Cardiovascular and sexual health effects of postmenopausal testosterone therapy

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Declaration of originality

I declare that the work presented in this thesis is my own, except where otherwise acknowledged.

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ABSTRACT

Introduction:

Testosterone is frequently used as part of postmenopausal hormone therapy (HT) but few preparations have been approved for use in women. The effects of androgens on the female cardiovascular (CV) system remain poorly understood and concerns exist over the long-term CV safety of testosterone therapy. This study aimed to investigate the CV effects of the transdermal testosterone patch (TTP) in postmenopausal women, on concomitant HT, using surrogate markers for CV disease.

Methods:

This open label pilot study investigated the effects of 12 weeks TTP on arterial stiffness and endothelial function in 21 postmenopausal women. Primary outcome measures were augmentation index (Alx), assessed by pulse wave analysis (PWA), reactive hyperaemic index (RHI) using peripheral arterial tonometry (PAT) and insulin resistance using the homeostasis model (HOMA-IR). Libido (brief profile of female sexual function (B-PFSF)), anthropometry, lipids and serum hormone levels were also assessed.

Results:

Testosterone was associated with significantly improved libido (increased B-PFSF score 5.05 points (p<0.0001)), increased total testosterone (1.3 nmol/L, p<0.0001) and free androgen index (2.0, p<0.001). Hip circumference significantly reduced (-0.74 cm, p<0.05) but there was no change in weight, body mass index, waist circumference or blood pressure.

Total cholesterol was unchanged, but there were small but significant decreases in high-density lipoprotein (HDL) cholesterol (-0.25 mmol/L, p<0.05) and lipoprotein (a) levels (-3.11mg/L, p<0.05). Fasting insulin, fasting glucose and insulin resistance were unchanged.
There was no change to AIx (1.07, 95% CI -3.85-1.72, p=0.43), or RHI (0.06, 95% CI 0.19-0.31, p=0.61) but there was a significant increase in salbutamol-mediated vasodilatation (p<0.05), assessed by PWA.

**Conclusion:**

These data suggest that short-term physiological testosterone does not adversely affect arterial stiffness and may improve markers of endothelial function. Testosterone use was associated with reductions in HDL cholesterol and lipoprotein (a), but whether this has significant long-term CV effects remains unclear.
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<td>CAD</td>
<td>Coronary artery disease</td>
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<td>CEE</td>
<td>Conjugated equine estrogen</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<td>CIMT</td>
<td>Carotid intima media thickness</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DHEA</td>
<td>Dihydroepiandrosterone</td>
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<td>DHEAS</td>
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<td>EFI</td>
<td>Endothelial Function Index</td>
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<td>FAI</td>
<td>Free Androgen Index</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GTN</td>
<td>Glyceril trinitrate</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>HOMA-IR</td>
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<td>HR</td>
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<td>HT</td>
<td>Hormone therapy</td>
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<td>HSDD</td>
<td>Hypoactive sexual desire disorder</td>
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<tr>
<td>IR</td>
<td>Insulin resistance</td>
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<td>IUS</td>
<td>Intra-uterine system</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LNG</td>
<td>Levonorgestrel</td>
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<tr>
<td>Lp(a)</td>
<td>Lipoprotein (a)</td>
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<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
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<tr>
<td>MT</td>
<td>Methyltestosterone</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>NETA</td>
<td>Norethisterone acetate</td>
<td></td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
<td></td>
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<tr>
<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
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<tr>
<td>POI</td>
<td>Premature Ovarian Insufficiency</td>
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<tr>
<td>PWA</td>
<td>Pulse wave analysis</td>
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<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
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<td>RHI</td>
<td>Reactive hyperaemia Index</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SHBG</td>
<td>Sex hormone binding globulin</td>
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<tr>
<td>SNRI</td>
<td>Serotonin noradrenalin reuptake inhibitor</td>
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<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
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<tr>
<td>TTP</td>
<td>Transdermal testosterone patch</td>
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<td>US FDA</td>
<td>United States Food and Drug Administration</td>
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<td>VTE</td>
<td>Venous thrombo-embolism</td>
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<td>WHI</td>
<td>Women’s Health Initiative</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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INTRODUCTION

Although commonly perceived as a male hormone, testosterone is the most prevalent active sex steroid in women (1) and it plays a crucial role in normal female physiology. Androgen levels decline gradually throughout life and deficiency can be associated with a multitude of symptoms. Improved awareness of the detrimental effects of androgen deficiency has led to increasing use of testosterone replacement within postmenopausal hormonal therapy regimens, particularly for women suffering from reduced sexual desire.

Despite significant evidence demonstrating the short-term benefits of postmenopausal testosterone therapy (2) there is a scarcity of products designed for use in women and few regulatory authorities have approved these preparations. Furthermore, although testosterone replacement has been used in women’s health for many years, few studies have examined the long-term safety of androgen therapy. Particular concerns have been expressed regarding the long-term effects of testosterone replacement on the cardiovascular system and breast (3).

Cardiovascular disease (CVD) is the leading cause of death in postmenopausal women (4) and optimising cardiovascular risk is a global health issue (5). The relationship between androgens and the cardiovascular system is complex, particularly in women. Although it was traditionally thought that androgens adversely affect cardiovascular risk, there are increasing data which challenge these views (6).

As with postmenopausal estrogen-progestogen hormone therapy (HT), there has been a move with female androgen replacement away from high dose parenteral preparations to physiological replacement via lower dose transdermal or subcutaneous preparations. There are many plausible reasons why this approach may avoid or reduce adverse effects, including the avoidance of first pass hepatic metabolism and through achieving lower, but more consistent, serum androgen levels. The benefits of transdermal compared to oral estrogen are well documented (7,8), however there has been a paucity of data investigating the benefits of the newer, physiological methods of female androgen therapy. Gold standard investigation of the long-term safety outcomes of
hormonal preparations requires large scale randomised or observational data. Such studies can take many years to achieve results and are an enormous financial burden. In their absence we can make use of surrogate markers for disease to try and obtain results in a realistic setting and timescale.

The present study explores the effects of postmenopausal transdermal testosterone on the cardiovascular system by assessing the effects of the transdermal testosterone patch on risk factors and surrogate markers for cardiovascular disease.
LITERATURE REVIEW

Androgen Physiology

Androgens are sex steroid hormones, produced in women by both the adrenal gland and the ovaries, with many important roles in female physiology. In premenopausal women, androgens such as testosterone and androstenedione are produced by ovarian theca cells, where they are either secreted directly into the circulation, or converted by granulosa cells into estrogens by the aromatase enzyme. Additionally, the adrenal gland produces the relatively weak androgens dihydroepiandrosterone (DHEA) and its sulfate (DHEA-S), which act as precursor hormones in the production of testosterone via peripheral conversion (Figure 1). It is estimated that ovarian and adrenal production each contribute 25% of circulating testosterone and the remaining 50% is contributed through conversion of androgen precursors in peripheral cells and target organs (9).

Two-thirds of testosterone circulates bound to sex hormone binding globulin (SHBG) and one-third bound to albumin, with only the remaining 1-2% circulating freely in a biologically active form (10). Testosterone only binds to albumin with relatively weak affinity and therefore 'bioavailable' testosterone, i.e. that with the ability to diffuse across cell membranes, is often considered as the free testosterone plus the albumin bound fraction. Bioavailable testosterone can therefore be strongly influenced by the many factors which affect SHBG levels, including obesity or exogenous estrogens.

Testosterone circulates in women at around 5% of the level found in men but is the most abundant active sex hormone (11). Women produce around 3-4 times more testosterone daily than estrogen, equating to approximately 100-400µg testosterone per day (12). Plasma levels of testosterone fluctuate during different phases of the menstrual cycle (13), being lowest in the early follicular phase with significant mid-cycle elevations (14). Levels also show circadian variation, being highest in the early morning.

The level of circulating androgens declines gradually with age, primarily owing to a reduction in adrenal production of androgens and androgen precursors. It is estimated that androgen levels decline by 50% between the ages of 20 and 40 years (15). Current
Data do not point to a precipitous drop in androgen production at natural menopause (16) and after menopause the ovaries remain an important source of androgen production (15). Atrophy of the adrenal cortex reduces the contribution from the adrenal gland to around 10% of circulating androgens, with 50% from the ovaries and 40% from peripheral conversion (17). This is in keeping with studies which demonstrate a 40-50% drop in testosterone following surgically induced menopause (18,19). As well as age-related decline and surgical oophorectomy, other causes for androgen deficiency include premature ovarian insufficiency (POI) (20), hypogonadotrophic hypogonadism (21), hypopituitarism (22), hyperprolactinaemia and oral estrogen or glucocorticoid therapy.

Changes in estradiol levels at menopause are also thought to influence androgen production, both by removal of the negative feedback and the reduction in SHBG production associated with the hypoestrogenic environment. The result is a relatively androgenic hormonal milieu in postmenopausal women, indeed some data suggest an increase in free androgen index in the perimenopausal years (23).

**Figure 1. Female androgen production.** Dihydroepiandrosterone sulphate (DHEAS), dihydroepiandosterone (DHEA), dihydrotestosterone (DHT). Percentages demonstrate contributing source for each androgen
The exact role of androgens in women remains poorly understood (24) due to the complex metabolic pathways involved. Testosterone is thought to exert its wide-ranging effects through 3 principal mechanisms (Figure 2). Its primary mode of action involves activation of the androgen receptor (AR). ARs are found in almost all tissues throughout the body including brain, skin, adipose, bone and the vascular tree. AR activation results in direct genomic effects in target cells which can be modulated by a variety of co-regulator proteins (25). Testosterone can also have more rapid, non-genomic effects by activating membrane bound receptors which exert their actions via activation of 2nd messenger pathways (26). Furthermore, as a proportion of testosterone is converted within target cells to estradiol by the aromatase enzyme, it is unclear to what extent the effects of testosterone are mediated directly or through the actions of estradiol.

**Figure 2. Mechanisms of testosterone action.** Modified from (25). (1) Direct activation of androgen receptor (AR) by testosterone (T) or dihydrotestosterone (DHT). (2) Rapid, non-genomic response via specific membrane binding sites (SMBS) and intracellular secondary messenger pathways. (3) Intracellular conversion to estradiol (E2) and activation of estrogen receptor (ER).
Androgen deficiency

Although free and total testosterone concentrations have not been shown to correlate well with measures of sexual function (27), exogenous testosterone has long been recognised to improve female sexual function and other aspects of physical and psychological well-being (28). Terms such as ‘relative androgen deficiency’ (15) or ‘female androgen insufficiency syndrome’ (29) have been proposed to characterize the collection of symptoms which may be associated with low testosterone (Table 1). However, the use of these terms has been controversial, due to the lack of a clear definition and often non-specific nature of symptoms (30).

Current recommendations suggest that androgen deficiency should be a clinical diagnosis, particularly in view of the inherent problems associated with serum testosterone measurement in women and the poor understanding of age-related normal reference ranges (31). Furthermore, data correlating testosterone levels with symptoms are lacking (32). Menopause symptom rating scales have been adopted for use in androgen deficiency and in clinical studies have proved a useful tool for assessing symptoms severity and response to treatment (33). It is hoped that further validation of these questionnaires will generate a standardised diagnostic tool for androgen deficiency. Although assessment of testosterone levels does not appear to have a role in diagnosis, it is generally recommended that baseline levels be established prior to treatment and to identify patients who may be at risk of developing supraphysiological levels (2).
Table 1. Symptoms associated with androgen deficiency (1,34).

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<td>Low sexual desire</td>
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<tr>
<td>Reduced sense of well-being</td>
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<td>Dysphoric mood</td>
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<tr>
<td>Low energy/reduced motivation</td>
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<td>Bone loss</td>
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<td>Reduced muscle strength</td>
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<td>Poor cognition and memory</td>
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<tr>
<td>Insomnia</td>
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<td>Vasomotor symptoms</td>
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<td>Joint pain</td>
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<td>Urinary dysfunction</td>
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Postmenopausal testosterone therapy

- Testosterone preparations

Testosterone has been used in the field of gynaecology for over 70 years, initially being used as a treatment for menorrhagia, dysmenorrhoea, mastalgia, and pelvic inflammatory disease (35). As early as the 1950s it was recognised that androgens may play a role in the management of climacteric symptoms (36), with studies demonstrating that combination estrogen-androgen therapy appeared to benefit sexual desire compared to estrogen alone. For several decades androgen replacement was administered orally and subsequently via intramuscular injection or subcutaneous implant, often in supraphysiological doses. Concerns consequently arose due to the high incidence of androgenic side effects associated with parenteral routes.

More recently, attention has turned towards transdermal testosterone replacement using patches or gel/cream. The transdermal route achieves a more constant,
physiological serum hormone level (37) and the avoidance of first pass hepatic metabolism may help reduce unwanted effects on hepatic proteins.

Several randomised placebo-controlled trials investigating the use of a transdermal testosterone patch (TTP) in treating hypoactive sexual desire disorder (HSDD) reported significant improvements in sexual desire and other domains of sexual function both with (38–42) and without (43) concurrent HRT use. This was also associated with an improved sense of well-being (38,39). The results of these trials led to the UK and European licensing of Intrinsa (300ug/day testosterone) (© Warner Chilcott) for the treatment of hypoactive sexual desire disorder (HSDD) in surgically menopausal women also taking estrogen. The Intrinsa license was voluntarily removed by Warner Chilcott in 2012 for commercial reasons.

Off-label use of testosterone gels/creams is common, but no preparations designed for use in women are currently recognised by the UK or European regulatory authorities or by the FDA in the US. The subcutaneous implants, which were licensed for 30 years for use in surgically menopausal women on concomitant estrogen are no longer available.

- **Post-menopausal testosterone replacement for low sexual desire**

Low sexual desire is a common problem amongst women of all ages, particularly in middle age. When associated with distress, it is termed hypoactive sexual desire disorder (HSDD), which is estimated to affect approximately 9-14% of postmenopausal women (44). HSDD has a significant impact on physical and psychological wellbeing, having been associated with low self esteem (45), depression (46), psychological distress (47), and reduced quality of life (48). Women with low desire are also more likely to experience lower arousal and pleasure, orgasmic difficulties and suffer dissatisfaction with their sex life and partner relationship (45). Healthy female sexual function is the result of complex interactions between a multitude of physiological, psychological and social factors, including estrogen or androgen deficiency (49).

Androgen replacement has long been recognised to play a role in improving sexual desire, particularly in postmenopausal women. In early observational studies, postmenopausal women using subcutaneous estradiol and testosterone implants
reported significant improvements in sexual function (50); findings that were later corroborated in randomised studies (51). Similarly beneficial effects on sexual desire were also reported in randomised studies involving oral testosterone, used in both naturally and surgically menopausal women (52,53). Although an effective treatment, oral therapy often resulted in supraphysiological doses and unacceptably high rates of androgenic side effects. Furthermore, concerns arose about the long term effects of treatment, particularly the risk of CVD, due to reported adverse effects to the lipid profile and to insulin resistance. This led to the search for other methods of delivery and most recent research has concentrated on achieving physiological replacement via transdermal or subcutaneous routes.

The development of the TTP led to a plethora of randomised controlled trials (RCTs) investigating the safety and efficacy of this product. In a study by Shifren et al., 75 surgically menopausal women, with impaired sexual function and who were also taking conjugated estrogens, were randomised to the TTP or placebo. The TTP was associated with significantly improved sexual function as determined by scores on the Brief Index of Sexual Functioning for Women (54).

A further randomized, placebo-controlled phase 2 study sought to examine the dose response effect of the TTP in surgically menopausal women on concomitant oral estrogen (41). Subjects were randomized to receive placebo (n = 119) or testosterone patches in dosages of 150 microgram/day (n = 107), 300 microgram/day (n = 110), or 450 microgram/day (n = 111) twice weekly for 24 weeks. The results showed that only the 300 microgram dose was associated with significant increases in sexual desire and frequency of satisfying sexual activity, but no significant benefit was derived from either the 150 or 450 microgram doses. The authors suggest 300 micrograms/day may be the top end of the dose response curve, which would explain the lack of additional efficacy of the 450 microgram dose. Furthermore, mean testosterone production in women is approximately 300 microgram/day and therefore the higher dose results in supraphysiological replacement, which may alter the in vivo effects.

These studies were then followed by two larger phase 3 studies (INTIMATE SM1 AND 2) (40,42) involving a total of 1094 surgically menopausal women. Again, the 300 microgram testosterone patch in addition to estrogen was associated with
significantly more satisfying encounters and increased sexual desire. Debate ensued as to whether a small increase in frequency of satisfying sexual activity (an additional 1-2 episodes over 4 weeks) was clinically meaningful, although crucially, these improvements in sexual function were associated with a reduction in distress levels.

The TTP only received a license for use in surgically menopausal women using systemic estrogen, however there are large scale RCT data to support its use out with this population. Significant increases in sexual desire, satisfying sexual activity and reductions in distress have also been observed in naturally menopausal women on HT (39), naturally postmenopausal women predominantly not on HT (55) and postmenopausal women not on concomitant HT (43). More recently, the benefits of the TTP in women suffering from selective serotonin reuptake inhibitor (SSRI) or serotonin noradrenalin reuptake inhibitor (SNRI) associated low libido have been observed. Administration of the TTP was associated with a significant increase in 4 week frequency of satisfying sexual episodes compared to placebo (2.3 vs 0.1, p=0.02) (56).

Testosterone creams (licensed in Australia) and gels (usually male products, used ‘off-label’) have also been gaining interest and data exist to support their use. In a randomized, placebo-controlled, cross over study in 53 postmenopausal women with low libido on HT, Nathorst-Boos et al. report that 10mg daily of testosterone gel was associated with significant improvements in desire, including frequency of sexual activity, fantasies and sexual interest (57). Although usually administered on a regular basis, there are pilot data which suggest benefit from using testosterone gel on an ‘as-required’ basis. In a small randomized double-blind cross over study, a single dose of testosterone gel administered 4-8 hours prior to intercourse in premenopausal women with HSDD resulted in increased arousal (58). Further data are required to confirm these findings, particularly as the use of testosterone in premenopausal women has not been well-studied.

A further area of interest is the potential effect of local vaginal testosterone application. A study by Witherby and colleagues (59) investigated the use of topical testosterone cream for the treatment of vulvovaginal atrophy in 20 postmenopausal patients with breast cancer who were taking aromatase inhibitors. Testosterone cream was applied to the vaginal epithelium daily for 28 days and was associated with a significant
improvement in dyspareunia, vaginal dryness and vaginal maturation index without increasing estradiol or testosterone levels.

The most recent systematic review and meta-analysis of postmenopausal testosterone use identified 35 RCTs (n=5053) and confirmed that testosterone therapy was associated with increased sexual activity, increased interest in sex, improved quality of sexual encounters and improved sexual self image (60). In subgroup analysis, testosterone was more effective than methyl-testosterone or testosterone undecanoate at improving sexual function. In further sub-group analysis, no significant differences were found dependent on route of testosterone, use of concomitant HRT, or type and route of estrogen use.

• **Systemic benefits of testosterone therapy**

  o **Well being and quality of life**

Although HSDD is the main indication for postmenopausal testosterone use, several other beneficial effects have been reported. Improved quality of life, mood and overall well being has consistently been noted (38,39,54,61). A recent observational study examined the use over 3 months of 75-160 mg testosterone implants, without concomitant estrogen, in 300 pre-menopausal and post-menopausal women reporting symptoms of androgen deficiency such as sexual problems, fatigue, mood disturbance, headaches, insomnia, memory loss and hot flushes. Testosterone implants were found to be effective for the relief of psychological, somatic and urogenital symptoms, in addition to sexual desire, with a dose-dependent response (33).

  o **Cognitive function**

Based on endogenous testosterone levels, a potential neuroprotective role for testosterone has been suggested (62,63). Recent data have suggested that transdermal testosterone may improve cognitive performance, although there are few high quality RCTs (2,64). The use of testosterone gel in postmenopausal women also using estrogen was associated with improvement in immediate and delayed verbal memory (65). In a
more recent RCT, 92 postmenopausal women, not on systemic estrogen therapy, were randomised to 300mcg testosterone gel or placebo. After 26 weeks of therapy there was significant improvements in verbal learning and memory in the testosterone group (66).

- **Bone density**

Androgens can also act to inhibit bone resorption and therefore it has been suggested that exogenous testosterone may benefit bone density. Although no studies have examined fracture rates as a clinical endpoint, there are data that testosterone implants (67) and combined oral estrogen-methyltestosterone (MT) therapy may improve bone density (68,69). However, conflicting data exist, as a further study found improvements in bone mineral density (BMD) in both estrogen and estrogen-androgen treated groups, with no significance difference observed between groups (70).

Few studies have examined the effect of transdermal testosterone on BMD in postmenopausal women. In a study investigating the effects of transdermal testosterone on bone density in 145 women with POI, the addition of 150mcg transdermal testosterone to 100mcg transdermal estradiol and sequential progestogen did not result in any additional benefit to BMD over the 3-year study period compared to placebo (71). In contrast, 300mcg TTP was associated with significant increases in BMD in women with hypopituitarism (72).

- **Safety Concerns**

There are compelling data highlighting the benefits of postmenopausal testosterone use and position statements from several endocrine and menopause societies support the use of androgens for postmenopausal women suffering from distressing low sexual desire (32,73–75). Despite this, the use of testosterone remains controversial and few regulatory authorities have approved its use. In the UK, implants and the TTP are licensed in surgically menopausal women, however the US Food and Drug Administration (FDA) has not approved any testosterone preparations for the treatment of low sexual desire in postmenopausal women. Of primary concern to
regulatory authorities is the lack of long-term safety data, particularly with regard to the risk of breast or endometrial cancer and CVD (3,30). Many of these concerns have been extrapolated from the findings from the Women’s Health Initiative (WHI) studies, due to the potential for testosterone to be converted to estradiol. To date, clinical trials have predominantly focused on safety data in terms of androgenic events, site reactions and basic blood parameters. Reviews of the subject have emphasised the need for improved long-term safety data (76).

- **Androgenic side effects**

Androgenic side-effects (hirsutism, acne, alopecia and rarely, virilisation) are associated with supraphysiological testosterone doses. Androgenic side effects from transdermal physiological therapy are generally mild and take several months to occur. They are usually dose dependent and resolve after treatment is discontinued.

The incidence of hirsutism in the TTP RCTs was estimated at 3-20%. A statistically significant increase in hirsutism was observed in only one study (43). This study was the longest in duration (52 weeks), however subjects were not on concomitant estrogen, which may have contributed to the findings.

In the majority of studies there was no observed increase in acne compared to placebo (incidence 4.6-7.5% participants). One study reported a significant increase in acne in TTP users, however both groups had a particularly high incidence of acne (18% of TTP users, 13% of controls) (41).

Nachtigall and colleagues performed a 4-year follow-up analysis of 1,094 subjects from two of the TTP RCTs with a mean duration of use of 1.1 years. Overall rates of androgenic events were 28.4% (0-12 months), 14.3% (12-24 months), 10.5% (24-36 months) and 3.5% (36-48 months). Reported events were mostly mild and did not result in discontinuation of treatment (77).

The more severe androgenic effect of virilisation (voice deepening, frontal hair loss and clitoromegaly) is extremely rare, and tends to only occur when sustained supraphysiological levels of testosterone occur via parenteral routes. It did not occur in any studies investigating the TTP.
Breast safety

Concerns have been raised about the risk of breast cancer with testosterone replacement (3). In fact, available data, both experimental and observational, point towards no increased risk in breast cancer from testosterone therapy (34). There are relatively few clinical trial data examining the effect of exogenous testosterone on breast cancer risk and no RCTs with breast cancer as a primary outcome. Available studies investigating non-oral routes of testosterone replacement have produced more reassuring results than those using oral or intramuscular, often high-dose, therapy.

Two studies have suggested an association with breast cancer and testosterone replacement. An early case–control study reported that the risk of breast cancer was increased in women using intramuscular testosterone in addition to estrogen or estrogen plus progestogen therapy (78). In the Nurses Health Study, 24 year follow-up contributing 1,359,323 person years of data and 4610 incident breast cancer cases, found an increased risk in combined estrogen and testosterone users compared with never users (RR: 2.48, 95% CI: 1.53–4.0) (79). In contrast, data from the WHI observational arm with a mean follow-up of 10 years reported no increased risk of breast cancer with CEE+MT (80). A further 6 six observational studies found no increased risk of breast cancer associated with postmenopausal testosterone use (81–86). Randomized, placebo-controlled studies showed no adverse effects from the TTP on breast cell proliferation (87) and mammographic density (88,89).

The effects of androgens may be mediated either directly via the androgen receptor, which has been demonstrated in breast tissue, or from conversion to estradiol by the aromatase enzyme which is found in breast tissue. It has also been suggested that testosterone may have further indirect effects on the breast by influencing SHBG levels and subsequently, the bioavailability of estradiol (90).

Experimental data investigating the effects of androgens on breast cell lines are conflicting, with both proliferative (91) and anti-proliferative (92–95) effects reported. Other studies have demonstrated that testosterone is pro-apoptotic and may inhibit gene expression in breast cells (92,96,97). This led to an interest in the potential protective effect of androgens on the breast (96).
There are also now studies reporting the beneficial effects of testosterone therapy in metastatic breast cancer (98) and combination testosterone-anastrazole therapy as treatment for hormone sensitive breast cancer (99). However, there is evidence to suggest differing effects of testosterone depending on tumour hormone status (100–102) and further studies are needed.

- **Endometrial safety**

Epidemiological data have reported an increased risk of endometrial cancer in association with raised free testosterone, however this association does not persist after adjustment for estrone and estradiol levels (103) and *in vitro* data suggest an inhibitory effect of androgens on endometrial cell proliferation (104).

There are a lack of clinical data investigating the effects of testosterone therapy on the endometrium. Only one randomized study has examined the endometrial effects of postmenopausal testosterone replacement as a primary outcome. In a study of 63 postmenopausal women randomised to either 2mg estradiol, testosterone undecanoate 40mg or combined therapy with both, there was no evidence of endometrial thickness or proliferation from androgen replacement as assessed by ultrasound and histopathology (105). The Aphrodite study investigated the effects of the TTP in women not on concomitant estrogen. 814 postmenopausal women using the TTP were followed up over 52 weeks and although vaginal bleeding was more common in the TTP group (10.6%) compared to placebo (2.6%), no subjects developed endometrial hyperplasia or carcinoma (43). In an observational case–control study, 8412 women were followed-up for a mean of 4.4 years. Of the 2103 testosterone users, there were no cases of endometrial cancer compared with five cases in 6309 controls (81).

Therefore although current data do not suggest any increased risk of endometrial cancer in association with testosterone therapy, further long-term studies are needed.
**Cardiovascular effects of testosterone**

- **Gender-based differences in cardiovascular disease**

Cardiovascular diseases (CVD), comprising coronary heart disease (CHD), stroke and venous thrombo-embolism (VTE), are the leading cause of mortality in women, responsible for almost a third of female deaths in the UK (4). Marked gender differences exist in the incidence of CVD with women tending to develop CVD around 10 years later than men (106,107), although with age, particularly after menopause, this gender difference disappears. This observation has led to much speculation surrounding the cardioprotective role of sex steroids, with early focus on the potential benefits of estrogens. More recently though, interest has been turning towards a potential role for androgens (6,108,109).

Both estrogens and androgens are implicated in the gender-based differences in CVD incidence. In men, high androgen levels have been associated with detrimental effects to important CV risk factors such as blood pressure, lipids and glucose metabolism. However, more recently it has been shown that low circulating testosterone levels are associated with higher mortality rates, mainly due to increased CVD (110–116) and metabolic syndrome or diabetes (117). This has subsequently led to much interest in the potential cardiovascular benefits of male androgen therapy (118,119).

In women, the CV effects of androgens are much less well understood (25,109). Several potential mechanisms have been suggested by which androgens may affect CV risk. These include direct effects of the vasculature, or indirectly via effects on insulin resistance, fat distribution and lipid profile.

Although many studies have investigated the effect of androgens on CV risk, results are frequently conflicting and, to date, there are only very limited data which can be applied to the postmenopausal use of testosterone. There is marked heterogeneity amongst available studies and many factors to consider when results of androgen studies are being interpreted (Table 2).
Table 2. Confounding factors which affect testosterone studies

- Endogenous vs exogenous androgens
- Physiological or supraphysiological doses
- Route of hormone administration
- Testosterone assay used
- Testosterone fraction used
- Concurrent use of estrogen/progestogen HT
- Presence of other CV risk factors

- Molecular studies

Atherosclerosis is the end result of chronic inflammation, due to vascular injury and endothelial dysfunction. Endothelial injury results in a procoagulant and inflammatory environment, with decreased endothelial production of vasodilators such as nitric oxide (NO) and prostacyclin, leucocyte and platelet adhesion and aggregation, and smooth muscle cell proliferation and migration (120). Persistent inflammation and morphological change results in the formation of atheromatous plaques which are characteristic of atherosclerosis. Many factors will affect the ability of the endothelium to respond to and compensate for injury but central to this process is the ability of the endothelium to produce NO. NO is a crucial regulator of endothelial function as it inhibits endothelial cell monocyte adhesion, vascular smooth muscle cell migration and adhesion and platelet aggregation.

Androgen receptors are present throughout the cardiovascular system, including endothelial and smooth muscle cells and myocardial fibres (25). Molecular studies to date have suggested various mechanisms by which androgens may affect CV risk. Older in vitro data associated testosterone with pro-atherogenic effects including increased apoptotic damage of endothelial cells (121), increased migration and proliferation of vascular smooth muscle cells (122), increased adhesion of mononuclear cells to the
endothelium (123), increased oxidation of low-density lipoprotein (LDL) cholesterol, and increased vascular cell adhesion molecule (VCAM-1) expression (123).

In contrast, there is now accumulating evidence that androgens exert a beneficial CV effect through effects on vascular function (124). Testosterone causes vasodilatation, due to production of endothelium-derived NO in and via effects on calcium and potassium channels in smooth muscle (125). In vitro data have shown that testosterone rapidly increases endothelial NO production; an effect not affected by an aromatase inhibitor and completely blocked by an androgen receptor antagonist, suggesting that this is a direct effect of the AR and independent of conversion to estradiol (126). Other beneficial effects of testosterone have been observed including reduced monocyte adhesion, reduced platelet aggregation (127), decreased VCAM-1 (128,129) and promotion of cholesterol efflux (130), which may reduce the formation of fatty streaks characteristic of early atherosclerosis.

- **Endogenous testosterone**

A deleterious effect of hyperandrogenaemia has long been suggested due to the association of polycystic ovarian syndrome (PCOS) and adverse CV risk profile. Women with hyperandrogenaemic PCOS, in contrast to those without elevated androgens, have increased CV risk factors including obesity, insulin resistance and metabolic syndrome (131). PCOS is associated with increased CV events as observed in longitudinal studies, event after adjustment for body mass index (BMI) (132).

The effects of endogenous androgen levels in postmenopausal women are less well understood as conflicting data exist (133). Raised endogenous testosterone has been linked to CVD and multiple CV risk factors (17). It has also been suggested that SHBG (which has a direct impact on free testosterone levels) has independent predictive power for CV risk (134,135).

Some (136–138) but not all studies (139,140) associate endogenous androgens with CV risk factors including increased BMI, increased abdominal obesity and raised systolic or diastolic blood pressure. Although a relationship between testosterone, SHBG and
obesity has been observed, data from the Study of Women’s Health Across the Nation suggests that weight gain across the menopause transition results in changes in hormone levels rather that the reverse (141).

Although the majority of observational data does not support a role for endogenous testosterone and lipid metabolism (17), some observational and clinical studies have demonstrated that increased androgenicity is associated with adverse changes to the lipid profile including increased total cholesterol, increased LDL cholesterol and reduced high density lipoprotein (HDL) cholesterol (142). Other studies showed that SHBG appears more important and that androgen levels are not independent predictors of risk factors for CVD (134).

Several cross-sectional studies have examined the effects of endogenous testosterone levels on risk of CVD using surrogate markers and CV events, but results are conflicting. An inverse relationship between endogenous testosterone and CV risk was found in studies assessing including carotid intima-media thickness (CIMT)(143,144) and carotid atherosclerosis on ultrasound (145). A case control study found that postmenopausal women with proven carotid artery atherosclerosis were found to have significantly lower levels of total testosterone compared to women with normal carotid arteries, independent of other CV risk factors (146). Wehr et al. (147) report that in 875 postmenopausal women referred for coronary angiography, low free testosterone was associated with CV mortality in diabetic women but not in non-diabetics.

In contrast, other studies have found high free or bioavailable testosterone to be associated with subclinical atherosclerosis as assessed by CIMT (148), and arterial stiffness, even after adjustment for estradiol and other CV risk markers (149). A further small study in 26 postmenopausal women found that high free androgen index (FAI) was associated with endothelial dysfunction as assessed by dorsal hand vein vasodilation (150). Polymorphism of the AR gene (CAG)n repeat was associated with severity of CAD and risk factor profiles in 131 postmenopausal women (151) with shorter length or repeats (suggesting increased AR activity) associated with increased severity of CAD. Other studies showed a correlation between high testosterone and presence of coronary artery disease at angiography (152). In a study by Munir et al., increased free testosterone was associated with increased calcified and non-calcified
coronary artery plaque but this association did not persist after adjustment for other CV risk factors (153).

Several other large cross section studies did not find a relationship between endogenous testosterone and surrogate markers including coronary artery plaques and CIMT (154), abdominal aortic calcification (155) and flow-mediated vasodilatation (156).

There are few data which link endogenous testosterone levels with CV events and mortality. A cross-sectional study in 344 older postmenopausal women by Patel et al. found that women in the top quartile of total testosterone levels had a 3 fold increased in risk of CHD (OR 2.95, 95% CI 1.2-73) and metabolic syndrome (OR 3.14, 95% CI 1.57-6.35), however this association was not true for free testosterone (157). In another study the association between FAI and increased CV risk did not persist after adjustment for BMI and other CV risk factors (158). Other studies also (139,159) showed no association with endogenous testosterone levels and CV events.

Five longitudinal studies investigating the effects of androgens in postmenopausal women were identified. Karim et al. demonstrated that that a higher total testosterone was associated with reduced subclinical atherosclerosis progression as assessed by CIMT over 2 years (160). In a cohort study of 438 Australian women, high free testosterone but not total testosterone was associated with increased 10 year risk of coronary event (161).

In the Rancho Bernardo study 651 Caucasian postmenopausal women not using HT were followed-up for 19 years. There was no association between free or total testosterone with death from CHD (139). A larger prospective cohort study found that in 2914 German women (of whom 1394 were postmenopausal), those in the lowest quintile of total testosterone had significantly increased mortality (HR 0.62, 95% CI 0.42-0.939) and risk of CV events (HR 0.68, 95% CI 0.48-0.97) (162). A further prospective population based study by Laughlin et al. followed 639 postmenopausal women for a mean of 12.3 years with 134 CV events. The results showed a U-shaped curve for CV risk with those in the highest (HR 1.79, 95%CI 1.03-3.16) and lowest (HR 1.
96 95%CI 1.13-3.41) quintiles for free testosterone having higher risk of incident CHD events (163).

Many of these studies contain heterogeneous populations which may explain the conflicting results. Some fail to adjust for estradiol levels and CV risk factors such as BMI. Furthermore it is unclear whether changes in sex steroid hormones may be an adaptation to a disease process or a marker of poor CV health. Overall the majority of data appear to suggest that both low and high endogenous testosterone may increase CV risk and it has therefore been suggested that optimisation of testosterone levels within the physiological range may be most beneficial for CV health (163).

- **Exogenous testosterone**

The suggestion that there may be CV benefit from maintaining testosterone within the physiological range has huge potential implications given the global burden of CVD. Whether this benefit can be derived from exogenous replacement remains unknown. Few studies have investigated the CV effects of postmenopausal testosterone therapy. Current data often involve pharmacological dosing and are limited to effects on CV risk factors or surrogate markers rather than CV events or mortality as a primary outcome.

  - **Animal data**

Testosterone has been shown to have vasodilatory effects in a variety of animal models (164,165). In a study by Chou et al. (165) testosterone treatment was associated with vasodilatation in canine coronary arteries in a sex-independent manner, an effect that was at least in part mediated by endothelium-derived nitric oxide. These findings have also been confirmed with physiological testosterone replacement in androgen deficient oophorectomised rats, which led to an improvement in the vasodilator response of the endothelium (166). More recently, physiological testosterone replacement in oophorectomised rats has been shown to enhance the cardioprotective benefits from estrogen replacement alone (167). Liu et al. demonstrated that estradiol and testosterone replacement alone or in combination reduced myocardial injury, increased
myocyte viability and improved contractile function. Combination treatment appeared to be more effective than either estradiol or testosterone replacement alone (167).

Although most animal studies point to a beneficial effect of testosterone on the CV system conflicting data exist. Testosterone has been associated with CV risk factors including activation of the renin-angiotensin system (168,169), elevated BMI and visceral fat (170). In hypercholesterolaemic rabbits, testosterone therapy was associated with impairment in endothelium-dependent vasodilation (171). Furthermore supraphysiological doses of testosterone were associated with doubling of atherosclerotic plaque burden in rabbit (172) and primate models (170).

- **Human Data**

  (1) **BMI/weight distribution**

  Initial studies investigating the effect of testosterone on body mass reported reductions in subcutaneous fat but increases in BMI and visceral fat, however these studies involved high-dose intra-muscular testosterone used in female-male transsexuals (173–175).

  Effects in postmenopausal women appear to show benefits in lean body mass, but may be dependent on the route of administration. Oral and subcutaneous implant therapy has been associated with increases in lean body mass (61,176,177) but also visceral fat mass (178). Treatment with the TTP was not associated with any changes in BMI over 24 weeks (39) but a study involving 39 postmenopausal women randomised to testosterone gel or placebo reported a significant reduction in BMI, total body weight and abdominal fat (179).

  (2) **Lipid profile & inflammatory markers**

  Several studies have investigated the effect of exogenous testosterone on lipids and the effects appear to be largely dependent on route of administration. Oral therapy has
consistently been associated with a beneficial effect on triglyceride levels and total cholesterol but with detrimental effect to HDL cholesterol (61,68,69,178,180–183) and a favourable effect on apolipoprotein C (183).

In contrast, no impact on lipid profile has been observed in associated with transdermal therapy including testosterone gel (179) and the TTP (77). In a 4 year follow-up study of 967 surgically menopausal women who had received at least 1 application of the TTP, no alterations in lipids were observed compared to the placebo group.

Only one RCT has investigated the effects of postmenopausal testosterone replacement on inflammatory markers. In 50 surgically menopausal women randomised to estradiol and placebo or testosterone undecanoate 40mg, testosterone was associated with counteraction of the estrogen-induced rise in C-reactive protein (184).

(3) Blood pressure

In a pharmacokinetic study of 12 postmenopausal women, on concomitant estrogen, inhaled testosterone was associated with an acute fall in systolic blood pressure (mean reduction of 10mmHg±12mmHg) 5 minutes after therapy (185). Other studies have no shown change in blood pressure in association with oral (178), subcutaneous (186) or transdermal therapy (39).

(4) Surrogate cardiovascular markers

There are only limited data investigating the effect of exogenous testosterone on surrogate markers for CVD. In a small study involving female-to-male transsexuals, high-dose intramuscular testosterone was associated with an impairment of vascular reactivity (187). Intramuscular testosterone and estrogen administration resulted in increased rates of severe aortic atherosclerosis in postmenopausal women, as demonstrated by radiographic detection of calcified aortic deposits in a trial by Hak et al. (188). Again this was a small study involving pharmacological testosterone doses but despite this only showed a small effect following 1 year of testosterone therapy. Penotti et al. (189) investigated the effects of 8 months of oral testosterone undecanoate versus placebo in addition to transdermal estrogen and medroxyprogesterone acetate on vascular reactivity in 40 postmenopausal women. They found a small but significant
(6%, p<0.05) increase in the pulsatility index of the middle cerebral artery, a reflection of the vascular reactivity of the cerebral arteries, in women receiving testosterone.

In contrast, testosterone has been associated with beneficial endothelial effects including acute vasodilatation with inhaled testosterone (185) and chronic vasodilatation with implant therapy (186). The study by Worboys et al. assessed the effect of 6 weeks of subcutaneous testosterone implant in 48 postmenopausal women also taking estrogen. Compared to the control group, testosterone was associated with an improvement in endothelium dependent (flow-mediated) and endothelium independent (GTN induced) brachial artery vasodilatation suggesting a beneficial role for exogenous testosterone in vascular reactivity and endothelial function (186). Additionally, DHEA administered to healthy postmenopausal women for 12 weeks was associated with improved endothelial function as assessed by brachial artery flow-mediated vasodilatation (190). Although DHEA has androgenic actions, it may exert its effects by a variety of metabolites and therefore the implications of this study cannot be applied directly to testosterone treatment.

In summary, although there is some evidence for the beneficial effect of testosterone on surrogate markers for CVD, no studies have examined the effect of physiological transdermal testosterone replacement in postmenopausal women.

(5) Cardiovascular events

Unfortunately there are no long-term prospective data investigating postmenopausal testosterone replacement with CV events as a primary outcome. A retrospective study of 293 female to male transsexuals who received long term high dose testosterone showed no increase in frequency of myocardial infarction (MI) or hypertension and no excess of deaths due CVD or other causes compared to the general population (191).

A pooled analysis of 5 phase III safety studies investigated the CV effects of the TTP. 2795 women were randomised to TTP or placebo for 24-52 weeks duration. There was no change in CV risk factors (including cholesterol, triglycerides, insulin, glucose and
blood pressure), although increases in HDL were slightly less in the TTP group compared to placebo group. There was no difference in the number of CV events reported during the treatment phase with (2 MIs and 2 strokes) were reported in placebo patients and 3 (2 MIs and 1 stroke) in the TTP group (192).

The results from a large randomised, placebo-controlled trial investigating the CV effects of transdermal testosterone gel which has a composite of CV events including death, non-fatal myocardial infarction, non-fatal stroke, coronary revascularization, hospitalized unstable angina, and venous thromboembolic events as a primary end-point are still awaited (193). It was hoped that this study would provide long-term outcome data but unfortunately it may not report due to commercial reasons.

Although there are data to suggest that high dose parenteral or oral replacement may have a negative impact on CV risk factors, these data cannot be applied to physiological replacement via subcutaneous or transdermal routes. Physiological replacement appears to have a beneficial effect on CV risk factors but its effect on surrogate markers and long term CV mortality remains unclear.
Testosterone and glucose metabolism/insulin resistance

Insulin resistance is central to the development of the metabolic syndrome which comprises insulin resistance, central obesity, hypertension and dyslipidaemia (194). The prevalence of obesity and metabolic syndrome in women increases around the time of the menopause and is a well-established risk factor for CV disease.

The relationship between endogenous androgens and glucose metabolism has long been suspected due the association between hyperandrogenism and insulin resistance seen in women with PCOS. Women with PCOS have significantly increased risk of type 2 diabetes compared to BMI matched controls (HR 1.75, 95% CI 1.51-2.02)(195).

Circulating testosterone levels appear to be higher in diabetics compared to non-diabetics (196,197) and higher circulating testosterone has been associated with raised fasting glucose (198). Analysis from the Rancho Bernardo study found marked sexual dimorphism in the relationship between endogenous testosterone and insulin resistance (199). In their analysis of 233 postmenopausal women not taking estrogen, there was a 3 fold increased risk of diabetes in women in the highest quartile of bioavailable testosterone (OR 2.9, 95% CI 1.1-8.4)(199). Other studies have also found a positive correlation between free and total testosterone and incident DM (200) and metabolic syndrome (201,202). In contrast, other longitudinal studies demonstrated that the association between testosterone and risk of DM did not persist after adjustment for confounding factors such as BMI and insulin resistance (203) (204).

Interpretation of these findings is further complicated by the effects of SHBG, which is known to be a strong predictor of insulin resistance, an effect which appears to be independent of estrogen and androgen levels (205). This has led to the suggestion that declining SHBG levels may be responsible for increasing insulin resistance rather than primary alterations in androgen levels (32).

The pathophysiology underlying the relationship between androgens and glucose metabolism remains poorly understood. Cause and effect is difficult to disentangle as insulin can act as a co-gonadotrophin with luteinizing hormone to stimulate androgen production from ovarian theca cells (206). Hyperinsulinaemia is associated with a decrease in hepatic SHBG production, resulting in higher bioavailable testosterone.
Conversely, it has also been suggested that hyperandrogenism may affect glucose metabolism and insulin sensitivity. In vitro studies have shown that adipocytes exposed to testosterone have reduced insulin mediated glucose uptake (207). Findings in oophorectomised rats treated with testosterone showed impaired whole-body insulin-mediated glucose uptake which was thought to be due to reductions in glycogen synthase expression (208). This is corroborated by a study investigating the treatment of PCOS patients with spironolactone, an anti-androgen, which led to reduced androgen levels and improved insulin sensitivity, independent of weight change (209).

Furthermore, androgens may have additional, indirect actions on glucose metabolism through effects on lipid profile and body fat composition. The most commonly accepted view is that there is a complex interplay between hyperandrogenism and hyperinsulinaemia with multiple mechanisms acting in vivo.

Although these data point to a detrimental effect from endogenous androgens on glucose metabolism, the implications of this for exogenous testosterone administration have not been well established. Several studies have shown that high-dose testosterone therapy in healthy young women can result in reduced peripheral glucose uptake (210), hyperinsulinaemia and impaired glucose tolerance (211). Oral testosterone replacement in postmenopausal women has shown both no impact on insulin sensitivity (178) and small reductions in insulin mediated glucose disposal (176).

Studies using the TTP have not reported any effect on carbohydrate metabolism, usually assessed by fasting glucose and insulin levels (39,40,43,212). In a pilot study investigating the effect of 6 months transdermal testosterone on functional capacity and insulin resistance in 36 elderly women with cardiac failure, testosterone was associated with improved exercise tolerance, muscle strength and insulin resistance as assessed by the homeostasis model (213).

The complex interplay between androgens, estrogens, SHBG and insulin resistance makes data interpretation in this area challenging. As with androgen levels and cardiovascular disease, it appears that both low and high endogenous androgen may adversely affect glucose homeostasis. In keeping with this, older studies involving pharmacological doses have suggested a detrimental effect from exogenous testosterone however studies involving physiological doses are much more reassuring.
Data involving transdermal replacement are encouraging but either limited to basic assessments of glucose metabolism or involving populations outwith the usual prescribing range for PM testosterone use and therefore further studies are needed.
Assessment of cardiovascular risk via surrogate markers

From a research perspective, the gold standard for investigating CV risk is using CV events as a primary outcome. However, this is often not feasible as it requires large-scale RCTs with many years follow-up. Therefore, other ways of assessing cardiac risk have been identified using surrogate markers which predict future CV events.

In clinical settings, assessment of CV risk is usually done using traditional CV risk factors identified from the Framingham study such as hypertension, hypercholesterolaemia and obesity (214) (215). Hypertension is one of the most important risk factors for CV disease in women but requires accurate measurement and in clinical practice often goes undiagnosed (215). Many biochemical markers of CV risk have been identified, including impaired glucose tolerance, elevations in total cholesterol, triglycerides, LDL, lipoprotein (a) and reduced HDL (215,216).

Despite their widespread use, it has been shown that these traditional risk factors do not correlate as well for women as for men and subsequently may underestimate risk (217)((218). Therefore there is a need to find more accurate ways to predict CV risk, particularly in women. The use of surrogate markers, or methods which detect subclinical disease, is increasing but at present is most commonly confined to research settings.

- **Endothelial function**

  o **Role of the endothelium**

Control of vascular tone in response to internal and external stressors is an essential regulatory function within the CV system. This may be mediated by the actions of hormones and the autonomic nervous system on vascular smooth muscle or may be influenced by local cytokine activation at the endothelium.

The endothelium is increasingly recognised as an important interface, both in health and disease. Vascular injury, and subsequent endothelial dysfunction, are well
established precursors to atherosclerosis (219), coronary artery disease and CV events (220,221).

The process of atherosclerosis is mediated by abnormalities of the vascular endothelium. Injury to endothelial cells may be secondary to recognised risk factors such as smoking, diabetes and hypertension but aging and genetic factors are also important (figure 3). Cumulative insults to the endothelial cells result in decreased NO production, increased platelet adhesion, and increased expression of adhesion molecules which lead to macrophage binding to the endothelial surface. In the presence of increased pro-inflammatory cytokines, the macrophages migrate into the vascular wall and engulf lipid to form foam cells. Smooth muscle cells migrate into the vascular intima and release pro-thrombotic cytokines which leads to the characteristic fibrous cap overlying a lipid core seen in advanced atherosclerosis. In addition, impaired endothelial cells are less able to perform fibrinolysis and produce increased quantities of vasoconstrictors such as endothelin-1 and angiotensin 2. As the plaque gradually occludes the vessel, angina may develop, whereas rupture of the fibrous plaque with subsequent exposure of the elements to the blood leads to acute thrombosis and myocardial infarction.

A crucial feature of an intact endothelium is the ability to vasodilate in response to NO. Endothelial dysfunction is characterised by a reduction in bioavailability of NO and subsequent reduction in endothelium dependent vasodilatation (222). NO is released from the endothelium in response to stress or insults and has several important anti-atherogenic mechanisms including the maintenance of normal arterial resting tone, as well as the inhibition of leucocyte adhesion, platelet aggregation and smooth muscle cell proliferation (219). Endothelial dysfunction is therefore associated with abnormal vascular tone and a pro-thrombotic, pro-inflammatory environment which predisposes to atheroma development (120).
Figure 3. Pathophysiology of atherosclerosis

- **Testing endothelial function**

Endothelial function can be tested by assessing the vasodilator response to physiological or pharmacological stimuli known to induce endothelial release of NO (223). Many methods for assessment of endothelial function have been identified (224). The gold standard has traditionally been assessment of coronary artery endothelial function. Coronary artery testing requires intra-arterial administration of pharmacological therapies such as acetylcholine, which induce production of endothelial NO, and the assessment of changes in artery diameter using quantitative coronary artery angiography. The invasive nature of these tests has limited their widespread use and led to the development of non-invasive methods to assess endothelial function.

Endothelial dysfunction is a systemic process, not just limited to coronary circulation and so non-invasive techniques to measure peripheral artery endothelial function have been developed. This is most commonly done by flow mediated vasodilatation (FMD) of the brachial artery (225). Brachial artery diameter is measured by ultrasound before and after reactive hyperaemia (RH), which stimulates local endothelial release of NO. This technique also has significant limitations including the requirement for specialist training particularly in sonography (226) and expensive equipment. FMD assessment is a complex technique, and is therefore susceptible to significant operator variability (225).
Peripheral Arterial Tonometry (PAT)

The recognition that changes in pulse wave amplitude can be measured to assess endothelial function led to the development of finger plethysmography to record pulse wave amplitude via peripheral arterial tonometry (PAT)(227). This offers a simple, non-invasive technique which measures digital volume changes in response to reactive hyperaemia (RH) in order to calculate an index of endothelial function. The finger probe plethysmography records the pulse wave volume during each pulsation. After a baseline recording a blood pressure cuff is inflated to supra-systolic pressures for 5 minutes (the occlusion phase) and then released. In a healthy individual the pulse wave volume will increase rapidly whereas a muted response will be observed in individuals with endothelial dysfunction (figure 4). From the recordings, an RH index (RHI) is calculated as the ratio of average amplitude during RH compared to baseline. Measurements taken in the contra-lateral arm are used as control data to take into account the systemic non-endothelial dependent effects of RH.

RHI shows good correlation with CV risk factors (228) and with other measurements of endothelial function (227,229). Bonetti et al. have reported that an RH-PAT index of <1.35 has a 80% sensitivity and 85% specificity to identify patients with coronary endothelial dysfunction as diagnosed by reduced coronary artery vasodilatation in response to acetylcholine infusion(229). Kuvin et al. (227) found a linear relationship between FMD and PAT-RHI in patients presenting with chest pain.

Impaired RH-PAT indices have been shown to predict coronary artery disease in women as assessed by coronary angiography (230) and CV events (231). In a sub-study from the Kronos Early Estrogen Prevention Study, PAT-RHI was used in recently postmenopausal healthy women where it was shown to be useful tool for CV risk stratification and suggested as a method of assessing response to hormonal therapy (232). A literature search did not find any other studies which have used RHI to assess endothelial response to hormone therapy in women.
Figure 4. Data showing recordings from PAT showing endothelial response in subjects with (a) high hyperaemic response and (b) reduced hyperaemic response. Reprinted with permission from reference (233).

(a)  
(b)  

Occlusion phase

• **Arterial Stiffness**

In addition to endothelial function, a further regulator of arterial blood flow is vascular elasticity. Arterial stiffness is increased in those with coronary artery disease (234,235) and is a predictor of risk of CV events (234,236) and has therefore been used as a surrogate marker to assess CV risk. An estimate of arterial stiffness can be made using the technique of pulse wave analysis (PWA), which uses radial artery applanation tonometry (Sphygmocor, AtCor, Sydney Australia). This records peripheral pulse waveforms from which central arterial (usually the ascending aorta) waveforms can be derived using a transfer function. Analysis of the aortic waveform can then be used to derive estimates of arterial stiffness. A commonly used method of quantifying a surrogate measure of arterial stiffness is by calculating the augmentation index (Alx).
The arterial pressure waveform achieves its characteristic shape from a combination of the forward pressure wave produced by ventricular systole and the pressure from a reflected wave returning from the peripheral vasculature. In the case of increased arterial stiffness, the pulse wave velocity is increased and so the pressure from the reflected wave occurs earlier within the waveform. This can be quantified by the Augmentation Index (Alx), which is defined as the difference between the second and the first systolic peaks expressed as a percentage of pulse pressure (figure 5).

Although several methods for measuring arterial stiffness exist (237), PWA is a simple, non-invasive technique which has shown high levels of repeatability (238). Changes in Alx have been used in many studies to assess the effect of pharmacological interventions (239), including HT studies (240). Furthermore, assessment of the changes in Alx in response to GTN and salbutamol can assess endothelial dependent and independent vasodilatation and so can be used as a further measure of endothelial function (241).

There are no prior studies which have used Alx to assess the effect of postmenopausal testosterone. However studies have used Alx to assess the effect of testosterone replacement in men (242) and estrogen replacement in women (243) over similar time periods (3-4 months).

**Figure 5: Representation of the arterial waveform and calculation of Augmentation Index (Alx).** SBP systolic blood pressure, DBP diastolic blood pressure. Modified from (237,239)
SUMMARY OF LITERATURE REVIEW

Androgens play a crucial role in female physiology, however their exact role is poorly understood. They may act directly via the androgen receptor, which is found in many tissues, or indirectly, through conversion to estradiol in peripheral tissues. Androgen levels decline gradually with age and surgical menopause is associated with a 40-50% fall in testosterone levels. Androgen deficiency has been particularly associated with reduced sexual desire and testosterone is increasingly used as part of hormone replacement regimens. There are data showing significant benefit from postmenopausal testosterone replacement in terms of sexual function, well-being, quality of life, cognitive function and bone density. Despite the well-documented benefits, safety concerns exist, particularly regarding the lack of long-term safety data.

Theoretical concerns about the increased risk of endometrial or breast cancer have been raised due to peripheral conversion of testosterone to estradiol. There are a lack of long-term RCTs with these conditions as primary outcomes however current data do not point to an increased risk of breast or endometrial cancer from testosterone replacement.

Of greater concern are the potential CV effects of testosterone as it has traditionally been assumed that androgens have a detrimental effect on the CV system. However, recent data have highlighted the complexities of the CV effects of testosterone. At present, epidemiological data appears to point to a U-shaped association between endogenous testosterone levels and cardiovascular disease. The implications of these findings for postmenopausal use of testosterone are not well understood.

Although older studies of testosterone replacement pointed to detrimental effects on lipid profile and surrogate markers for CV risk, these studies involved high dose parenteral treatment. As with estrogen replacement there has been a move towards low dose, physiological hormone replacement via the transdermal route. Few studies have investigated the CV effects of postmenopausal testosterone, particularly via the transdermal route. Available data point to no adverse effect from physiological testosterone replacement on risk factors for CV disease such as blood pressure, lipid metabolism or fat distribution.
There are no studies investigating the effects of postmenopausal testosterone with CV events as a primary outcome. Pooled analysis of 5 phase 3 safety studies showed no increase in CV events (192). In the only study to investigate subcutaneous testosterone in PM women, implant therapy was associated with an improvement in endothelial function, potentially suggesting a beneficial CV effect (186).

A further important marker of CV risk is impaired glucose metabolism. Older studies using high dose parenteral testosterone have suggested a detrimental effect from androgens on glucose metabolism. Data involving transdermal physiological replacement have been more reassuring but have been limited to basic assessment of insulin resistance.

A summary of all studies identified involving transdermal or subcutaneous testosterone replacement can be found in table 3.

**Assessment of CV risk via surrogate markers**

Although a RCT with CV events as a primary endpoint would be considered the gold standard for assessing the CV effect of postmenopausal testosterone, this approach has inherent disadvantages, particularly the need for large-scale long-term follow-up. Surrogate markers for CVD are therefore frequently used as a more practical alternative.

The traditional risk factors for CVD, such as blood pressure and lipid profile, do not predict CV events as accurately in women as in men. Many other surrogate markers for CVD have been identified including measures of endothelial function and arterial stiffness, both of which have been shown to correlate well with future CV events.

Simple non-invasive methods of measuring endothelial function and arterial stiffness have been developed which correlate well with the gold standard techniques. The techniques used in the present study for assessing arterial stiffness (radial artery applanantion tonometry, Sphygmocor) and endothelial function (peripheral artery tonometry, Endopat) have not previously been used to assess the effect of
postmenopausal testosterone replacement but have been used to assess the effect of other hormonal therapies in both men and women.
Table 3. Summary of studies of exogenous transdermal/subcutaneous testosterone replacement

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Study type</th>
<th>Population</th>
<th>Primary route of T treatment</th>
<th>Primary outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braunstein (2005)</td>
<td>RCT</td>
<td>447 SM on E</td>
<td>TTP 24 weeks 150/300/450μg doses</td>
<td>Increased sexual activity and desire (PFSF) in 300ug dose</td>
<td>41</td>
</tr>
<tr>
<td>Buster (2005)</td>
<td>RCT</td>
<td>533 SM on E</td>
<td>TTP 24 weeks</td>
<td>Increased sexual activity and desire (PFSF), reduced distress</td>
<td>42</td>
</tr>
<tr>
<td>Davis (2006)</td>
<td>RCT</td>
<td>77 SM on E</td>
<td>TTP 24 weeks</td>
<td>Improved sexual desire on PFSF</td>
<td>38</td>
</tr>
<tr>
<td>Davis (2008)</td>
<td>RCT</td>
<td>814 NM and SM, not on HT</td>
<td>TTP 24 weeks 150/300μg doses</td>
<td>Increased sexual activity and desire (PFSF), reduced distress for 300μg dose</td>
<td>43</td>
</tr>
<tr>
<td>Davis (2009)</td>
<td>Retrospective cohort</td>
<td>631 PM women</td>
<td>Transdermal or implant</td>
<td>No increased risk breast cancer. RR 1.35 (95% CI 0.76-2.38)</td>
<td>86</td>
</tr>
<tr>
<td>Davis (2009b)</td>
<td>RCT</td>
<td>279, no HT</td>
<td>TTP 24 weeks 150/300μg doses</td>
<td>No effect on mammographic density</td>
<td>89</td>
</tr>
<tr>
<td>Fooladi (2014)</td>
<td>RCT</td>
<td>44 pre and PM women with SSRI/SNRI-emergent loss of libido</td>
<td>TTP 12 weeks</td>
<td>Increased satisfactory sexual events</td>
<td>56</td>
</tr>
<tr>
<td>Hofling (2007a+b)</td>
<td>RCT</td>
<td>99 PM on CEE/NET</td>
<td>TTP 24 weeks</td>
<td>Reduction in breast cell proliferation, no effect on mammographic density</td>
<td>87, 88</td>
</tr>
<tr>
<td>Iellamo (2010)</td>
<td>RCT</td>
<td>36 older PM with heart failure</td>
<td>TTP 24 weeks</td>
<td>Improved functional capacity, insulin resistance and muscle strength</td>
<td>213</td>
</tr>
<tr>
<td>Panay (2010)</td>
<td>RCT</td>
<td>272 NM, mainly not on HT</td>
<td>TTP 24 weeks</td>
<td>Increased sexual activity and desire (PFSF), reduced distress</td>
<td>55</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Participants</td>
<td>Intervention</td>
<td>TTP (weeks)</td>
<td>Outcome</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>Popat (2014)</td>
<td>RCT</td>
<td>145 POI</td>
<td>Transdermal gel, 150µg daily</td>
<td>12 weeks</td>
<td>No additional benefit from T at 12 weeks, but projected benefit at 3 years using repeated measures model</td>
</tr>
<tr>
<td>Shifren (2000)</td>
<td>RCT</td>
<td>75 SM on CE</td>
<td>Transdermal gel, 150/300µg doses</td>
<td>12 weeks</td>
<td>Increased sexual activity and pleasure-organism and psychological well-being</td>
</tr>
<tr>
<td>Shifren (2006)</td>
<td>RCT</td>
<td>549 NM on HT</td>
<td>Transdermal gel, 150/300µg doses</td>
<td>24 weeks</td>
<td>Increased sexual activity and desire (PFSF), reduced distress</td>
</tr>
<tr>
<td>Simon (2005)</td>
<td>RCT</td>
<td>562 SM on E</td>
<td>Transdermal gel, 100µg daily</td>
<td>24 weeks</td>
<td>Increased sexual activity (x2 episodes/wk) and desire (PFSF), reduced distress</td>
</tr>
<tr>
<td>Vitale (2008)</td>
<td>Pooled analysis on 5 RCTs</td>
<td>2795 NM and SM, with and without HT</td>
<td>Transdermal gel, 150/300µg doses</td>
<td>24-52 weeks</td>
<td>No effect on CV events or risk factors (lipids, insulin, glucose, BP), except reduction in HDL increases</td>
</tr>
</tbody>
</table>

**Gel/cream**

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Participants</th>
<th>Intervention</th>
<th>TTP (weeks)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davis (2014)</td>
<td>RCT</td>
<td>92 PM women, no HT</td>
<td>Transdermal gel, 300µg daily</td>
<td>26 weeks</td>
<td>Improved verbal learning and memory</td>
</tr>
<tr>
<td>Gruber (1998)</td>
<td>RCT</td>
<td>39 NM women not on HT</td>
<td>Transdermal gel, 24 weeks</td>
<td></td>
<td>Reduction in BMI, total body weight and abdominal fat</td>
</tr>
<tr>
<td>Nathorst-Boos (2006)</td>
<td>RCT, cross over</td>
<td>53 PM on HT</td>
<td>Transdermal gel, 10µg daily</td>
<td>24 weeks</td>
<td>Increased sexual activity, desire, orgasm and arousal. Improved psychological well-being</td>
</tr>
<tr>
<td>Shah (2006)</td>
<td>RCT</td>
<td>76 PM on E</td>
<td>Transdermal gel, 400µL daily</td>
<td>16 weeks</td>
<td>Improved immediate and delayed visual and verbal memory, unaffected by AI</td>
</tr>
<tr>
<td>Study</td>
<td>Study Design</td>
<td>N</td>
<td>Interventions</td>
<td>Outcomes</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
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<td>-------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>White (2012)</td>
<td>RCT</td>
<td>3-4000</td>
<td>Transdermal gel, 0.22g/d, &gt;12 months</td>
<td>Composite of CV events - results not yet reported.</td>
<td></td>
</tr>
<tr>
<td>Witherby (2011)</td>
<td>Open label pilot</td>
<td>21 PM women with BC and AI induced vaginal atrophy</td>
<td>Vaginal cream 300/150μg doses daily for 4 weeks</td>
<td>Improvement on dyspareunia, dryness, vaginal pH and maturation index with 300ug dose</td>
<td></td>
</tr>
<tr>
<td><strong>Implant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burger (1987)</td>
<td>RCT</td>
<td>22 PM</td>
<td>T 50mg implant, 6 weeks</td>
<td>Improved libido</td>
<td></td>
</tr>
<tr>
<td>Cardozo (1984)</td>
<td>Observational</td>
<td>120 PM</td>
<td>E(50mg) + T(100mg) implants</td>
<td>Improved vasomotor symptoms, libido and mood</td>
<td></td>
</tr>
<tr>
<td>Davis (1995)</td>
<td>RCT</td>
<td>34 PM, on E</td>
<td>T implant 50mg 3-monthly 24 months</td>
<td>Improvement in bone density and sexual function</td>
<td></td>
</tr>
<tr>
<td>Davis (2000)</td>
<td>RCT</td>
<td>33 PM on HT</td>
<td>50mg T implants every 3 months 2 years</td>
<td>Increased lean body mass</td>
<td></td>
</tr>
<tr>
<td>Dimitrakakis (2004)</td>
<td>Retrospective observational</td>
<td>508 PM</td>
<td>T Implants 5-150mg, 5monthly</td>
<td>No increased rates of breast cancer. Incident rates E+T 115/100,000, E/P+T 293/100,000</td>
<td></td>
</tr>
<tr>
<td>Garnett (1992)</td>
<td>RCT</td>
<td>50 PM on E</td>
<td>T implant 100mg 6-monthly for 12 months</td>
<td>No increase in bone density c/t E alone</td>
<td></td>
</tr>
<tr>
<td>Glaser (2011)</td>
<td>Cohort study</td>
<td>300 pre- and PM, no HT</td>
<td>T implant 75-160mg</td>
<td>Improvement in psychological, somatic and urogenital symptoms</td>
<td></td>
</tr>
<tr>
<td>Van Staa (2009)</td>
<td>Observational case-control</td>
<td>2103 T users, 6309 controls</td>
<td>Implant (72.2%), oral (18.4%), IM (7.9%)</td>
<td>No increased risk of breast cancer. RR breast cancer 0.78 (95% CI 0.44-1.37)</td>
<td></td>
</tr>
<tr>
<td>Worboys (2001)</td>
<td>Case-control</td>
<td>33 PM women on HT</td>
<td>T implant 50mg 6 weeks</td>
<td>Improvement in endothelium dependent and independent vasodilation</td>
<td></td>
</tr>
</tbody>
</table>
AI aromatase inhibitor, BC breast cancer, BP blood pressure, CEE conjugated equine estrogens, CI confidence interval, CV cardiovascular, E estrogen, HDL high density lipoprotein, HT hormone therapy, IM intramuscular, NM naturally menopausal, NET norethisterone, PFSF profile of female sexual function, PM postmenopausal, POI premature ovarian insufficiency, RCT randomised controlled trial, RR relative risk, SM surgically menopausal T testosterone, TTP transdermal testosterone patch.
STUDY AIMS AND OUTCOME MEASURES

The present study aimed to investigate the effect of the TTP on CV health and libido.

The primary outcome measures were defined as changes in arterial stiffness (AIx), endothelial function (AIx in response to GTN and salbutamol, and RHI) and insulin resistance (HOMA-IR).

Secondary outcome measures were defined as changes in sexual function (B-PFSF), blood pressure, body composition (weight, BMI, waist and hip circumference), and lipid metabolism (total cholesterol, HDL cholesterol, lipoprotein (a)).

HYPOTHESES

- Transdermal testosterone, in conjunction with HRT, will not have adverse effect on insulin resistance or vascular function in postmenopausal women.

- Transdermal testosterone will significantly improve sexual well-being in postmenopausal women.
METHODOLOGY

Trial participants

The study was approved by the West London Research Ethics Committee, UK medicines regulatory authorities (MHRA) and NHS trusts. It was registered with the National Agency for Medicine (EudraCT 2009-013275-21). The study was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice. All participants gave written informed consent prior to any study procedures.

Subjects were recruited from hospital endocrinology/menopause clinics between February 2011 and November 2011 and all study visits took place at the Royal Brompton NHS Foundation Trust, London. Participants were healthy menopausal women aged between 45-70 years, on a stable HT regimen and complaining of symptoms of low libido. Women with a history of recent testosterone use (<3 months transdermal or <12 months subcutaneous) were excluded.

Other exclusion criteria included dyspareunia, medications which may interfere with the study (Selective Serotonin Reuptake Inhibitors (SSRIs), anti-androgens, Phosphodiesterase type 5 (PDE5) inhibitors, dihydroepiandrosterone (DHEA), selective estrogen receptor modulators (SERMS)), current warfarin use, significant psychiatric co-morbidity, history of breast cancer, diabetes mellitus, thrombo-embolic disorders, cardiovascular disease, any condition affecting carbohydrate metabolism, uncontrolled hypertension and uncontrolled hyperlipidaemia, current use of tibolone (due to its androgenic effect) or hypersensitivity to testosterone (table 4).
Table 4. Study exclusion criteria.

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyspareunia</td>
</tr>
<tr>
<td>Medications which may interfere with the study</td>
</tr>
<tr>
<td>SSRIs, anti-androgens, PDE5 inhibitors, DHEA, SERMS, tibolone</td>
</tr>
<tr>
<td>Current use of warfarin</td>
</tr>
<tr>
<td>Significant psychiatric co-morbidity</td>
</tr>
<tr>
<td>History of medical co-morbidities:</td>
</tr>
<tr>
<td>• breast cancer    diabetes mellitus</td>
</tr>
<tr>
<td>• thrombo-embolic disorders cardiovascular disease</td>
</tr>
<tr>
<td>• uncontrolled hypertension uncontrolled hyperlipidaemia</td>
</tr>
<tr>
<td>• any condition affecting carbohydrate metabolism</td>
</tr>
<tr>
<td>Hypersensitivity to testosterone</td>
</tr>
</tbody>
</table>

Following the baseline visit, participants received the 300mcg transdermal testosterone patch (Intrinsa®, Warner Chilcott) for 12 weeks, with study visits after 6 weeks and 12 weeks of testosterone use. The TTP is worn on the abdomen and patches are replaced every 3.5 days.

Visits were performed in a temperature and noise controlled environment at the same time of day. Subjects were fasted for 12 hours prior to visits and were asked to abstain from caffeine or alcohol for 6 hours prior to study visits. Measurements of endothelial function and arterial stiffness were performed prior to blood samples and anthropometric measurements. All participants had a normal physical, pelvic and breast examination at baseline visit. All study assessments were carried out by one investigator to avoid inter-observer variability. Full details of investigations carried out at each visit can be seen in table 5.
Table 5. Details of procedures undertaken at each study visit.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Visit 1 (Start)</th>
<th>Visit 2 (Week 6)</th>
<th>Visit 2 (Week 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement of blood pressure, temperature, heart rate, weight, waist and</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>hip measurements, height.</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Demographic data recorded.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Medical and gynaecological history.</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Examination of facial and scalp hair, acne and voice depth.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Physical examination including breast and pelvic examination.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Hormone profile and lipid profile.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Liver function test and full blood count</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Fasting glucose and insulin.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Arterial compliance measurements.</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Endothelial function assessment</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>BPFSF completed.</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>General assessment of well-being. Asked about any current medication and</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>any side effects.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dispensing of patches.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Return unused patches.</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

1Only on Visit 1.

**Radial artery applanation tonometry**

KM performed all measurements, which were undertaken in a quiet, temperature-controlled room. Blood pressure was measured at the brachial artery, in the supine position of the contra-lateral arm being used for using for assessment of vascular function, after a 10 minute rest.

Peripheral pressure waveforms were captured using radial artery applanation tonometry via the SphygmoCor apparatus (AtCor Medical Ltd., Sydney, Australia; software version 8.0). The central (ascending aortic) pressure waveform was then
derived from an averaged peripheral waveform using a validated, transfer function (244). The augmentation index (Alx), which gives a composite measure of wave reflection and systemic arterial stiffness can then be calculated by analysis of the central waveform. Alx was defined as the difference between the first and second systolic peaks of the central pressure waveform, expressed as a percentage of the central pulse pressure. Only measurements which achieved a quality index of 90% or greater were included in the analysis. Baseline measurements were repeated and an average of 3 measurements taken. As Alx is dependent on heart rate, it was normalized to a heart rate of 75 beats per minute for comparisons.

Pulse wave analysis was also used to assess endothelial function. This was carried out by assessing the changes in Alx in response to glyceryl trinitrate (GTN), an endothelium-independent vasodilator, and salbutamol, an endothelium-dependent β2-adrenoceptor agonist, using a validated method (242). Endothelial dysfunction is characterized by attenuation of endothelium-dependent vasodilatation.

Subjects were given sublingual GTN (250 µg) and recordings for Alx were taken from baseline to 20 minutes post administration. Following a 30 minute washout period, salbutamol 400µg was given by supervised inhalation (Ventolin Rotacap, Allen & Hanbury’s, Uxbridge, United Kingdom) after initial demonstration. The empty cap was inspected after each inhalation to ensure complete drug delivery. Alx measurements were then recorded again from baseline to 20 minutes after inhalation. The response to salbutamol or GTN was defined as the maximum change in Alx after drug administration as per previous studies (245,246). An endothelial function index was then calculated, expressing endothelium-dependent vasodilatation (salbutamol) as a percentage of endothelium-independent vasodilatation (GTN).

**RH-PAT**

Endothelial function was further assessed by measuring digital pulse volume changes in response to reactive hyperaemia, using PAT technology (EndoPAT® 2000, Itamar Medical Ltd, Israel). KM performed all measurements in a quiet, temperature controlled
environment with subjects in fasted state. The RH-PAT technique was carried out as described in prior studies (229,247).

Subjects lay in a supine position and a PAT finger probe was placed on each index finger with both hands at the same level (figure 6). Finger probes consist of inflatable latex air-cushions within a rigid cover which apply constant counter pressure to the fingertip. Volume changes were detected by a pressure transducer and transferred to computer. A blood pressure cuff was placed on one upper arm while the other arm acted as the control arm. After an initial stabilisation period, a baseline measurement was taken for at least 5 minutes, followed by a 5 minute occlusion period, where a blood pressure cuff was inflated to 60mmHg above the systolic blood pressure. After cuff release, post-occlusion monitoring continued for a further 5 minutes. From the data, the Reactive Hyperaemia Index (RHI) was calculated automatically by the EndoPAT 2000 computer algorithm from the ratio of pulse wave amplitude before and after ischaemia, compared to the control arm. A lower RHI was indicative of endothelial dysfunction.

Figure 6. Representation of the Endopat finger probes and study set-up. Reprinted with permission from reference (233).
**Glucose metabolism and HOMA-IR**

Blood samples were taken for fasting glucose and insulin levels. From these results, insulin resistance was then estimated using the updated homeostasis model assessment method for insulin resistance (HOMA-IR) computer algorithm (248,249).

The HOMA-IR model, first described in 1985 (250), uses fasting glucose and insulin concentrations to derive a surrogate index of insulin resistance. It has been shown to correlate well with the more invasive gold standard technique for assessing insulin resistance – the hyperinsulinaemic euglycaemic glucose clamp method (249). The hyperinsulinaemic euglycaemic clamp method requires insertion of 2 intravenous catheters, trained staff and is time consuming (251). Several other methods of assessing insulin sensitivity exist (252) but the HOMA-IR model provides an easy, inexpensive tool by which to assess longitudinal changes to IR in a clinical trial setting.

**Hormone, biochemical and haematological parameters**

All blood samples were performed in a fasted state.

Hormones, including testosterone, were measured by an electro-chemiluminescent assay (Roche Diagnostics Ltd, Burgess Hill, UK). Inter- and intra-assay percentage coefficients of variation were <5% for testosterone, <5% for insulin, <7% for estradiol and <4% for SHBG. Free androgen index (FAI) was calculated using the equation: FAI = (testosterone (nmol/l)/SHBG (nmol/l)) x 100.

Total cholesterol, HDL cholesterol and liver function tests were assayed using a Beckman DxC600 analyser (Beckman Coulter, High Wycombe, Bucks, UK) and lipoprotein (a) on a Beckman Immage nephelometer (Beckman Coulter, High Wycombe, Bucks, UK).
Assessment of female sexual function

Libido was assessed at each visit using the Brief profile of female sexual function (B-PFSF), a validated self-administered questionnaire (253) for identifying HSDD. The B-PFSF is based on 7 questions (see table 6). Each question is scored on a 6-point scale from ‘always’ to ‘never’. A total score is calculated from a sum of all questions. Previous studies have identified a score of less than 20 as suggestive of HSDD (253).

Table 6. The brief profile of female sexual function questionnaire

<table>
<thead>
<tr>
<th></th>
<th>Never</th>
<th>Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>Very often</th>
<th>Always</th>
</tr>
</thead>
<tbody>
<tr>
<td>I felt like having sex</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I was unhappy about my lack of interest in sex</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Getting aroused took forever</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>I felt sexually numb</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>I lacked sexual desire</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>I felt disappointed by my lack of interest in sex</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>I reached orgasm easily</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Anthropometric measurements and adverse events

Anthropometric measurements were performed in light clothing without shoes. Waist circumference was measured to the nearest 0.5 centimetres (cm) with a tape measure placed at the midpoint between the lower margin of the last palpable rib and the iliac crest, as per World Health Organisation (WHO) guidance (254). Hip circumference was measured to the nearest 0.5cm at the maximal circumference over the buttocks with the
tape parallel to the floor. Weight was measured in light clothing to the nearest 0.1 kilograms (kg) on a digital scale. Height was measured to the nearest centimetre using a wall-mounted stadiometer. Body mass index was calculated as weight (kg) divided by height (m) squared.

At each visit an assessment of adverse effects and androgenic side effects was performed. Facial acne was graded using the Palatsi scale from 0 (none) to 4 (severe acne) (appendix 1). Assessment of facial hirsutism was performed using the Lorenzo Scale (appendix 2) with grades 0 (no hair) to 4 (heavy hair growth) at the upper lip and chin. Subjects were questioned as to whether they had noticed any changes in their voice, patch site reactions, unscheduled bleeding or other adverse events.

Validation of data

- **Arterial Stiffness**

PWA using radial artery applanation tonometry is operator dependent, although previous studies have shown high levels of intra- and inter-observer repeatability, even in those with limited experience (238)

As one investigator performed all measurements, no assessment of inter-observer variability was performed. Intra-observer variability was assessed by performing repeated AIx measurements on 5 healthy controls, prior to subject recruitment, and compared to previously reported results. The mean difference ± SD between repeated AIx measurements was 0.3 ± 0.8 (figure 7) and coefficient of variation 21.05%.

This compared favourably to previous studies where intra-observer variability of 0.1±8 (238), 1.5±7.0 (255), and 1.5 ± 1.1 (256) have been observed.

- **RH-PAT**

EndoPAT measurements are analysed by a computerized automated algorithm in an operator independent manner and therefore inter-observer variability is minimal
Prior studies have confirmed a high repeatability of EndoPAT measurements, with repeat testing on two days showing high correlation under controlled conditions (257,258).

**Figure 7. Bland-Altman plot of Alx repeatability data.** The difference between paired Alx measurements is plotted against their average. The mean difference and 2 standard deviations are marked.

**Statistical Analyses Plan**

- **Sample Size Calculation**

There were a lack of previous data on which to base accurate power calculations. No previous studies had examined the effect of testosterone replacement in women using Alx or RHI. A pilot study investigating the effect of 3 months of transdermal testosterone gel in hypogonadal men on Alx found no significant change (242). The
present study was therefore designed to provide data to aid power calculations for future studies.

Sample size calculations estimated that recruitment of 18 participants into the study would provide over 80% power to detect a shift of 1 standard deviation at the 5% level. These calculations were based on data from McEniery et al. (255) who showed a mean Alx ± standard deviation of 33 ± 9 in 495 50-59 year old women and Kuvin et al. (227) who demonstrated mean RHI of 1.6 ± 0.2 in 20 postmenopausal women. We therefore aimed to recruit at least 20 subjects to allow for possible dropouts.

- **Data analysis**

Analyses of efficacy and safety data were performed after all participants had completed their last visit.

Analysis was performed using SPSS v20.0. All demographic data have been summarised for the participants. For categorical variables, the number and percent of patients in each category is presented. Continuous variables such as age, hormone levels and vascular assessments are summarised by presenting the sample size, mean and standard deviation or median and range.

Baseline and post treatment outcomes have been compared by paired Student’s t tests on normalised data or Wilcoxon’s signed rank test for non-parametric comparisons. Inter-dependencies between variables, for example the association between age, body mass index (BMI), hormone levels, lipids, Alx and RHI, were explored using Spearman’s linear correlation analysis (for non-parametric data) or Pearson’s correlation analysis (parametric data) and linear regression analysis. For the primary outcomes (Alx and RHI) p< 0.05 was considered statistically significant.
RESULTS

Baseline Characteristics

22 subjects were recruited into the study. One subject withdrew consent following the first visit and did not commence treatment. 21 subjects completed the study. The data presented are therefore based on analysis of 21 subjects unless otherwise stated.

The baseline characteristics of subjects are presented in Table 7. No subjects were currently using anti-hypertensives. One subject was using a lipid-lowering agent and one was a current smoker of 15-20 cigarettes per day.

Mean BMI was within the normal range based on WHO classification. Mean baseline HOMA-IR was 0.78 (range 0.4-1.6) with only 4 subjects having a very mild impairment of insulin sensitivity.

Although symptoms of low libido were required to participate in the study, based on the B-PFSF scores, one third of subjects did not fulfil the diagnostic criteria for HSDD (usually considered to be a score <20).

Baseline arterial stiffness and endothelial function compared similarly to data in age matched populations. McIneiry et al. (255) have previously demonstrated a mean Alx of 33±9 in 495 50-59 year old women. Kuvin et al. (227) have shown a mean RHI of 1.7±0.1 in 35 women with a mean age of 53 years.
Table 7. Clinical and demographic characteristics of participants at baseline (n=21). Values are expressed as mean (SD) or median (range).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.0 (6.7)</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>1 (4.8%)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.2 (11.1)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24.5 (4.7)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>85.6 (11.4)</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>101.6 (8.5)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>114.6 (11.8)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73.2 (9.5)</td>
</tr>
<tr>
<td>B-PFSF</td>
<td>15.3 (9.1), 15 (0-33)</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>332.0 (296.0)</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>0.39 (0.31)</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>76.0 (36.3)</td>
</tr>
<tr>
<td>FAI</td>
<td>0.56 (0.40)</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>40.38 (24.62)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.05 (0.66)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.78 (0.40)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.65 (0.79)</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.83 (0.59)</td>
</tr>
<tr>
<td>Tot chol:HDL</td>
<td>3.31 (0.96)</td>
</tr>
<tr>
<td>Lipoprotein(a) (mg/dL)</td>
<td>29.9 (41.9)</td>
</tr>
<tr>
<td>RHI</td>
<td>1.79 (0.36)</td>
</tr>
<tr>
<td>AIx (%)</td>
<td>24.21 (10.70)</td>
</tr>
<tr>
<td>Fall in AIx after GTN</td>
<td>-18.55 (6.3)</td>
</tr>
<tr>
<td>Fall in AIx after salbutamol</td>
<td>-2.9 (5.2)</td>
</tr>
<tr>
<td>Endothelial function index</td>
<td>14.43 (29.82)</td>
</tr>
</tbody>
</table>

- **HT preparations**

A summary of the HT preparations used by subjects can be seen in table 8. No patients were using CEE. Of the 21 patients included for analysis, five were using estradiol alone due to prior hysterectomy (three using estradiol transdermal patch, two using estradiol implant). Of those using combined EP HT this was mainly transdermal estradiol (gel or patch) with the levonorgestrel intrauterine system (LNG-IUS) (seven patients) or
micronized progesterone (six patients). Only three patients were using other progestogens (Kliovance® (oral estradiol/NET), Femseven conti® (transdermal estradiol/LNG), Elleste duet® (oral estradiol/NET)). Only two patients were using oral estradiol.

Table 8. Summary of HRT preparations used by subjects

<table>
<thead>
<tr>
<th>Type of HRT preparation</th>
<th>Estradiol only</th>
<th>Estradiol + progestogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transdermal</td>
<td>3</td>
<td>Transdermal E₂ + levonorgestrel 8</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>2</td>
<td>Transdermal E₂ + progesterone 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral E₂ + norethisterone 2</td>
</tr>
</tbody>
</table>

Impact of testosterone on:

- Sexual function

Compared to baseline measurements, scores on the B-PFSF questionnaire improved significantly by a mean of 3.7 points by 6 weeks (p<0.05) and 5.05 points by 12 weeks (p<0.0001). Significant improvements in B-PFSF score were also seen when subjects with and without HSDD were analysed separately (table 9).

There was no correlation observed between change in B-PFSF score and changes in FAI, total testosterone or SHBG, either in the total population, or when the subjects with HSDD were analysed separately.

Additionally, there was no correlation between baseline B-PFSF score and total testosterone/FAI/SHBG. However, when the HSDD group was analysed separately there was an inverse relationship between B-PFSF score and total testosterone (r 0.56, correlation coefficient (cc) -0.665, p<0.01) but not FAI.
Figure 8. Box plot showing median + interquartile range for B-PFSF at each visit.

![Box plot showing median + interquartile range for B-PFSF at each visit.](image)

Table 9. Change in B-PFSF in subgroups with and without HSDD

<table>
<thead>
<tr>
<th></th>
<th>Mean difference in B-PFSF</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSDD (n=14)</td>
<td>5.36</td>
<td>1.9-8.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>No HSDD (n=7)</td>
<td>4.43</td>
<td>0.7-8.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
• **Hormone levels**

A summary of the observed changes in various serum hormone levels can be seen in table 10. These results show that 12 weeks of transdermal testosterone resulted in significantly increased total testosterone (p<0.0001) and FAI (p<0.001). Levels were significantly increased by 6 weeks of testosterone therapy (p<0.001 for both total testosterone and FAI). No significant changes were observed in estradiol, FSH or SHBG levels.

**Table 10. Change in serum hormone levels following 12 weeks of testosterone therapy.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean difference</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (U/L)</td>
<td>9.60</td>
<td>-1.1 to 20.3</td>
<td>0.077</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>-89.4</td>
<td>-245.0 to 67.2</td>
<td>0.349</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>-5.50</td>
<td>-16.5 to 5.5</td>
<td>0.099</td>
</tr>
<tr>
<td>Total Testosterone (nmol/L)</td>
<td>1.26</td>
<td>0.8 to 1.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FAI</td>
<td>2.04</td>
<td>1.1 to 3.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

• **Anthropometric and metabolic characteristics**

No significant change was observed in weight, BMI or waist circumference over the duration of the study. Hip circumference significantly reduced (p<0.05). No changes were observed in systolic or diastolic blood pressure (table 11).

Although total cholesterol and total cholesterol:HDL ratio were unchanged, there was a small but significant decrease in HDL cholesterol (p<0.05). There also appeared to be a small but significant reduction in lipoprotein (a) levels (mean difference -3.11, p<0.05)

Fasting glucose and insulin measurements were used to derive an estimate of insulin resistance using the HOMA-IR. No changes to fasting insulin, fasting glucose or insulin resistance were observed.

There were no alterations in liver enzymes following testosterone therapy.
Table 11. Effect of 12 weeks testosterone therapy on anthropometric and metabolic characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Mean difference</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>0.443</td>
<td>-0.18 to 1.07</td>
<td>0.211</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.159</td>
<td>-0.38 to 0.07</td>
<td>0.218</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>-0.262</td>
<td>-1.38 to 0.86</td>
<td>0.661</td>
</tr>
<tr>
<td>Hip Circumference (cm)</td>
<td>-0.738</td>
<td>-1.42 to -0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>-2.52</td>
<td>-7.02 to 1.98</td>
<td>0.265</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>1.81</td>
<td>-2.80 to 6.42</td>
<td>0.342</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/L)</td>
<td>0.053</td>
<td>-0.12 to 0.23</td>
<td>0.533</td>
</tr>
<tr>
<td>Fasting Insulin (pmol/L)</td>
<td>5.809</td>
<td>-10.66 to 22.27</td>
<td>0.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.106</td>
<td>-0.20 to 0.41</td>
<td>0.705</td>
</tr>
<tr>
<td>Total cholesterol (nmol/L)</td>
<td>-0.0053</td>
<td>-0.24 to 0.23</td>
<td>0.964</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>-0.251</td>
<td>-0.51 to 0.0071</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total chol:HDL</td>
<td>-0.776</td>
<td>-0.54 to 0.38</td>
<td>0.689</td>
</tr>
<tr>
<td>Lipoprotein (a) (mg/L)</td>
<td>-3.11</td>
<td>-76.32 to 13.75</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Bilirubin (umol/L)</td>
<td>-0.714</td>
<td>-3.56 to 2.13</td>
<td>0.888</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>-0.619</td>
<td>-6.90 to 5.66</td>
<td>0.42</td>
</tr>
</tbody>
</table>

- Endothelial function and arterial stiffness
  - Pulse wave analysis

20 subjects had complete arterial stiffness data as obtained by radial artery applanation tonometry and were therefore included in the analysis as presented in table 12. There was no difference between baseline AIx and AIx following 3 months of testosterone therapy (mean difference 1.067, 95% CI -3.85 to 1.72, p=0.433). A fall in AIx was noted after both salbutamol and GTN administration and there was a significant increase in salbutamol-mediated vasodilatation by 3 months (p=0.029).

Change in AIx after GTN and salbutamol administration was used to derive a further measure of endothelial function using the formula:

$$EFI = \frac{AIx \text{ before salbutamol administration} - AIx \text{ after salbutamol administration}}{AIx \text{ before GTN administration} - AIx \text{ after GTN administration}} \times 100\%$$
Although there was an increase in EFI (improved endothelial function) of 11.3% at month 3, this did not reach statistical significance (95% CI -3.175 to 25.79, p=0.62).

Table 12. Summary of results obtained from radial artery applanation tonometry

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 months</th>
<th>Change</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alx</td>
<td>23.30</td>
<td>24.37</td>
<td>1.067</td>
<td>-3.85 to 1.72</td>
<td>0.433</td>
</tr>
<tr>
<td>EFI</td>
<td>14.43</td>
<td>25.74</td>
<td>11.31</td>
<td>-3.18 to 25.79</td>
<td>0.62</td>
</tr>
<tr>
<td>Δ Alx salbutamol</td>
<td>-2.90</td>
<td>-5.07</td>
<td>2.167</td>
<td>-0.49 to 4.83</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Δ Alx GTN</td>
<td>-18.55</td>
<td>-20.47</td>
<td>1.917</td>
<td>-1.26 to 5.09</td>
<td>0.126</td>
</tr>
</tbody>
</table>

- RH-PAT

17 subjects had valid data for assessment of endothelial function using RHI. Due to machine error 4 subjects did not have both pre- and post- treatment readings. There was no observed change in baseline RHI following 3 months of testosterone therapy (mean difference 0.06, 95% CI -0.19 to 0.31, p=0.612)

- Subgroup Analysis

Subgroup analysis by time since menopause showed no significant difference between change in Alx, EFI or RHI between those ≤ 5 years postmenopausal (n=16) and those > 5 years postmenopausal (n=5) (table 13) The small numbers in each of these subgroups limit definitive conclusions.

Subgroup analysis by type of menopause (surgical n=5), natural (n=16) showed no significant difference between Alx (p=0.129), EFI (p=0.132) or RHI (p=0.557) but analysis was also limited by the small subgroup numbers.

Analysis was also performed on women using combined HT (E+P, n=16) and estrogen alone (E, n=5)(table 14). Change in B-PFSF score remained significant in the E+P group but not E alone. None of the outcome measures reached statistical significance in the E
alone group as the numbers were limited. A significant improvement in endothelial function as assessed by applanation tonometry was noted in the E+P group when analysed separately. This is unexpected and therefore needs to be interpreted with caution due to the small numbers.

Table 13. Comparison of vascular changes in sub groups menopause ≤ 5 years (n=16) and >5 years (n=5).

<table>
<thead>
<tr>
<th></th>
<th>Mean difference</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIx</td>
<td>-3.02</td>
<td>-9.49 - 3.44</td>
<td>0.339</td>
</tr>
<tr>
<td>EFI</td>
<td>20.47</td>
<td>-12.50 - 53.44</td>
<td>0.209</td>
</tr>
<tr>
<td>RHI</td>
<td>0.542</td>
<td>-0.55 - 0.65</td>
<td>0.849</td>
</tr>
</tbody>
</table>

Table 14. Comparison of B-PFSF and vascular changes depending on type of HT

<table>
<thead>
<tr>
<th></th>
<th>E+P (n=16)</th>
<th>E only (n=5)</th>
<th>Between group p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>B-PFSF</td>
<td>6.19</td>
<td>3.34 to 9.04</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Total chol</td>
<td>0.157</td>
<td>-0.83 to 1.14</td>
<td>0.736</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.06</td>
<td>0.51 to 0.38</td>
<td>0.777</td>
</tr>
<tr>
<td>Lipo (a)</td>
<td>-5.071</td>
<td>-38.28 to 28.14</td>
<td>0.747</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.193</td>
<td>-0.33 to 0.72</td>
<td>0.4404</td>
</tr>
<tr>
<td>AIx</td>
<td>-0.06</td>
<td>-3.4 to 3.3</td>
<td>0.973</td>
</tr>
<tr>
<td>EFI</td>
<td>17.53</td>
<td>0.99 to 34.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RHI</td>
<td>0.02</td>
<td>-0.3 to 0.3</td>
<td>0.916</td>
</tr>
</tbody>
</table>
**Correlation/Regression**

- **Baseline**

There were no correlations with baseline Alx or RHI and hormonal, metabolic or vascular parameters.

There was a significant correlation between total cholesterol and FAI, \( r = 0.49, \) \( cc = -0.236, \) \( p < 0.05 \)(figure 9). There was also an inverse relationship observed between baseline SHBG levels and fasting glucose \( r = 0.44, \) \( cc = -0.564, \) \( p < 0.05 \).

The 2 measures of endothelial function (EFI and RHI) were positively correlated \( r = 0.56, \) \( cc = 0.496, \) \( p < 0.05 \)(figure 10).

- **Post treatment**

Changes in FAI, total testosterone and SHBG did not correlate with changes in RHI, EFI or salbutamol or GTN mediated vasodilatation.

There was no correlation with change in FAI/TT and anthropometric measurements, metabolic parameters or vascular assessments. There was a correlation \( r = 0.56, \) \( cc = -0.610, \) \( p < 0.005 \) between change in Alx and SHBG (figure 11), such that a reduction in SHBG was associated with worsening of Alx, but with no other vascular, metabolic or hormonal parameters. There was also an inverse association between change in SHBG levels and change in waist circumference \( r = 0.28, \) \( cc = -0.454, \) \( p < 0.05 \).
Figure 9. Correlation of baseline total cholesterol with FAI.

Figure 10. Correlation of the 2 baseline measures of endothelial function
Additional effects and adverse events

Subjectively, following 12 weeks of testosterone therapy 11 (52%) participants reported improved energy levels, 8 (38%) reported improved libido, 11 (14%) reported improved mood, 2 (9.5%) improved concentration and 1 improved headaches (4.8%). 6 (29%) participants reported that their overall effect from the testosterone patches was neutral during the study period. 6/14 (42.9%) of those with HSDD versus 2/7 (28.6%) without reported a subjective improvement in libido. This difference was not statistically significant (p=0.66), however the small subgroup numbers limit the analysis.

7 (33%) participants experienced adverse events (AE), all of which were classified as mild AEs and did not result in discontinuation of treatment (table 15). 4 (19%) reported
skin irritation due to the patches, 2 (9.5%) reported an increase in facial hair (both gained 1 point on Lorenzo scale from baseline), 1 (4.8%) increased acne (classified as mild on Palatsi scale), 1 (4.8%) per vagina spotting (transvaginal ultrasound showed thin endometrium of less than 4mm) and 1 (4.8%) patient had 2 episodes of blepharitis.

Table 15. Subjective effects of 12 weeks of transdermal testosterone. n (%)
DISCUSSION

CVD is the leading cause of mortality in post-menopausal women and the increase in CVD after menopause has led to much interest in the role of sex steroids in its pathogenesis. The potential cardioprotective role of androgens is being increasingly recognised, however the implications for exogenous androgen therapy have not been well studied and the effects of androgens remain poorly understood, particularly in women. This is the first study to investigate the effects of the transdermal testosterone patch, in conjunction with HT, on endothelial function and arterial stiffness as surrogate markers of cardiovascular risk.

- Vascular effects

In the present study, transdermal testosterone was associated with no significant change in arterial stiffness as assessed by PWA or endothelial function as assessed by RH-PAT. However, PWA demonstrated a significant improvement in endothelium-dependent (salbutamol-mediated) vasodilation after 12 weeks treatment and an 11.3% improvement in overall endothelial function, although this was not statistically significant.

In the only previous study investigating the effect of postmenopausal testosterone on endothelial function by Worboys et al, significant improvements in both endothelium dependent and independent vasodilatation were observed after 6 weeks of testosterone implant therapy as assessed by FMD(186). This study involved the use of parenteral testosterone resulting in supraphysiological doses, which may act to explain in part why physiological transdermal replacement in the present study showed lesser vascular effect.

Testosterone has been recognised to act as a vasodilator in both animal and human models but the underlying mechanisms are complex and conflicting data exist. In the present study the improvement of vasodilatation following salbutamol rather than GTN after testosterone therapy points to an endothelium dependent mechanism.
Animal data suggest that the vasodilatory effects of testosterone are mainly through endothelium independent mechanisms, mediated for example via changes in smooth muscle cell ion channels or genomic effects via the androgen receptor (259–266). Endothelium dependent effects, mediated for example by nitric oxide, have also been reported (164,267,268). It has been suggested that the effect is dose dependent, with endothelial dependent vasodilatation occurring with physiological testosterone levels and endothelium-independent vasodilatation at supraphysiological levels (124).

There are limited data in humans exploring the mechanism of this effect and it is unclear whether there may be gender differences. Studies in men with coronary artery disease suggested both endothelium dependent (269) and independent mechanisms (270). There is evidence to support a gender difference in the effects of testosterone as men have greater AR expression in the vasculature than women (271), therefore results from male studies cannot necessarily be applied to women.

In summary, available data point towards a beneficial vascular effect of testosterone but further large-scale data are required. Reassuringly, in view of the concern regarding the potential deleterious CV effects of testosterone as cited by regulatory authorities (3), physiological replacement appears to have no adverse impact on endothelial function or arterial stiffness.

- **Biochemical effects**

This study found a significant reduction in lipoprotein (a) levels at the expense of a small but significant reduction in HDL cholesterol. Lipoprotein (a) is a strong, independent risk factor for cardiovascular disease (272) acting as a pro-atherogenic, pro-thrombotic molecule. In contrast to LDL, lipoprotein (a) levels are less amenable to dietary intervention or traditional treatment with statins (273). Few studies have examined the effect of testosterone on lipoprotein (a) levels. A similar study investigated the effect of 6 months of transdermal testosterone gel (5mg daily) in 36 surgically postmenopausal women using estradiol (274). They reported a significant reduction in LDL cholesterol with no effect observed in triglycerides, HDL or lipoprotein (a). The observed reduction in HDL cholesterol in the present study is consistent with
prior studies investigating oral testosterone (68,180,181), but has not been observed with transdermal therapy (179).

Administration of transdermal testosterone had no effect on fasting glucose or insulin levels in this, or other, TTP studies (39, 54, 55). This is the first study to report on the effect of TTP used in conjunction with HT for low libido on IR as assessed using the HOMA-IR model, which is a more accurate assessment of IR than fasting glucose or insulin alone, and reassuringly no adverse effects on insulin sensitivity were found. One previous study has used the TTP in elderly women (mean age 68.2 years) with cardiac failure, not on HT, and found a significant improvement on IR as assessed by HOMA-IR (213).

This study was also able to examine the effect of endogenous androgen levels by exploration of baseline results, which showed an inverse correlation between baseline FAI and total cholesterol. This is in contrast to some epidemiological data (142) linking increased androgenicity with adverse lipid alterations, but in keeping with other data (163) suggesting that low androgen levels are associated with CV risk.

- **Systemic effects**

No change was observed in weight, BMI, waist circumference, or blood pressure, which corresponds to previous studies investigating the safety and efficacy of the TTP. Alterations in body composition from testosterone may occur due to changes in visceral fat or muscle mass. The effect on hip circumference was surprising, as prior studies have demonstrated an effect from testosterone on abdominal (178) but not hip circumference. Previous data shows that the effect of testosterone on body composition is very route dependent and the majority of TTP studies have reported no effect from physiological replacement.

The improvement in B-PFSF scores reached significance at 6 weeks and increased further by 12 weeks of treatment. This improvement remained statistically significant when subjects with and without HSDD were analysed separately. These results suggest that even in a group of postmenopausal women who do not necessarily fulfil the
diagnostic criteria for HSDD but subjectively complain of low libido, the TTP may have a role. Prior studies have generally only included women who have HSDD (39–41,43,55,212) and therefore it may be interesting to investigate the effect of testosterone further in women whom, as in this study, have a subjective complaint of distressing low sexual desire. The previous Endocrine Society guidelines (30) suggested that androgen therapy should generally only be recommended in women who have undergone bilateral oophorectomy, however the recently updated guidance (32) no longer restricts use to surgically menopausal women. This is in keeping with the findings from the current study in which the majority of women were naturally menopausal and still derived significant improvement in libido from the TTP.

Over 50% of patients reported a subjective improvement in energy levels and fatigue. Improvements in physical and mental exhaustion have also been observed in women receiving testosterone implants with particular benefit in women with high BMI (33). Although in the present study the improvement in fatigue was a subjective assessment and not evaluated using a validated rating scale, it is another potential benefit from postmenopausal testosterone therapy which should be investigated in future studies. Other reported benefits were improved concentration and headaches, findings which have also been demonstrated in other studies (33,275). Androgen receptors are found in many tissues throughout the body and therefore there are plausible mechanisms as to why the effects of testosterone therapy may be widespread. Unfortunately, the systemic effects of transdermal testosterone other than libido have not been well studied in placebo-controlled trials and therefore more data are needed to confirm these findings.

- **Hormone levels**

Although androgen levels have not been shown to correlate well with symptoms of low libido, of note all but one subject had total testosterone levels in the lower third of the reference range at baseline (i.e. <1nmol/L) and all subjects had FAI in lower third of reference range. There was no correlation between baseline FAI and B-PFSF score, even
in the HSDD group, reiterating the recommendations that androgen insufficiency is a clinical rather than biochemical diagnosis.

Following 12 weeks of treatment, there were significant increases in both total testosterone and FAI. Total testosterone rose slightly above the physiological range in only one subject and FAI remained within physiological range in all participants. These data help confirm that the TTP delivers physiological testosterone levels. FAI at visit 3 did not correlate with incidence of androgenic side effects, as mean FAI was lower in the group with androgenic side effects. The majority of women involved had adequately replaced estrogen at baseline, with only 3 having low estradiol levels (<100 pmol/L). As in previous studies of the TTP (55), no changes in SHBG and estradiol levels were observed over the course of this study.

- **Role of SHBG**

In this study a reduction in SHBG was associated with a rise in Alx and waist circumference. In addition, at baseline, there was an inverse relationship between SHBG and fasting glucose.

The relationship between SHBG and fasting glucose and insulin sensitivity has previously been recognised (203,276). Furthermore, from studies of endogenous hormone levels, it has been suggested that CV risk may be more affected by SHBG than testosterone (134) although it is unclear whether the effects are truly independent of testosterone (17).

Whether some of the effects of exogenous testosterone may be mediated through changes in SHBG remains unknown. However, of note in this study, 12 weeks TTP was not associated with any changes in SHBG despite significant rises in free and total testosterone. By avoiding first pass hepatic metabolism, transdermal testosterone would be expected to have minimal impact on SHBG which is largely regulated by hepatic production.
Study Limitations

- Study design and population

This was a pilot study and therefore has several limitations. Subjects’ baseline measurements acted as a control data and therefore the lack of a separate placebo control arm is a significant limitation. The original study protocol was designed as a randomised placebo-controlled trial but unfortunately the manufacturers ceased production of the placebo patch and so the control arm was withdrawn, as no alternative placebo was available. As such it is difficult to fully distinguish between potential androgenic effects and effects from continuing hormone therapy.

As this was a pilot study we had only a limited sample size. However, even with the small numbers, adequate number of recruits were obtained to detect a 1 SD change in the primary outcome measures. The small sample size limited the findings from subgroup analyses that may have been relevant, including time since menopause, type of menopause (i.e. natural or surgical) and type of HT, all of which may have an impact on endothelial response to sex steroids and androgen physiology. The study population involved was not diverse. The women were predominantly Caucasian (21/22), of normal BMI, with minimal co-morbidities and overall low CV risk. These findings cannot therefore be extrapolated into other populations, although they would still be applicable for the majority of HT users. We chose to recruit subjects with a subjective complaint of distressing low libido, rather than a formal diagnosis of HSDD. Recruits were all women who would have been offered testosterone therapy within normal clinical practice, and as CV measures were the primary outcome we felt that heterogeneity in sexual function would not adversely affect CV outcomes.

A further limitation was the variable types of concomitant HT, although all subjects were using estradiol and the majority were using transdermal or subcutaneous preparations. Due to the small study population it was not possible to limit data analysis to those on transdermal/subcutaneous estradiol or particular progestogens. Worboys et al. (186) have previously demonstrated that 6 weeks of subcutaneous testosterone (50mg implant) in addition to HT resulted in improved endothelial function to a control group of non-HT users. Their study population also used a variety of routes and types of
concomitant HT. Subgroup analysis in their study found no difference in change of FMD based on route of estrogen administration.

Ideally the study would have been able to perform subgroup analysis in a larger number of subjects on estrogen alone to remove the potential confounding effects of progestogens. Subgroup analysis was limited to 5 subjects on estrogen only therapy and therefore it is not possible to draw meaningful conclusions. Additionally, there was also variation in the use of progestogens amongst subjects which is of particular relevance as different progestogens can exhibit anti-androgenic (cyproterone acetate, dienogest, drospironone), androgenic (LNG, medroxyprogesterone acetate, NET) or anti-mineralocorticoid (drospironone) effects which may influence the effects of studies such as this. Of the 16 women using progestogens, only 2 were using oral NET. The others were all using either micronized progesterone or non-oral LNG (transdermal or IUS), which would be anticipated to have less CV influence than oral NET. Future studies should aim to standardise concomitant HT whilst examining the effects of the TTP.

Subjects in the present study had a wide age range of 46-66 years. It is not clear if testosterone, like estradiol, may have differing effects on the vasculature depending on the duration post menopause and extent of vascular damage present. Subgroup analysis of the vascular effects on those less than 5 years postmenopausal and those greater than 5 years postmenopausal demonstrated no difference in change in AIx and RHI between subgroups, although the analysis was limited by small numbers. Studies have explored the effects of subcutaneous testosterone implants in pre- and post-menopausal women (33), but there are no data investigating whether the ‘timing hypothesis’, now proven for estrogen replacement (277–280), also exists for testosterone replacement.

- Outcome measures

For the purposes of this study we chose to use surrogate markers for CVD. The gold standard for RCTs is to use CV events, however this is only possible in large scale studies with long follow-up. These are both costly and time-consuming and take many years to provide results. The use of surrogate markers is well established, and in the case of this study, both RHI (230) and AIx (234) have been shown to correlate well with risk of CVD in women. Both these methods provide a means of detecting impaired
endothelial function before abnormalities may be seen at coronary angiography and have previously been used in short term HT studies to investigate response to treatment (232)(240). There are several other methods for assessing arterial stiffness and endothelial function, however these methods were chosen as they are simple, non-invasive and reproducible (229,281).

RH-PAT has several limitations; the finger-probes are extremely sensitive and are thus prone to artefact from movement (although software enables the removal of small sections of artefact). Endothelial function is affected by various environmental factors, which were controlled for as much as possible in the present study. Finger probes are expensive and cannot be re-used. Unfortunately, due to machine error, 4 subjects had only incomplete RH-PAT data.

We chose to use the B-PFSF as a tool for assessing libido. As the B-PFSF only contains 7 questions it does not provide a detailed assessment of a woman's sexual functioning, however it has been shown to discriminate accurately between those who have HSDD from those who do not (253) and is useful for assessing response to treatment. It has been validated against more in depth assessments such as the Profile of Female Sexual Function and has the advantages of being a brief, self-administered questionnaire which is easy to interpret (253). As sexual function was a secondary outcome measure we felt that the B-PFSF provided an appropriate tool for the purposes of this study. The study did not require in depth assessment of the individual domains of female sexual function and, as such, a brief global assessment to investigate response to treatment was felt to be suitable.

The use of fasting glucose and insulin as markers of IR is not well validated, particularly in healthy populations (251) and so we used the homeostasis model for assessing IR. HOMA-IR has previously been used to detect changes in IR in elderly women with cardiac failure using the TTP for 6 months (213) and is a more accurate method than using fasting indices alone. HOMA-IR does have some limitations, particularly with the older, simplified mathematical formula (248) and so IR was calculated using the updated computer model, which has incorporated results from newer insulin assays. As insulin undergoes pulsatile secretion, ideally the study would have used the mean value obtained from 3 samples taken over 5 minutes (249) however this would have been
significantly more invasive for the study subjects. Use of 3 samples has been reported to improve the intra-subject coefficient of variance from 10.3% for a single sample to 5.5% when 3 samples are used (249).

• **Study Strengths**

Only 1 patient withdrew from the study prior to commencing treatment, therefore biases associated with subject withdrawal should be negligible. Even though study numbers were small, recruitment was adequate as based on the power calculations and missing data were minimal. Subjects were highly motivated to take part and no study visits were missed. Use of the TTP was associated with significant increases in free and total testosterone level, implying good compliance and appropriate application amongst subjects.

As only 1 investigator carried out all study visits there was no inter-observer variability in the arterial stiffness and anthropometric measurements. The validation study showed that intra-observer variability with PWA was comparable to published data. RH-PAT is not susceptible to intra-observer variability but endothelial function has shown to be acutely susceptible to many environmental factors, which were well controlled for in this study. All visits were performed in controlled environment, with standardised testing time, appropriate resting period, in a constant position and a fasted state, with no caffeine/alcohol as specified in the protocol. Vascular measurements in this study compared similarly to published data in age-matched populations, and there was good correlation between the 2 methods of endothelial function testing, helping verify the accuracy of the data.

• **Androgen research**

Androgen research is fraught with difficulties. It remains unclear whether the effect of testosterone on the vasculature is mediated directly through the androgen receptor, alternative non-genomic pathways or through the effects of local conversion to estrogen
via the aromatase inhibitor. Animal studies in male mice suggest that both androgen receptor dependent and independent pathways may be responsible for the cardioprotective role of testosterone (282,283), however further human data are required.

Studies involving testosterone are also highly susceptible to many confounding factors, for example, the increased aromatase activity which occurs due to age, obesity, alcohol, insulin resistance, breast cancer, medication, processed diet and a sedentary lifestyle.

Of further difficulty are the inherent challenges associated with testosterone measurement, as there has been a lack of reliable assays and age-defined reference ranges. Studies into androgens have used a multitude of different assays of varying accuracy and the need for sensitive and specific testosterone assays has been recognised (32).

Mass spectometry is usually considered the most accurate method for assessing testosterone levels but is labour intensive and relatively expensive. The direct immunoassays (both radio and chemiluminescence) are cheaper and faster but have the disadvantage that they may be less accurate, particularly at the lower levels in women. The assay used in this study has been validated by the Centre for Disease Control and Prevention as part of their Hormone Standardization Program.

There is also debate as to whether total, free or bioavailable testosterone provides the most clinically useful testosterone fraction and the measurement of free testosterone is subject to significant challenges (284). The most accurate way of measuring free testosterone is by either equilibrium dialysis or ultrafiltration (32) and there has been criticism of the use of FAI (285) although in women it has shown good correlation with physical separation methods (284).
**Future Studies**

This study has demonstrated a useful protocol for the investigation of the vascular effects of transdermal testosterone via surrogate markers of CVD. Larger scale, placebo controlled studies would be very beneficial to examine further the CV effects of testosterone. Whether testosterone acts through direct effects on the vasculature, indirectly via changes in body composition or through its conversion to estradiol remains unanswered, and data in humans which help us better understand its mode of action would be valued.

Future studies should aim to explore the effects of longer term physiological replacement, both with and without concomitant HT, and quantify more accurately the other benefits of postmenopausal testosterone beyond libido. Future research should also address the other safety concerns surrounding postmenopausal testosterone including breast and endometrial effects.
CONCLUSION

Unfortunately few preparations are currently licensed for postmenopausal testosterone replacement in the UK. The TTP used in the present study was licensed for surgically postmenopausal women with HSDD on concomitant estrogen by the European Medicines Agency in 2006. The patch has subsequently been withdrawn from the market, due to lack of commercial viability rather than any safety concerns. Transdermal gels designed for men are currently used off label and a testosterone cream is available in Australia. A survey of prescriptions by US physicians showed 21% of prescriptions for male testosterone products were used by women (286), therefore there is a great unmet need for the development of products designed for use in women. In recognition that many women are having to use these products designed for men off-license, current guidance has recommended that testosterone levels be checked at baseline and at 3-6 weeks and every 6 months to avoid supra-physiological levels (32).

A significant barrier to product development has been the safety concerns raised by regulatory authorities. It is now clear now that findings from older studies investigating supraphysiological oral testosterone cannot be applied to modern methods of postmenopausal androgen replacement, i.e. physiological replacement via the transdermal route. This study has shown the beneficial effect of the TTP on libido and certain markers of CV health. This, along with previous studies, has not suggested any deleterious CV effects from physiological testosterone replacement. Furthermore there are much published data in support of the many benefits of androgen replacement. This greater understanding of the benefits and long-term effects of testosterone will hopefully stimulate the development of products designed specifically for use in women.
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I am extremely grateful to my supervisors, Nick Panay, John Stevenson and Peter Collins for their encouragement and unflinching support at all stages of the project. It has been a privilege working with you and I have learnt so much from each of you over the last few years.

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Finally, I cannot express my gratitude enough to the women who took part in the study and therefore made it all possible. Their commitment and enthusiasm for the study was impressive and it was a pleasure working with all of them.

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Kate Maclaran
April, 2015
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Appendix 1.

**Palatsi Scale: Degree of Facial Acne Vulgaris**

**Severity of Acne**

Grade 0: The facial area is perfectly clear or contains only a few small lesions.

Grade 1: Mild acne. A few pustules and about 10 papules are present.

Grade 2: Moderate acne. About half the face is affected and numerous lesions are present.

Grade 3: Severe acne. Numerous lesions and general inflammation of the facial skin is present.
Appendix 2.

**Lorenzo Scale for assessment of facial hirsutism**

### Upper Lip

- **0**  No Hair
- **1**  A few scattered hairs
- **2**  A small moustache emerging from the outer margins
- **3**  A moustache extending halfway from the outer margins
- **4**  Heavier growth with fusion in midline

### Chin

- **0**  No hair
- **1**  A few scattered hairs
- **2**  Scattered hairs with small concentrations
- **3**  Complete cover light
- **4**  Complete cover heavy
Appendix 3. Permissions

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