortisol and cortisone. In subjects with

Table 1 Characteristics of subjects: Subcutaneous abdominal adipose tissue arteriovenous difference studies.

Characteristic	Median	Range: min–max
Age (years)	45	19–65
Body mass index (kg/m ²)	32.7	20.4–77.1
Waist:hip ratio		
Women	0.93	0.75–1.1
Men	1.01	0.85–1.2
Percentage body fat (%)	39.2	7.8–61.1
Total body fat (kg)	34.4	5.6–119.1
Blood pressure (mmHg)		
Systolic	139	94–200
Diastolic	85	60–140

both deep venous and abdominal venous samples these were taken simultaneously.

Blood flow measurement

Blood flow in the subcutaneous abdominal wall was measured by ¹³³Xe washout (Nielsen, 1972). At least 45 min after the injection of 5MBq of liquid ¹³³Xe (Malinkrodt, UK) into the subcutaneous fat of the anterior abdominal wall, a CsI gamma radiation detector (Mediscint, Oakfield Instruments Ltd, Witney, UK) was used to detect ¹³³Xe radioactivity at the injection site. The rate of decline of radioactivity is proportional to the adipose tissue blood flow (Nielsen, 1972). For all subjects a xenon adipose/blood partition coefficient of 10 was used (Coppack *et al.*, 1992). Some authors have suggested that the partition coefficient is greater in obese subjects (Bulow *et al.*, 1987). Such an assumption would increase the local production rates in the adipose tissue of obese relative to lean subjects. However, not all workers have found such changes in partition coefficient (Jansson & Lonnroth, 1995), so we have adopted the more conservative assumption that the partition coefficient is the same in lean and obese.

Blood flow in the forearm from which venous samples were taken was determined by plethysmography (Butler & Home, 1987) using a mercury strain gauge (Hokansen system, PMS Instruments, Maidenhead, UK). Plasma flows were calculated as the blood flow multiplied by (1-haematocrit) for each subject. The haematocrit was measured in arterial, forearm and epigastric venous samples.

Assays

Serum was analysed by radioimmunoassay for cortisol (Moore *et al.*, 1985) and cortisone (Wood *et al.*, 1996). Serum samples were extracted with chloroform prior to cortisone

radioimmunoassay (Wood *et al.*, 1996). The cross-reactivity of cortisone antisera with cortisol was less than 0.1%. All samples from an individual subject were assayed within the same batch. Intra-assay CVs for cortisol were 8.2% and 5.6% at cortisol concentrations of 156 and 410 nmol/l, respectively. Intra-assay CVs for cortisone were 8% and 6% at cortisone concentrations of 50 and 80 nmol/l, respectively.

Calculations and statistics

Estimated whole-body corticosteroid interconversion by adipose tissue. The product of local plasma flow (ml/100 g tissue/min) and A-V difference in cortisone (nmol/l) yields the local clearance rate (pmol/100 g adipose tissue/min) according to the Fick principle (Butler & Home, 1987). Cortisone net clearance rates for the whole body fat mass (WBFM) (nmol/day) were estimated by multiplying local clearance rate by WBFM as measured by bioimpedance. Cortisol net production rates were assumed to be the same on a molar basis as cortisone clearance rates (conversion factor for cortisol nmol to mg = 3.6×10^{-4}). These calculations assume homogeneity of 11 β -HSD1 activity in all adipose tissue depots. It is likely that such activity is heterogeneous (Bujalska *et al.*, 1997) and these figures serve to illustrate the potential WBFM cortisol production, but should be interpreted with caution.

Statistical analysis

Non-parametric tests were used because of skewness in the data. Data are reported as median (interquartile range). Wilcoxon's paired test was used to assess whether the A-V difference in cortisol and cortisone concentrations significantly differed from zero. Spearman's rank correlation coefficients were applied to assess the relationships between the A-V difference/clearance rate of cortisol/cortisone to age, sex, blood pressure and indices of adiposity.

Results

A-V differences of cortisone and cortisol

In the 34 subjects who underwent superficial epigastric vein and radial artery cannulation, the arterial concentration of cortisone was 40 (32–58) nmol/l compared to a venous concentration of 39 (28–50) nmol/l. There was a significant ($P < 0.001$) cortisone A-V difference of 4 (1–7) nmol/l reflecting net cortisone clearance in abdominal adipose tissue (Fig. 1). There was a trend for abdominal adipose venous cortisol concentrations [225 (152–263) nmol/l] to be higher than arterial [203 (142–292) nmol/l] but the A-V difference was not statistically significant.

In the 29 subjects who had forearm studies, there were no significant A-V differences for cortisol [arterial 255 (180–293) nmol/l, deep venous 270 (176–315) nmol/l] or cortisone [arterial 54 (48–64) nmol/l, deep venous 53 (48–64) nmol/l]. In the subset of 10 subjects with samples available from artery, deep vein and abdominal vein the median A-V difference across the superficial abdominal adipose tissue bed was 4.5 (0.8–7.0) nmol/l compared with -1.0 (-6.5 – 7.25) nmol/l across the deep forearm compartment, but neither reached statistical significance. The haematocrits in arterial, forearm and epigastric venous blood for each subject were equal, excluding any dilutional effects of sampling.

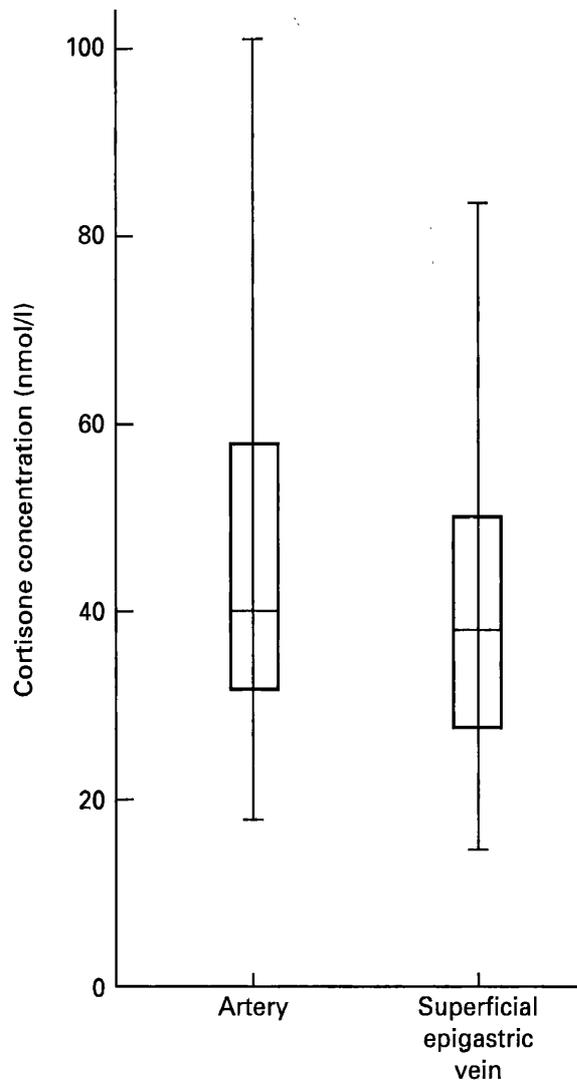


Fig. 1 Arterial and superficial epigastric venous concentrations of cortisone (nmol/l) in the 34 subjects who underwent arterial and abdominal vein cannulation. Minimum, maximum, 25th, 50th and 75th centiles are shown for arterial and venous samples.

Cortisol:cortisone ratios

The cortisol:cortisone ratios demonstrated a significantly higher ratio ($P < 0.05$) in adipose venous blood [5.2 (4.1–7.1)] when compared to arterial [4.8 (3.7–6.8)] blood. There was no significant difference between the cortisol:cortisone ratio in arterial and forearm venous blood [4.7 (3.5–5.5)]. In the subset of 10 subjects with samples available from both artery, deep vein and superficial epigastric vein the cortisol:cortisone ratios did not differ significantly between the different sites.

Net clearance/production rates of cortisone and cortisol

The local subcutaneous adipose tissue cortisone clearance rate was 3.62 (0.36–6.21) pmol/100 g/min. The local adipose tissue cortisone clearance rate correlated significantly with WBFM ($r = 0.35$, $P = 0.05$), but not significantly with percentage body fat ($r = 0.32$) or with BMI ($r = 0.20$). Similarly, there were no significant relationships between local cortisone clearance and age, sex or blood pressure. WBFM cortisone clearance was 2000 (150–5400) nmol/day. There was a significant correlation between WBFM cortisone clearance and BMI ($r = 0.47$, $P < 0.01$). Assuming cortisone underwent oxo-reduction in adipose tissue, the median WBFM cortisol production was approximately 0.7 mg/day.

Discussion

We have demonstrated a significant net A-V difference of cortisone across the subcutaneous abdominal adipose tissue bed. This suggests that the 11 β -HSD1 oxo-reductase enzyme is active in subcutaneous abdominal adipose tissue. Although there is no detectable A-V difference in cortisol, this is likely to be due to limitations of assay precision. Measuring a 4 nmol/l A-V difference with a median arterial cortisol level of 200 nmol/l would require an assay precise enough to detect a 2% difference in cortisol concentrations between arterial and venous samples. We found no significant A-V difference of cortisone or cortisol across the deep forearm tissue. Although other studies (Van Uum *et al.*, 1997) suggest there is 11 β -HSD activity in forearm skin, the deep forearm cannulas in our subjects sampled blood draining muscle rather than skin.

Previous studies indicate that the oxo-reductase activity of 11 β -HSD (Bujalska *et al.*, 1997) is greater in visceral than in subcutaneous adipose tissue. However, when cortisol was added to the culture medium in these studies there was a significant increase in both subcutaneous and visceral 11 β -HSD oxo-reductase activity. Our estimate of the WBFM cortisol output (median 0.7 mg) assumes that all adipose tissue has the same oxo-reductase activity as subcutaneous adipose tissue. However, *in vitro* studies (Bujalska *et al.*, 1997) show

that oxo-reductase activity in visceral fat is greater than in subcutaneous abdominal adipose tissue. Thus it is likely that our WBFM cortisol output is an underestimate. *In vivo* studies of the visceral adipose tissue bed would be advantageous, but would require portal venous sampling.

Cortisol production in adipose tissue may have paracrine implications. The role of cortisol in enhancing lipolysis (Divertie *et al.*, 1991; Samra *et al.*, 1996) is well described, but an excess of cortisol more commonly leads to central fat accumulation (Bjorntorp, 1991; Hauner & Entenmann, 1991) as in Cushing's syndrome. Central fat accumulation is partly mediated by lipoprotein lipase action, which is increased by insulin and cortisol (Bjorntorp, 1991; Ottosson *et al.*, 1994; Ottosson *et al.*, 1995). Adipose stromal cells cultured from superficial abdominal adipose tissue show a greater response to cortisol than do femoral adipose stromal cells (Hauner & Entenmann, 1991). Thus, cortisol production in adipose tissue could serve a paracrine role (Bujalska *et al.*, 1997), favouring central fat accumulation and contribute to the adverse metabolic consequences of central obesity. It has been postulated that cortisol-mediated vascular reactivity increases peripheral resistance and contributes to hypertension (Walker *et al.*, 1992), but such reactivity has not been studied in the adipose tissue bed where we have identified local 11 β -HSD activity.

We have assumed that xenon partition coefficients are the same in lean and obese subjects. This assumption will reduce the local cortisone clearance rates of obese subjects relative to those of the lean subjects. Even with this assumption, we have shown that local cortisone clearance rates (pmol/100 g tissue/min) are increased in those subjects with a higher WBFM, suggesting upregulation of 11 β -HSD1 oxo-reductase activity in obesity. This is compatible with a cortisol-cortisone shuttle promoting obesity through paracrine mechanisms. However, the equivalent relationships between cortisone clearance rate and BMI or percentage body fat were less strong.

It has previously been shown that basal and 24 h mean cortisol plasma levels are normal in obesity (Kopelman, 1998). However, cortisol production rates are increased in obesity as reflected in raised 24-h urine cortisol metabolites. A recent study showed the urinary ratio of 11-oxo/11-hydroxy cortisol metabolites (Em/Fm) correlated positively with total body fat (Weaver *et al.*, 1997), but there was no shift in urine free cortisol:cortisone ratios, suggesting that renal 11 β -HSD2 activity is unaltered in obesity.

The alteration seen in the urinary Em/Fm ratio in obesity reflects net increased whole body conversion of cortisol to cortisone. The explanation for this finding until recently had been that in obesity adipose tissue converts cortisol to cortisone, depleting plasma cortisol levels which are replenished by hypothalamo-pituitary-adrenal feedback mechanisms (Marin

et al., 1992). Our data and Stewart's data suggest that adipose tissue is a site of net 11 β -HSD oxo-reductase, rather than dehydrogenase, activity. The kidney is the main site of dehydrogenase activity in man (Whitworth *et al.*, 1989), but renal 11 β -HSD2 activity is not altered in obesity (Weaver *et al.*, 1997). There is also significant 11 β -HSD2 activity in the salivary glands (Edwards *et al.*, 1988; Wood *et al.*, 1997). It is possible that salivary gland 11 β -HSD2 activity increases with the excess food intake characteristic of obesity. Since it would appear that neither renal nor adipose 11 β -HSD2 activity accounts for the increased cortisol clearance of obesity, the role of salivary gland 11 β -HSD2 activity in this context needs further study.

In conclusion, our data support the conclusions of Stewart's group (Bujalska *et al.*, 1997) that adipose tissue is a site of 11 β -HSD oxo-reductase activity. However, we diverge on the question of oxo-reductase activity in subcutaneous abdominal adipose tissue, which is significant in our study. *In vivo* data comparing visceral and subcutaneous adipose tissue oxo-reductase activity is lacking. 11 β -HSD1 oxo-reductase is active in subcutaneous abdominal adipose tissue. This oxo-reductase activity may be upregulated in obesity.

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PERIPHERAL TESTOSTERONE PRODUCTION IN PREMENOPAUSAL WOMEN WITH AND WITHOUT POLYCYSTIC OVARY SYNDROME

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In this study, we apply the in-vivo method of arteriovenous (AV) difference sampling to measure the conversion of androstenedione (A) to testosterone (T) in subcutaneous adipose tissue (SAT) and forearm muscle (FM). Previous research has estimated the daily testosterone production rate (TPR) in women to be 0.4 mg/day, increasing to 1-2 mg/day in women with polycystic ovarian syndrome (PCOS). We studied 13 women with PCOS: median {interquartile range} body mass index (BMI) 33 {27 - 41} kg/m², total body fat (TBF) 34 {18 - 47} kg, lean body mass 29 {26 - 30} kg, Ferriman Gallway Score (FGS) 5 {2 - 7}; and 8 non-PCOS women: BMI 46 {31 - 48} kg/m², TBF 61 {27 - 73} kg, LBM 29 {23 - 31} kg, FGS 0 {0}. The hospital ethical committee approved the study. All subjects were premenopausal and studied in the follicular phase of the menstrual cycle. After an overnight fast, cannulae were inserted into a radial artery, a superficial epigastric vein (draining abdominal SAT) and a deep forearm vein (draining muscle) and simultaneous serum samples were taken from 3 sites for assay of A, T and oestradiol (E2). Blood flow was measured by ¹³³Xe washout in SAT and by mercury strain gauge plethysmography in the forearm. In the PCOS group, a hyperinsulinaemic euglycaemic clamp was then carried out with insulin infused at 20 $\mu\text{m}^2/\text{min}$ for 2 hours and at 40 $\mu\text{m}^2/\text{min}$ for a further 2 hours, with further sampling at the end of both stages of the clamp. Arteriovenous (AV) differences and SAT/FM clearance/ production rates of A, T and E2 were calculated. In-house radioimmunoassays were used with intra-assay c.v.s of 5 to 7%. In SAT, but not FM, there was net production of T and E2 with clearance of A, with no relationship of production rates to adiposity or presence of PCOS. Median basal WBFM TPR was 0.04 mg/day and E2PR was 5.9 mcg/day. In the PCOS group, insulin infusion increased the SAT production rate of T to 0.08 mg/day and resulted in significant FM clearance of A (0.19mg/day), and M production of T (0.02 mg/day).

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