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Investigating the Aetiology of Hot Flushing in Postmenopausal Women and Hypogonadal Men

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Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

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Abstract

Hot flushes are the most commonly reported symptom in postmenopausal women, occurring in approximately 73% of women and causing significant morbidity in 25%, affecting social life and even the ability to work. With improved healthcare and increased life expectancy (death rates decreased by 19% in the last 10 years), women spend a considerable proportion of their lives (30 years on average) in the menopause. At present 36% of the women in the UK are over 50 years of age. If left untreated, hot flushes resolve within one year, or less, in the majority of postmenopausal women. A third will report symptoms that last up to 5 years after natural menopause, and in 20% hot flushes persist for up to 15 years. This equates to as many as 1.5 million women in the UK. Despite this the mechanism of flushing is still poorly understood.

A hot flush resembles a heat dissipation response, in that both are characterised by sweating and peripheral vasodilation. It follows that the underlying mechanism may involve some dysfunction in thermoregulation, which in humans is controlled by the medial preoptic area of the hypothalamus (MPOA), and effectors include cutaneous vessels for vasodilation and vasoconstriction.

Therefore a dysfunction in thermoregulation may lie within the control centre (MPOA), its messengers (adrenergic neurones controlling vasoconstriction and cholinergic neurones controlling vasodilation) or the effectors (cutaneous vessels).

Studies by Freedman et al, using an ultrasensitive temperature probe, suggest that hot flushes are triggered by small elevations in core body temperature (T_c) acting within a narrowed thermoneutral zone, mainly due to a lowering of the sweating threshold, in symptomatic postmenopausal women.

However, the trigger remains unknown.

Oestrogen is likely involved as these changes occur at times of relative oestrogen withdrawal; however, there is little correlation between hot flushes and circulating oestrogen levels. This suggests that other mechanisms are involved.

Noradrenaline is thought to be the primary neurotransmitter responsible for lowering the thermoregulatory set point and triggering hot flushes. Animal studies have shown that intrahypothalamic injection of noradrenaline acts to narrow the thermoregulatory zone and hot flushes can be provoked in symptomatic postmenopausal women with the α_2 -adrenergic antagonist yohimbine, and ameliorated with clonidine, an α -adrenergic agonist. Furthermore, clonidine has been shown to widen the thermoregulatory zone in humans.

Serotonin or 5-hydroxytryptamine (5-HT) is involved in many bodily functions including mood, anxiety, sleep, sexual behaviour and eating, and is thought to play a key role in thermoregulation. Oestrogen withdrawal is associated with decreased blood serotonin levels, which is returned to normal with oestrogen therapy. Furthermore, selective serotonin reuptake inhibitors (SSRI), designed to increase the available serotonin at the serotonergic synapse, have been shown in placebo-controlled trials to be effective in reducing the number and severity of hot flushes.

It has also been shown that flushing women have a diminished vasoconstrictor response to cold and that they have increased blood flow to the forearm and hand during a flushing episode. Alterations in skin blood flow during a flushing attack have also been demonstrated in castrate men, and, as with women, improvements in symptoms are seen with hormone replacement.

The aim of this thesis was to better understand the mechanism of flushing in postmenopausal women and hypogonadal men by assessing the role of cutaneous vessels.

I measured cutaneous microvascular perfusion, using LASER Doppler imaging with iontophoresis, in postmenopausal women who flush and compared it with postmenopausal women with no flushing, and found that perfusion responses to vasoactive agents were increased in women with flushing. Paradoxically, these women with apparently 'better' endothelial function had evidence of serum cardiovascular risk factors.

In a double-blind longitudinal cross over study, the role of the alpha-adrenergic system in the pathophysiology of flushing was investigated, by treating women with clonidine and placebo. There was an increase in perfusion responses with both clonidine and placebo. Clonidine was not shown to be superior to placebo in reducing the number and severity of flushes.

The role of serotonin, both peripherally and centrally was studied by treating postmenopausal women, who experienced severe flushing, with venlafaxine (a serotonin and noradrenaline reuptake inhibitor that acts as a selective serotonin reuptake inhibitor at low doses). Flushing symptoms, as assessed by hot flush diaries and Greene climacteric scale (GCS) scores, were reduced, as were skin blood flow perfusion responses. Central serotonin transporters (SERT) were assessed in vivo using SPECT (single photon computed tomography) imaging and a radioligand, [^{123}I] -beta-carbomethoxy-3- β -(4 iodophenyl)tropane ([^{123}I] beta-CIT), with a high affinity for serotonin transporters. [^{123}I] beta-CIT binding was significantly reduced, and this was associated with a significant reduction in BDI scores; in a group of non-depressed women.

Adiposity is associated, both, with an increased risk of postmenopausal flushing and impaired endothelial function, but in this study, there were no differences demonstrated between obese and lean participants at baseline, despite significant differences in serum markers of endothelial dysfunction.

Oestrogen receptors are also present on endothelial cells and as the most commonly used, and most effective, treatment for vasomotor symptoms, cutaneous microvascular perfusion was assessed following 8 weeks of HRT, and demonstrated an increase in both endothelium dependent and independent vasodilation.

Hot flushes are also common in men on luteinising hormone releasing hormone (LHRH) agonists for prostate cancer therapy. Perfusion responses in these men were assessed prior to commencement of therapy (baseline), and after 8 and then 24 weeks of therapy. No differences were detected at baseline, between those who developed flushing as a result of treatment, and those who did not. At 8 weeks, those with flushing demonstrated increased skin blood flow compared to those without flushing. At 24 weeks, 2 gentlemen with flushing had

kept diaries and these demonstrated an improvement in flushing, but no alteration in perfusion responses.

ACh- and SNP-stimulated vasodilation was, however, reduced when compared to healthy controls.

In this thesis, the data appear to support a role of skin blood flow in the mechanism of hot flushing in both postmenopausal women and hypogonadal men, which may be controlled or altered via neurotransmitters at a local level. The placebo response was significant, but alterations in skin blood flow do not appear to have mediated the adiposity effect.

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Preface

Herein, co-authors of the publications included in this thesis and members of the research team are named.

Dr. Helen Fox MBChB began a BSc project entitled; A Study to Investigate the Value of LASER Doppler Iontophoresis as a Biomarker for Vasomotor Symptoms funded by the Translational Medicine Research Collaboration. This project was not complete when Dr Fox took up other employment, upon which time; I completed the participant assessments and all analysis of the data prior to commencement of the work funded by Wellbeing of Women. Dr. Fox is a co-author of two of the three publications submitted with this thesis, in acknowledgement of her contribution to the study as described above.

Professors Ferrell and Lumsden are my supervisors and have contributed to both the design of the project and editorial aspects of the publications. Professor Sattar contributed to the editorial aspects of the first two publications.

Drs. Krishanadas and Cavanagh have contributed to the statistical analysis of the third publication, and Dr. Nicol completed the analysis of the SPECT images. Dr. Pimlott was responsible for the production of the radioligand required for the SPECT imaging.

Mrs Anne Brown instructed me on the ELISA technique, but I have completed all ELISAs noted in this thesis.

List of Accompanying Material

Full publications

Sassarini J, Fox H, Sattar N, Ferrell W, Lumsden MA. (2011) Vascular function and cardiovascular risk factors in women with severe flushing. *Clinical Endocrinology* 74(1):97-103.

Sassarini J, Fox H, Ferrell W, Sattar N, and Lumsden MA (2012) Hot flushes, vascular reactivity and the role of the α -adrenergic system. *Climacteric* 15 (4): 332-338.

Sassarini J, Krishnadas R, Cavanagh J, Nicol A, Pimlott S, Ferrell W, Lumsden MA (2014) Venlafaxine alters microvascular perfusion, [123 I] -beta-CIT binding and BDI scores in flushing

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I would like to acknowledge Dr Helen Fox, who undertook some of the initial work, and who took the time to teach me the methods that were fundamental to this project. Thank you to Mrs Ann Brown for teaching me the way of the ELISA, and thank you to Mrs Fiona Jordan for allowing me into her lab to do it. I am also grateful to them, and everyone else in the department, for keeping me sane when nothing seemed to be going right, and for their invaluable advice on how to make it so.

Thank you to vascular biochemistry who have analysed samples for the study, and everyone involved in the SPECT imaging at the Southern General Hospital Neurological Institute. Mrs Mary Hansen for ensuring it all ran smoothly, Dr Jim Patterson for overseeing it, until his retirement, and Dr Alice Nicol for reading all of the scans. Not forgetting Sally Pimlot, who was responsible for the production of the radioisotope, and who was kind enough to show me how it was made. Many thanks also go to Dr Rajeev Krishnadas and Dr Jonathan Cavanagh, for their assistance in analysis of the results.

Thank you to all of the women, and men, who participated in the study, by generously donating of your time. I truly enjoyed your warm and witty conversation throughout the study, which didn't make it seem like work at all,

and thank you to everyone at the Tennent Institute, where all the study visits took place.

Particular thanks must go to Dr David Russell, whose assistance in recruitment of the men was invaluable, and of course, thanks must go to Wellbeing of Women, for funding this work.

I would also like to thank my family. To my parents, for their endless love, support and encouragement, you have made all of this possible. Special thanks, also, for much needed babysitting, often at the last minute, and to mom for her spellcheck and grammar corrections. To my long-suffering siblings, thank you for lending ears and time, rarely acknowledged, but always appreciated.

And to my husband, who has quite literally had to pick me up at times, thank you for your patience, kindness, encouragement and support, I could not have done this without you. Finally, this acknowledgement would not be complete without extending a welcome to two amazing little people who have punctuated one great achievement with two others.

It is to them that I dedicate this thesis; to my wonderful son, Rowan, and to my beautiful daughter, Grace.

Author's Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

This thesis has been composed by me, and I have been responsible for patient recruitment, tissue collection, laboratory studies and analysis unless otherwise acknowledged.

Jenifer Sassarini, December 2013.

List of Abbreviations

[¹²³ I]β-CIT	[¹²³ I] -beta-carbomethoxy-3-β-(4 iodophenyl)tropane
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
AAAD	Aromatic amino acid decarboxylase
ACh	Acetylcholine
ADT	Androgen deprivation therapy
AMH	Anti-Müllerian Hormone
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
AUC	Area under the curve
BDI II	Becks Depression Inventory II
B _{max}	Transporter availability
BMI	Body mass index
CAD	Coronary artery disease
CBT	Cognitive behavioural therapy
CEE	Conjugated equine estrogens

cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
COMT	catechol-O-methyltransferase
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
DAT	Dopamine transporter
DBP	Diastolic blood pressure
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DSM	Diagnostic and statistical manual of mental disorders
DVT	Deep vein thrombosis
E ₁	Oestrone
E ₂	Oestradiol
E ₃	Oestriol
ECG	Electrocardiograph
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoassay
EMA	European Medicines Agency

eNOS	endothelial nitric oxide synthase
FDA	Food and Drug Administration
FMD	Flow mediated dilation
FMP	Final menstrual period
fMRI	Functional magnetic resonance imaging
FSH	Follicle stimulating hormone
GCS	Greene Climacteric Scale
GLM	General linear model
GnRH	Gonadotrophin releasing hormone
GP	General Practitioner
GS	Gleason score
Gy	Gray
HADS	Hospital anxiety and depression scale
Hct	Haematocrit
HDL	High density lipoprotein
HERS	The Heart and Estrogen/Progestin Replacement Study
HFRDIS	Hot flush related daily interference scale
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy

HT	Hormone therapy
ICAM-1	Intercellular adhesion molecule
IDDM	Insulin dependent Diabetes Mellitus
IL-6	Interleukin-6
IV	Intravenous
keV	electron volt
kg	Kilogram
LASER	Light Amplification by Stimulated Emission of Radiation
LDI + ION	LASER Doppler imaging and iontophoresis
LDL	Low density lipoprotein
LH	Luteinising hormone
LHRHa	Luteinising hormone releasing hormone agonist
LiHep	Lithium heparin
LMP	Last menstrual period
MANCOVA	Multivariate analysis of covariance
MDD	Major depressive disorder
MDMA	3,4-methylenedioxymethamphetamine
MDT	Multi-disciplinary meeting
MHPG	Methoxy-4-hydroxyphenylglycol

ml	milliliter
MOAO	Monoamine oxidase
MPA	Medroxyprogesterone acetate
MPOAH	Medial preoptic area of the hypothalamus
MWS	Million Women's Study
NA	Noradrenaline
NaCl	Sodium chloride
NICE	National Institute for Health and Care Excellence
NO	Nitric oxide
NTA	Night time awakening
OCD	Obsessive compulsive disorder
PSA	Prostate specific antigen
PU	Perfusion units
PVAT	Perivascular adipose tissue
PVADRF	Perivascular adipose tissue derived relaxing factor
QoL	Quality of life
RCT	Randomised controlled trial
ROI	Region of interest
rpm	Rotations per minute

SBP	Systolic blood pressure
SERT	Serotonin transporter
SHBG	Sex hormone binding globulin
SNP	Sodium nitroprusside
SPECT	Single photon emission computed tomography
SSRI	Selective serotonin reuptake inhibitor
STRAW	Stages of Reproductive Ageing Workshop
SWAN	Study of Women's health Across the Nation
T _c	Core temperature
TNF- α	Tumour necrosis factor α
TPH	Tryptophan hydroxylase
U.K.	United Kingdom
U.S.	United States
VACURG	Veterans Administrative Cooperative Urological Research Group
VCAM-1	Vascular cell adhesion molecule
VIP	Vasoactive intestinal peptide
VMAT	Vesicular monoamine transporters
VOP	Venous Occlusion Plethysmography
WHI	Women's Health Initiative

WHO World Health Organisation

WHR Waist Hip Ratio

Chapter 1

Introduction

History

The Greek philosopher Aristotle (384-322 BC) noted that women stop giving birth after the age of 50 and he believed that the apparent abundance of menstrual blood compared with seminal fluid underpinned the ascendancy of the male in almost every species. Life, he thought, is dependent on heat and moisture and over a lifespan an animal can use up its innate heat, becoming colder and drier and nearer to death.

To Hippocrates (460-370 BC), the cessation of bleeding meant the reassimilation of the unruly female body with the male, knowable and supposedly more orderly body.

In sixth century Byzantium, Aëtius Ameidenus, a physician, stated “the menses do not cease before the thirty fifth year nor appear after the fiftieth; rarely some menstruate until the sixtieth year. Those who are very fat cease early”. But there was still no term for it and it was discussed rarely before the seventeenth century.

In 1816, ‘*la menespausie*’ was described by a French physician, C.P.L. de Gardanne. *Meno* derived from the Greek word for month, and *pause* from the Greek word *pauses* or halt, literally meaning halting of monthly cycles.

But, it was not until the late nineteenth century, alongside the introduction of anaesthesia, antisepsis and germ-theory, that there was research into the secretions of endocrine glands. In 1538, Vesalius, an anatomist, described the ovaries as female testicles, however, Stensen pointed out that as they contained ova, that they should be called ovaries. However, it would be another 200 years before surgeons noted cessation of menstruation following removal of the ovaries, leading to a conclusion that menstruation was due to a ‘peculiar condition of the ovaries’.

Reference to symptoms that we would now commonly associate with the climacteric are difficult to find. In 1628, Robert Burton’s *The Anatomy of Melancholy* refers to hot flushes and other symptoms and a cooling diet was recommended as therapy in 1675. Thomas Sydenham notes the tendency of

women aged 45-50 to develop ‘hysterical fits’ and suggests bloodletting as a treatment in 1701 and in 1899, in an article entitled ‘*Epochal Insanities*’, under the heading ‘*Climacteric Insanity*’, physicians were invited to treat the menopause as a syndrome in need of attention (1).

In this same year, the Merck Manual featured several treatments, including a course brownish powder available in pills flavoured with vanilla or in a tablet form, called Ovarin, which was derived from dried and pulverised cow ovaries. Other routes of administration and formulations were available, including self-injections of an extract of testicles of dogs and guinea pigs reported by Charles Edouard Brown-Sequard (2).

Interestingly, whilst these treatments sound unpalatable, they are arguably more acceptable than those suggested by William Tyler Smith some 50 years earlier. He advocated a course of injections of ice water into the rectum, the introduction of ice into the vagina, and the application of leeches to the labia and cervix for a disorder that he describes as;

“attended by vivid sensations of heat, flushing of the face and neck, with giddiness, almost amounting to insensibility...followed by relaxation of the neck, great coldness or chills, and faintness, with perspiration over the whole of the body. The paroxysms are so violent as to wake patients out of their sleep. ...these paroxysms can occur many times in twenty-four hours”.

In the early twentieth century Edward Sharpey-Schafer (1850 - 1935) laid down the physiological foundations of endocrinology and F.H.A. Marshall (1878 - 1949) and William Jolly (1873 - 1945) made detailed investigations of ovarian endocrinology. The secretions now known as hormones were named on the suggestion of a Cambridge classicist, from the Greek ‘*I excite*’, and the term was first used in Britain in 1905 by Ernest H. Starling, Professor of Physiology and University College London.

After 1910 the existence of sex hormones was no longer being questioned and the Association for the Study of Internal Secretions was founded in June 1917, becoming the Endocrine Society in 1952. Edward Doisy, a biochemist and Edgar Allen, a zoologist, had devised the criteria which established it as an acceptable field of scientific study by 1936. Ovarian hormones were now being isolated and

synthesised; oestrin in 1923, progesterone in 1929, oestriol in 1930, oestrone in 1930 and oestradiol in 1936.

Bernhard Zondek (1891 - 1966) found small quantities of oestrone in the urine of human males and discovered that the urine of pregnant mares was another rich source leading to commercialisation. The manufacture of the first synthetic oestrogen stilboestrol (DES, diethylstilboestrol) in 1938 at the University of Oxford was quickly followed by another, dienioestrol.

From 1940 to 1970, DES was given to pregnant women in the mistaken belief it would reduce the risk of pregnancy complications and losses, but in 1971, it was shown to cause a rare vaginal cancer in girls and women who had been exposed to this drug in utero and use in this group was stopped. Approval for use in the treatment of menopausal symptoms was granted by the FDA in 1941.

By 1947, there were 53 formulations sold by 23 companies for the treatment of menopausal disorders (2) and the use of oestrogens increased dramatically until reports in 1975 of increased endometrial carcinoma caused a decline in its use. The addition of a progestagen, in those with a uterus, saw an increase in use again through the 1980s to 1990s, until the publication of the Women's Health Initiative (WHI) trial in 2002 and the Million Women Study (MWS) in 2003.

Over the last 11 years, there has been extensive research and reanalysis, and we are beginning to understand a little more about the treatments for symptoms of the menopause, however there is still a way to go. Our knowledge of the underlying mechanisms of the symptoms that women experience during this time is limited; and this is the foundation of directed, appropriate and safe treatments.

Reproductive Physiology and Endocrinology

The main functional unit of the ovary is the ovarian follicle. At birth, all follicles are composed of a germ cell (oocyte) surrounded by a single layer of granulosa cells. This is known as a primordial follicle. The number of these are maximal at the time of birth, approximately one to two million, and decrease with age (see figure 1) (3).

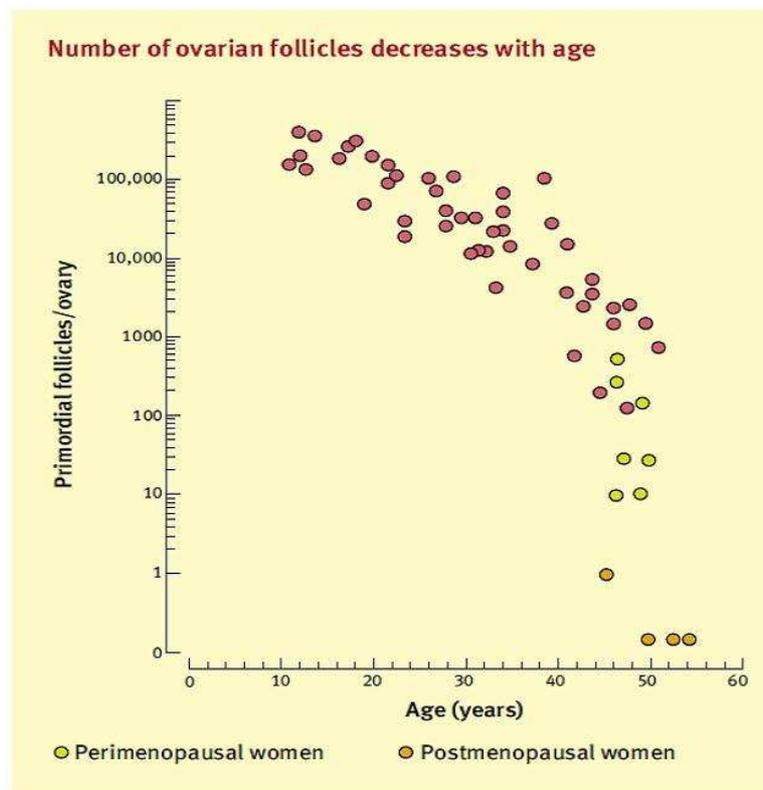


Figure 1: Decreasing numbers of primordial follicles with age
Richardson SJ, Senikas V, Nelson JF. Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. *J Clin Endocrinol Metab* 1987; 65: 1231-7 (3).

Primordial follicles are housed in the cortex of the ovary and are surrounded by stroma called tunica albuginea (see Figure 2, P26). Prior to puberty a number of these primordial follicles will undergo atresia, so that at the time of first ovulation, there may be as few as 400 000 remaining.

At the time of puberty, under the influence of increasing levels of hypothalamic gonadotrophin releasing hormone (GnRH), the normal pituitary releases pulses of

luteinising hormone (LH) and follicle stimulating hormone (FSH) with increasing amplitude and frequency (Figure 3).

Primordial follicles are recruited into a process termed folliculogenesis, during which follicle growth occurs. Granulosa cells increase in number, an antrum develops within the follicle (adjacent to the oocyte) and cells of the stroma (theca cells) surround the follicle. A mature follicle is termed a Graafian follicle (see Figure 2 and 4).

Granulosa cells are responsible for production of oestrogens through aromatisation of androgens produced in theca cells (follicular phase) (see Figure 4). At a critical level of oestradiol, there is initiation of the positive feedback on LH, leading to the mid-cycle LH-surge and the first ovulation.

Luteinisation of the granulosa cells of this dominant (Graafian) follicle occurs, forming the corpus luteum (luteal phase), which generates high volumes of progesterone and oestradiol.

Granulosa cells also produce inhibin A and B. Inhibin A is produced by the granulosa cells of the dominant follicle and levels are high during both the follicular and luteal phase. Inhibin B is produced by granulosa cells of the follicles in the recruited cohort and levels are highest in the follicular phase but decrease following the LH surge.

Ovarian steroids inhibit LH and FSH through a negative feedback mechanism (see figure 3), except midcycle when positive feedback leads to the gonadotrophin surge. The inhibins (particularly Inhibin B) also inhibit FSH.

During the ovarian cycle, there are also changes to the endometrium, designed to support pregnancy should fertilisation occur. During the follicular phase (see Figure 5), there is proliferation of the stroma and glands. By day 12 the glands are large and dilated and blood vessels are also more prominent.

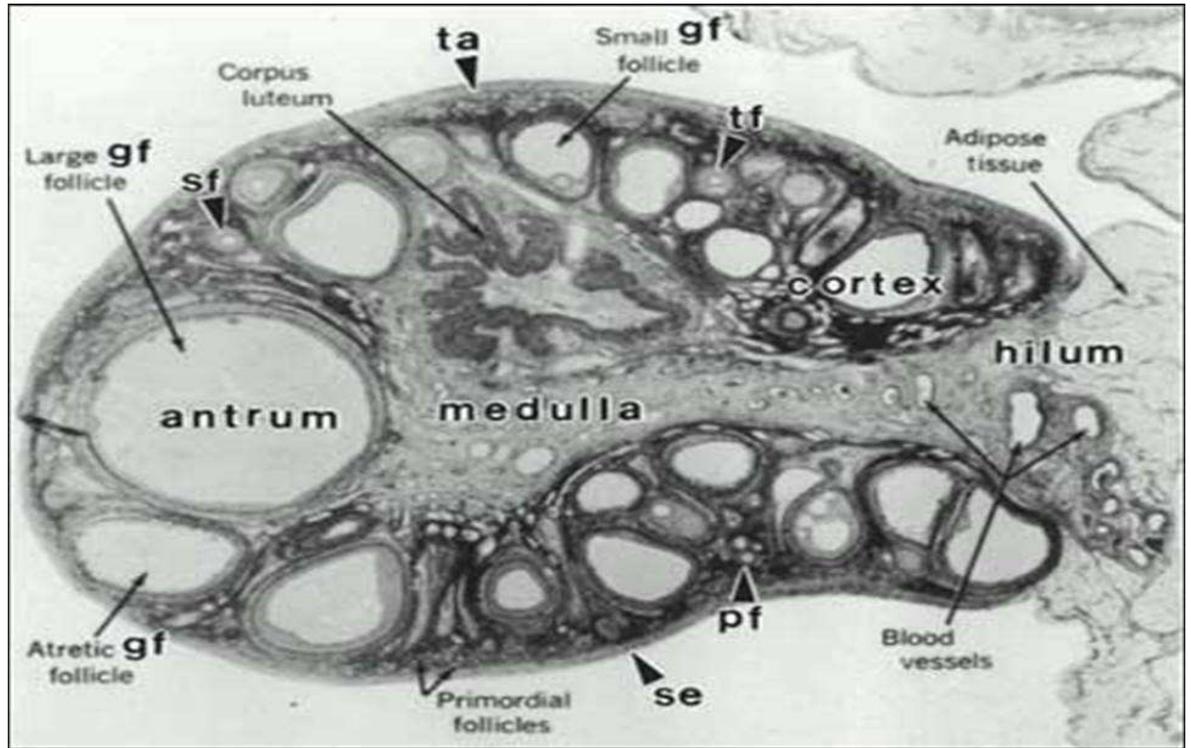


Figure 2: Histology of an ovary
 ta; tunica albuginea. gf; Graafian follicle. tf; follicle. pf; primordial follicle. se; surface epithelium. sf; secondary follicle

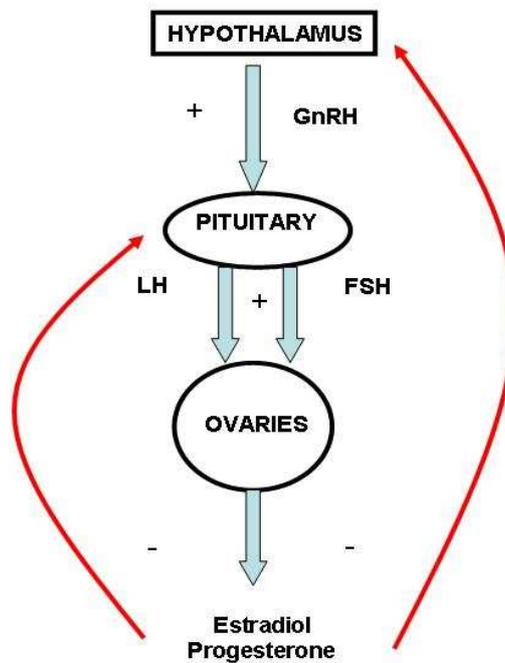


Figure 3: Hypothalamic Pituitary Ovarian Axis

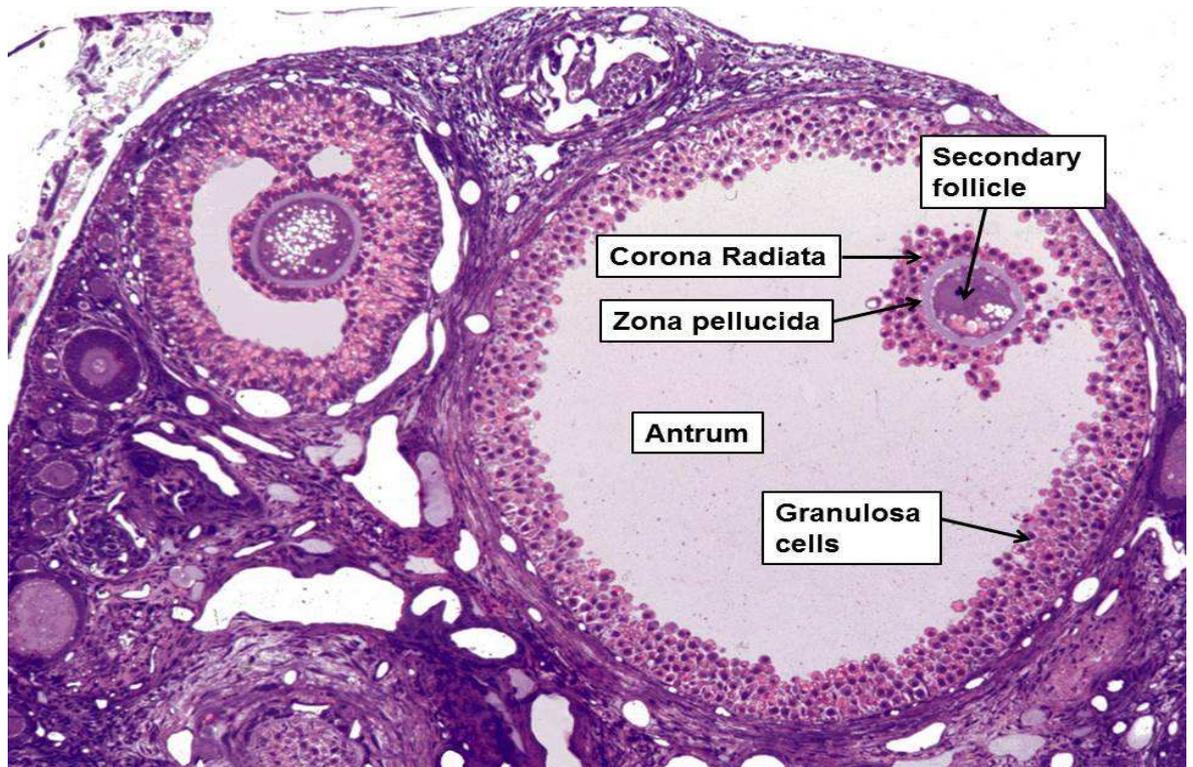


Figure 4: Graafian follicle

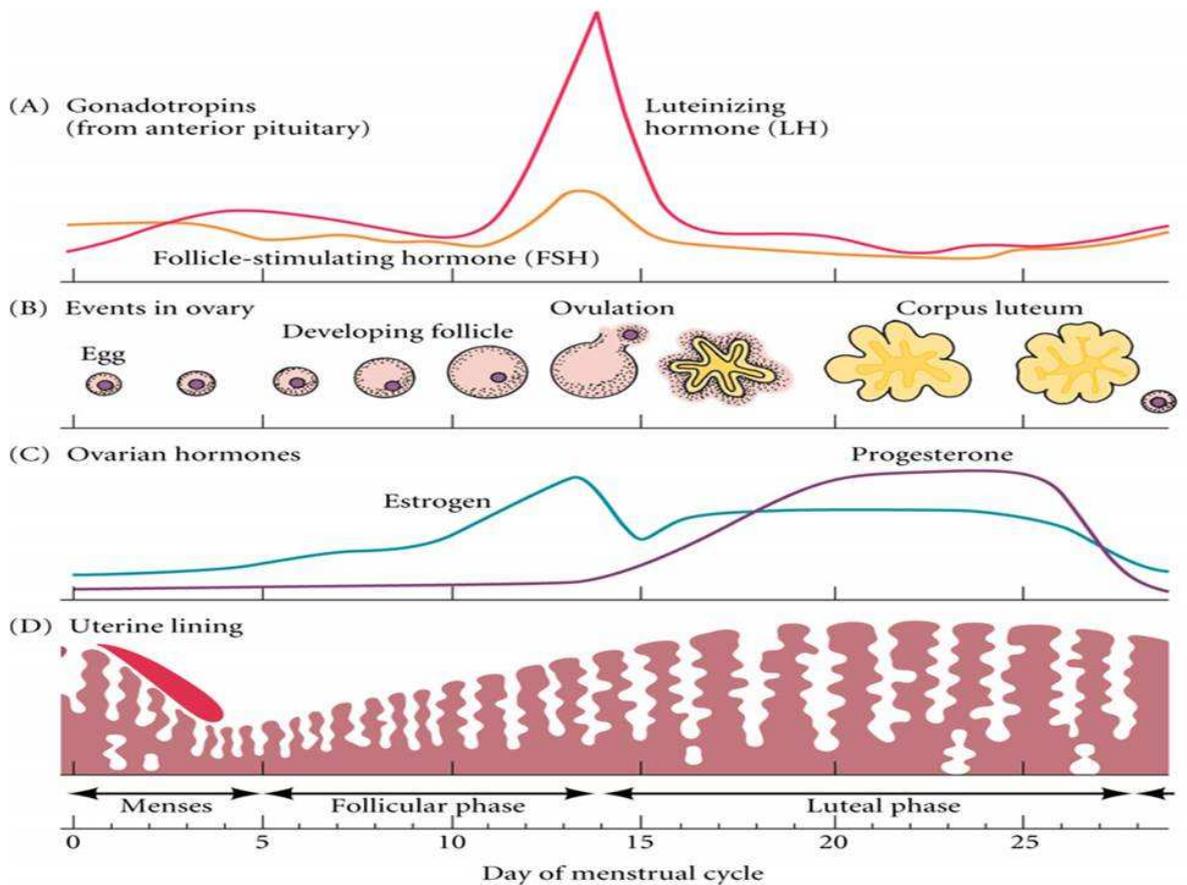


Figure 5: Human menstrual cycle
Developmental Biology, Chapter 17; Hormones and mammalian egg maturation (4)

Following ovulation and during the luteal phase (see Figure 5), the large quantities of progesterone induce secretory changes in the glands and swelling of the stromal cells. There is now a rich blood supply and the capillaries become sinusoidal.

In the event that fertilisation does not occur, there is breakdown of the corpus luteum with a resultant fall in oestradiol and progesterone, leading to break down and shedding of the endometrium.

Reproductive Ageing

The average age of menopause in the UK is 51.15 years (5), however the change in gonadotrophins and sex steroids actually starts in the late 30s as the rate of decrease in the ovarian follicle numbers escalates.

Levels of serum FSH begin to increase in women still having regular cycles. Figure 1 shows a rapid decrease in the number of primordial follicles after the age of 40 years. Fewer follicles result in decreased secretion of inhibin B, with a subsequent increase in FSH that can maintain oestradiol until follicle depletion (6).

In women whose menses occur at intervals of more than 3 months, FSH levels are increased and there is a substantial decrease in circulating levels of oestradiol and the inhibins (6). However, repetitive sampling in an individual woman may show various patterns, with high or low FSH, oestradiol and inhibin (7).

Anti-Müllerian Hormone (AMH), secreted by granulosa cells, however, is unique amongst female sex hormones in that it is stable within and across menstrual cycles (8). Concentrations decline with increasing reproductive age (9) and it correlates with ovarian primordial follicle number (10); it is therefore used as a determinant of ovarian (follicular) reserve, particularly in assisted conception settings.

The Menopause

Definition

Menopause is an inevitable event for women in the process of normal ageing. Ageing can be defined as the natural progression of changes in structure and function that occur with the passage of time in the absence of known disease. The 2001 Stages of Reproductive Ageing Workshop (STRAW) Working group describes the female reproductive axis to be composed of the hypothalamic-pituitary axis and the Mullerian-derived structures. Menopause (non-functioning reproductive axis) occurs at a time when a woman is otherwise healthy as a result of oocyte depletion in the ovary (11).

Menopause has been defined by the World Health Organisation and STRAW Working Groups as the permanent cessation of menstrual periods that occurs naturally or is induced by surgery, chemotherapy or radiation (12).

Natural menopause is a retrospective diagnosis that can only be made after 12 months of amenorrhoea, that is not associated with some other physiological (e.g. lactation) or pathological cause and occurs on average between the ages of 50 and 51 (13).

The STRAW working group viewed reproductive ageing as a process, not an event, with the end (menopause) easily identifiable, but the beginning not so, and that is the purpose of the staging system. A number of terms including 'climacteric', 'perimenopause', 'menopausal transition', 'postmenopause' and 'menopause' have been used to refer to the stages of reproductive ageing surrounding the final menstrual period (14).

The World Health Organisation (WHO) recommended the use of 'perimenopause' and 'menopausal transition' in place of 'climacteric' in 1996 and a model was developed in 2001 to describe the stages of the menopausal transition (see figure 6) (11). This model identifies seven stages of reproductive life and is primarily based on the characteristics of the menstrual cycle and secondarily on follicular phase Follicle Stimulating Hormone (FSH) levels. As women progress through the menopausal transition, the menstrual cycle length

becomes irregular and FSH levels are raised in response to decreased ovarian hormone concentrations. Menstrual cycles are then missed and ultimately stop, as does ovulation.

		Final menstrual period (FMP)										
		-5			-4		-3	-2	-1	0	+1	+2
Terminology		Reproductive					Menopausal transition			Postmenopause		
		Early	Peak	Late	Early	Late				Early	Late	
							Perimenopause					
Duration of stage		variable					variable			a 1 year	b 4 years	Until demise
Menstrual cycles		Variable to regular	regular		Variable cycle length (>7 days different from normal)	≥2 skipped cycles and an interval of amenorrhea (≥60 days)		a m e n - s t r e a l a m e n o r r h e a	none			
Endocrine		Normal FSH		↑ FSH	↑ FSH			↑ FSH				

Figure 6: Proposed staging and revision of nomenclature
 Reproduced from the Executive Summary, Stages of Reproductive Ageing Workshop (STRAW), Soules MR et al, J Womens Health Gend Based Med. 2001 Nov;10(9):843-8 (11).

In 2007, ReSTAGE collaborators proposed modifications of this STRAW staging system, which included simplifying the definition of the bleeding criterion for late transition to amenorrhoea of at least 60 days, inclusion of the FSH criteria of a serum FSH value ≥ 40 IU/l to facilitate prediction of proximity to the final menstrual period (FMP), and adoption of the persistent 7-or-more-day difference in cycle length as the bleeding criterion for onset of early transition (see figure 7) (15).

Stages	Menarche n			Final menstrual period (FMP) 0				
	-5	-4	-3	-2	-1	+1	+2	
Terminology	Reproductive			Menopausal transition		Postmenopause		
	Early	Peak	Late	Early	Late	Early	Late	
				Perimenopause				
Duration of stage	variable			variable		1 year	4 years	Until demise
Menstrual cycles	Variable to regular	Regular		Variable cycle length (persistent 7 or more day difference in length of consecutive cycles)	An interval of amenorrhoea ≥ 60 days	amenstruals	none	
Endocrine	Normal FSH		\uparrow FSH	\uparrow FSH	\uparrow FSH (≥ 40 IU/l)	\uparrow FSH		

Figure 7: ReStage proposed modifications of STRAW staging system
 Reproduced from Recommendations from a multi-study evaluation of proposed criteria for Staging Reproductive Aging. Harlow SD, Crawford S, Dennerstein L, Burger HG, Mitchell ES, Sowers M-F for the ReSTAGE Collaboration. *Climacteric* 2007, 10 (2): 112–119 (15).

And in 2011, STRAW+10 provided a more comprehensive basis for assessing reproductive aging in both the research and clinical context. It simplified bleeding criteria for the early and late menopausal transition, recommended modifications to criteria for the late reproductive and early postmenopause stage, provided information on the duration of the late transition and early postmenopause, and recommended the application regardless of women's age, ethnicity, body size, or lifestyle characteristics (see Figure 8) (16).

Stage	Menarche n				Final menstrual period (FMP) 0							
	-5	-4	-3b	-3a	-2	-1	+1a	+1b	+1c	+2		
Terminology	Reproductive				Menopausal transition		Postmenopause					
	Early	Peak	Late		Early	Late	Early		Late			
Duration	variable				variable	1-3 years	2 years (1+1)		3-6 years	Remaining lifespan		
PRINCIPAL CRITERIA												
Menstrual cycle	Variable to regular	Regular	Regular	Subtle changes in flow/length	Variable cycle length (persistent 7 or more day difference in length of consecutive cycles)	An interval of amenorrhoea \geq 60 days						
SUPPORTIVE CRITERIA												
Endocrine			Low	Variable	↑ Variable	↑ \geq 25IU/l	↑ variable	Stabilises				
FSH			Low	Low	Low	Low	Low	Low	Low			
AMH				Low	Low	Low	Low	Low	Low			
Inhibin B												
Antral Follicle Count			Low	Low	Low	Low	Very Low	Very Low				
DESCRIPTIVE CHARACTERISTICS												
Symptoms						Vasomotor symptoms likely	Vasomotor Symptoms most likely			Increasing symptoms of urogenital atrophy		

Figure 8: STRAW + 10
 Reproduced from Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. Harlow SD, Gass M, Hall JE, Lobo R, et al for the STRAW + 10 Collaborative Group. *Climacteric* 2012; 15:105–114 (16).

Epidemiology

Studies estimating age at natural menopause are mostly based on samples of Caucasian women in Western societies (17-19), however, there is one international study of 18,997 women from 11 countries which found that the median age at natural menopause was 50 years (range, 49-52 years) (20). There are reports of younger ages at menopause in non-Caucasian women; Africans (21, 22), African Americans (23, 24), and Hispanics (25, 26) of Mexican descent may have an earlier age at menopause than Caucasian women, while Japanese (27, 28) and Malaysian (29-31) women report a median age at menopause similar to that for women of European descent (13).

It is widely known that for smokers, menopause occurs 1-2 years earlier than for non-smokers (32). It has also been found that an earlier natural menopause is associated with less education, low social class, nulliparity or having fewer children, never having used oral contraceptives, and low relative weight (17, 33). Most of these factors may affect the hypothalamic-pituitary-gonadal axis and its regulation of gonadotropins and sex steroid hormones.

Smoking is associated with elevated FSH levels (34), however a study investigating the relationship between smoking and changes in AMH, found no statistically significant association (35), however smoking may be associated with a greater rate of follicular decline (36). Both animal (37) and human (38) studies have demonstrated that polycyclic aromatic hydrocarbons in cigarette smoke are toxic to ovarian follicles, and there may also be alterations in sex steroids, including lower concentrations of oestrone, oestradiol and oestriol (39).

Data from a large multicenter study (SWAN, Study of Women's Health Across the Nation) of a multiracial/multiethnic sample of 14,620 women (40) was analysed to examine the independent association of a number of factors with age at natural menopause. The results demonstrated that current smoking, lower educational attainment and nonemployment were related to earlier age at natural menopause and that prior use of oral contraceptives and parity were associated with later age at menopause. However, the results demonstrated

that Japanese women have a later menopause than Caucasian, African-American, Hispanic and Chinese (13).

A further large study of 95,704 ethnically/racially diverse women found that Japanese-American race/ethnicity was independently associated with relatively late natural menopause and Latinas were found to experience relatively early natural menopause, with those born in the United States experiencing the event later than those born outside the United States (41). The results were also consistent with previously published results regarding smoking, low body mass, age at menarche and parity.

Menopausal Transition

There are a number of symptoms associated with perimenopause and decreasing oestrogen levels, although some women will experience none of these. They include hot flushes and night sweats (vasomotor symptoms), vaginal symptoms, depression, anxiety, irritability and mood swings (psychological effects), joint pains, migraines or headaches, sleeping problems and urinary incontinence.

With improved healthcare and increased life expectancy (death rates decreased by 19% in the last 10 years), women spend a considerable proportion of their lives (30 years on average) after the menopause (42). At present 36% of the women in the UK are over 50 years of age (42). It is estimated that approximately 75% of women will experience some symptoms related to oestrogen deficiency during the menopausal transition, and 40% will seek medical advice for the management of menopausal symptoms.

Hot Flushes

The terms hot flush (U.K.), hot flash (U.S.), and vasomotor symptom are commonly used synonymously. Hot flushes are characterised by a feeling of intense warmth, often accompanied by profuse sweating, anxiety, skin reddening and palpitations, and sometimes followed by chills.

Hot flushes are the most common, occurring in approximately 73% of women (43) causing significant morbidity in 25%, affecting social life and even the ability to work (44). They are the most common indication for the prescription of hormone replacement therapy (HRT) since it is effective in over 80% of cases. In 1995, 37% of American women took HRT, principally for this purpose.

Flushing commonly occurs when hypoestrogenism follows a period of oestrogen exposure. If left untreated, hot flushes resolve within one year, or less, in the majority of postmenopausal women (45). A third will report symptoms that last up to 5 years after natural menopause, and in 20% hot flushes persist for up to 15 years(45) or longer. Menopause induced by surgery is associated with about a 90% probability of hot flushes during the first year (46) and symptoms associated

with surgical menopause are often more abrupt and severe and can last longer than those associated with a non-surgical menopause (47).

Hot flushes can occur in both men and women after acute gonadal steroid withdrawal, such as ovariectomy in young women or orchidectomy in men; in men or women who are hypogonadal; or in those who take gonadotrophin-releasing hormone (GnRH) agonists, antagonists or other anti-gonadal agents which cause oestrogen (in women) or testosterone (in men) levels to fall (48).

Physiology of a hot flush

Skin temperature changes during a hot flush are minimal over most of the body, with large temperature changes recorded only in the digits (temperature increase from around 28°C to 33°C) (49). Although the greatest temperature changes have been found in the fingers and toes, the symptom of flushing is usually experienced mainly in the face, neck and upper trunk, and it seems that the subjective sensation of heat is out of proportion to the actual temperature increase, which in these areas may be only about 1°C. Furthermore, the temperature increase often persists for several minutes after the sensation of warmth has passed, indicating that the flush is only experienced while the skin temperature is increasing (50). The perceived severity of a hot flush is therefore probably related more to the rate of temperature change than to actual temperature increase.

The increase in skin temperature results from a sudden peripheral vasodilatation which precedes the subjective sensation of the flush by at least 1 min and persists for several minutes afterwards, thereafter declining slowly (51). A rise in heart rate coincides with the sensation of a hot flush and usually returns to normal quickly once the flush has resolved (52, 53). There is no significant change in blood pressure either during or following a menopausal hot flush (53). Although many women report palpitations during a hot flush, no change in cardiac rhythm has been found during a hot flush during electrocardiograph (ECG) recording (52).

Pathophysiology/Mechanism of a hot flush

The exact pathophysiology of flushing is not known, although it is generally accepted that falling oestrogens play a main role. As already stated, flushes generally occur at times of relative oestrogen withdrawal and replacing it will result in improvement in most women. However, oestrogen concentrations remain low after the menopause whereas most vasomotor symptoms will diminish with time, and therefore a fall in oestrogen concentration would not seem to provide the complete answer. It has also been found that circulating levels of oestrogen do not differ significantly between symptomatic and asymptomatic postmenopausal women (54).

Furthermore, oestrogen withdrawal, rather than low circulating oestrogen levels, is thought to be the central change that leads to hot flushes. There are several observations to support this theory. The abrupt oestrogen withdrawal due to bilateral oophorectomy in premenopausal women is associated with a higher prevalence of flushes than in those women who experience a gradual physiological menopause. Also, young women with gonadal dysgenesis, who have low levels of endogenous oestrogen, do not experience hot flushes unless they receive several months of oestrogen therapy and then abruptly discontinue its use (55).

As a hot flush closely resembles a systemic heat dissipation response; both are characterised by sweating and cutaneous vasodilatation and can be provoked by heating (51, 56); it is thought that there may be some dysfunction in thermoregulation in symptomatic postmenopausal women.

Core body temperature (T_c) in humans is regulated by thermoregulatory nuclei within the medial preoptic area of the hypothalamus, through the activation of mechanisms that conserve or dissipate heat, such as vasodilatation or vasoconstriction (55, 57, 58). It has therefore been widely hypothesised that a hot flush represents an alteration in the central thermoregulatory set-point in the hypothalamus (51, 59, 60).

It is thought that T_c in humans is maintained within a thermoneutral zone within which major thermoregulatory responses do not occur, and above and below which thermoregulatory mechanisms come into action.

Studies by Freedman et al, using an ultrasensitive temperature probe, suggest that hot flushes are triggered by small elevations in core body temperature acting within a narrowed thermoneutral zone in symptomatic postmenopausal women (see figure 9) (61). This group found that small but significant elevations in T_c precede most (76%) hot flush episodes (59, 62, 63). These same investigators subsequently found that postmenopausal women with hot flushes had a narrower thermoregulatory zone (0°C) compared with postmenopausal women who do not flush (0.4°C). This narrowing was mainly due to a lowering of the sweating threshold in symptomatic women (64). Since heat loss mechanisms can be triggered by a 0.01° elevation of core body temperature above the regulatory zone, the subtle changes in temperature before a hot flush, coupled with a narrow homeostatic temperature zone, may trigger the heat loss mechanisms that lead to hot flush symptoms.

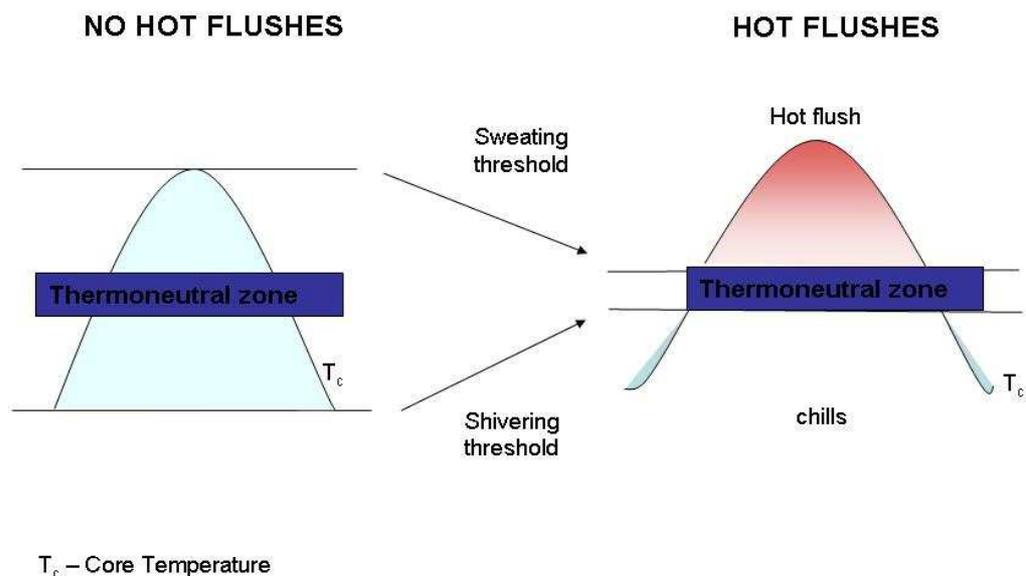


Figure 9: Thermoneutral zone
 Freedman RR, Krell W. Reduced thermoregulatory null zone in postmenopausal women with hot flushes. *Am J Obstet Gynecol* 1999; 181:66-70. (61)

Subsequently, the same group found that oestrogen (E_2) replacement in symptomatic postmenopausal women raised the T_c sweating threshold and reduced hot flush occurrence without any affect on fluctuations in T_c . Elevation of the sweating threshold may be responsible for improvement of vasomotor symptoms with oestrogen therapy since it would reduce the likelihood that T_c fluctuations would trigger a hot flush (65).

It is known that the T_c sweating threshold in premenopausal women varies with the menstrual cycle, being lower in the follicular phase when oestrogen and progesterone levels are low, and higher in the luteal phase, when levels are high (66, 67). Despite this lowering of the sweating threshold in the follicular phase, premenstrual women without premenstrual syndrome rarely experience hot flushes (68). It has been shown that both symptomatic and asymptomatic postmenopausal women undergo fluctuations in T_c (69). Freedman's group subsequently went on to demonstrate that symptomatic postmenopausal women are distinguished from asymptomatic postmenopausal women and from premenopausal women in the luteal and follicular phases by significantly lower T_c sweating thresholds and higher maximum sweat rates (66).

Using functional magnetic resonance imaging (fMRI), this group also demonstrated different areas of activation in postmenopausal women during a hot flush compared to sweating in asymptomatic amenorrhoeic women (70). Most recently, they have demonstrated a rise in brainstem activity, in symptomatic postmenopausal women, preceding the detectable onset of a hot flush, followed by increased activity in the insular and prefrontal areas (71), leading them to conclude that pre-hot flush activity may reflect the functional origins of internal thermoregulatory events and insular, prefrontal and striatal activity may be associated with the phenomenological correlates of hot flushes.

Whilst there has been extensive study investigating the central control of this altered thermoregulation, little consideration has been given to the possibility that peripheral vascular reactivity may also play an important role in the pathophysiology of hot flushing. It has been shown that women experiencing severe flushing exhibit a diminished vasoconstrictor response to cold stimulation, and that with improvement in vasomotor symptoms the vasoconstrictor response returns to normal (72). Symptomatic postmenopausal women also exhibit

consistently poor peripheral vascular control, which shows a dose-related improvement with oestrogen therapy (73). This impairment of vasomotor control affects thermoregulation, and although it can be postulated that this all occurs due to a central disorder of thermoregulation, the possibility of peripheral vascular dysfunction cannot easily be dismissed

It is clear that the mechanism behind these thermoregulatory changes is not fully accounted for, although it is likely that there are changes in core body temperature prior to a hot flush and this precipitates alterations in neuroendocrine pathways involving the steroid hormones, noradrenaline, the endorphins and serotonin (57).

Oestrogen

Oestrogen is a steroid hormone whose name comes from the Greek *estrus* (sexual desire) and *gen* (to generate). The three most commonly occurring are oestrone (E1), oestradiol (E2) and oestriol (E3). Oestradiol is the primary oestrogen from menarche to menopause, after which oestrone predominates.

Oestrogens in premenopausal non-pregnant women are formed by aromatisation of androstenedione and testosterone in the granulosa cells of the ovary (see figure 10). Receptors are located in the cell nucleus and are members of the superfamily of receptors known as nuclear ligand-activated transcription factors. Activated oestrogen receptors bind to oestrogen response element genes, resulting in gene expression. The genes subsequently expressed may then result in the synthesis of receptors, growth factors, enzymes, brain-derived neurotrophic factors and other proteins which act to regulate cellular functioning.

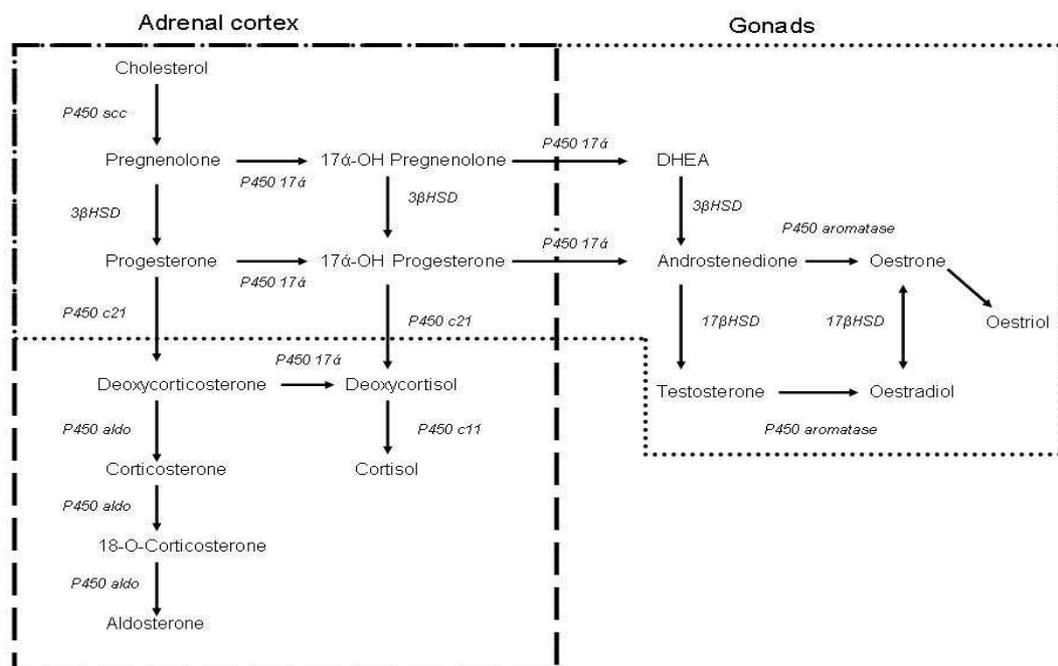


Figure 10: Steroid synthesis

In addition, steroid receptors may also be present in mitochondrial membranes and over the last 20 years, studies have focused on non-genomic effects of sex

steroids regulating cell function through the rapid activation of multiple signalling cascades and second messenger systems (74).

There are other sites of oestrogen biosynthesis throughout the body and these become important after the menopause. These include adipose tissue and skin (75), osteoblasts (76) and chondrocytes in bone, vascular endothelial (77) and aortic smooth muscle cells (78) as well as a number of sites in the brain, including the medial preoptic/anterior hypothalamus, the medial basal hypothalamus and the amygdala (79).

It has been suggested that the oestrogen synthesised in these compartments is probably only biologically active at a local tissue level in a paracrine fashion (80). Thus the total amount of oestrogen synthesised by these extragonadal sites may be small, but the local tissues concentrations are probably quite high and exert significant biological influence locally (81).

The effect of decreasing levels of oestrogen has been extensively studied. Changes in mood, anxiety, depression, insomnia, headaches/migraine and alterations of cognitive functions are thought to be linked to changes in serotonergic, noradrenergic and impairment of opioidergic peptide synthesis and secretion in postmenopausal women. HRT has also been shown, in most cases, to restore levels of these neurotransmitters and that this is accompanied by an improvement in mood, psychological and cognitive disturbances (82).

Changes in core temperature may also be associated with alterations in neuroendocrine pathways involving steroid hormones, noradrenaline, the endorphins and serotonin (57). Noradrenaline and serotonin are thought to be the key neurotransmitters involved.

Peripheral vasodilation and sweating is the peripheral response to centrally controlled temperature regulation and are also hallmarks of flushing. Peripheral vasodilation occurs as a result of withdrawal of vasoconstriction (10-20%) and activation of sympathetic vasodilator nerves in the skin (80-90%). This active vasodilation is mediated by cotransmission from sympathetic cholinergic nerves, with a moderate role for nitric oxide (83).

All blood vessels are lined by a single layer of cells called endothelial cells, which act as the interface between the rest of the vessel wall and the circulating blood. Amongst their many roles, they are responsible for release of nitric oxide which acts as a potent vasodilator. One of the best characterised examples of rapid sex steroid signalling is the activation of endothelial nitric oxide synthase in endothelial cells by oestradiol, a process initiated by the activation of membrane bound oestrogen receptors (84).

Administration of HRT to postmenopausal women with Type 2 diabetes has also been shown to improve vascular reactivity, but it is not known if this contributes to its efficacy in treating flushing (85).

Noradrenaline

Noradrenaline (NA) is thought to be the primary neurotransmitter responsible for lowering the thermoregulatory set point and triggering hot flushes (55, 64).

Animal studies have shown that intrahypothalamic injection of noradrenaline acts to narrow the thermoregulatory zone (86). Noradrenergic stimulation of medial preoptic area in monkeys (87) and baboons (88) by microiontophoretic application of noradrenaline causes peripheral vasodilation and heat loss and a drop in core body temperature.

It has also been shown that plasma levels of the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) are significantly increased both before and during hot flush episodes in postmenopausal women (59).

This theory is supported by clinical studies showing that clonidine, which is a α_2 -adrenergic agonist that reduces brain NA, significantly reduces hot flush frequency (89, 90), in particular by decreasing the intensity of the flushes. It has also been demonstrated that clonidine acts to widen the thermoregulatory zone in humans (91, 92).

It has also been shown, in a controlled laboratory investigation, that yohimbine (α_2 -adrenergic antagonist that elevates brain NA) provokes hot flushes in symptomatic women (93).

Castration in rats is associated with an increase in hypothalamic noradrenaline turnover (94) and oestrogen replacement decreases this activity (95).

Postmortem studies have shown that most α_2 -receptors in the human brain are inhibitory, presynaptic receptors. Given that oestrogens modulate brain adrenergic receptors (96, 97), it is possible that the oestrogen withdrawal at menopause is associated with the deficit of the inhibitory α_2 -receptor function in symptomatic postmenopausal women (withdrawal of tonic inhibition). This could lead to increases in noradrenaline and a subsequent narrowing of the thermoneutral zone.

Oestrogen and testosterone can also stimulate natural endorphin production and thus may exert a modulatory effect on noradrenaline release both directly and indirectly (see figure 11) (98).

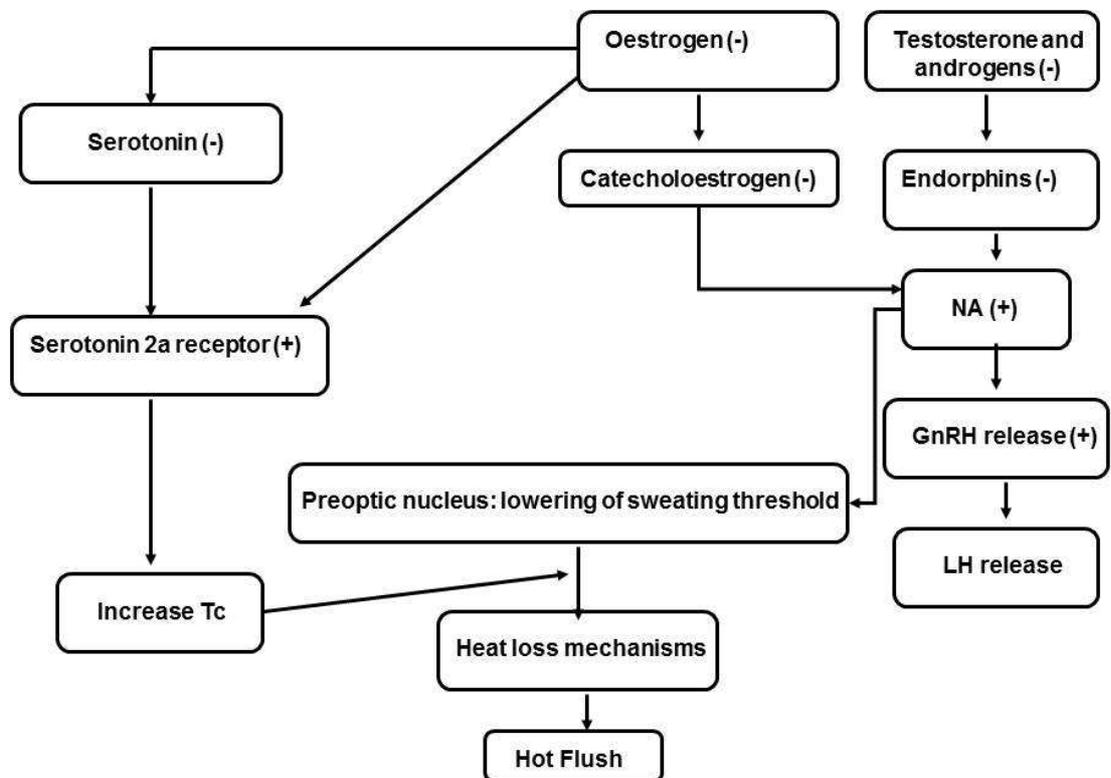


Figure 11: Model of pathogenesis leading to alteration of sweating threshold
This is a model proposed by Shanafelt (58), integrating and summarising the neurotransmitters that are thought to play a role in hot flushing. (+) increased, (-) decreased.

Whilst the hypotensive effect of clonidine is thought to be mediated mainly through selective stimulation of presynaptic alpha-adrenergic receptors in the region of the vasomotor centre in the medulla (99), a direct action of clonidine, with constriction of skin though not of muscle vessels, has been demonstrated in the human forearm after intra-arterial injection of the drug (100).

It has also been suggested that increased forearm blood flow, induced by intravenous infusion of adrenaline, angiotensin and noradrenaline, was significantly less in symptomatic postmenopausal women when treated for at least 6 weeks with clonidine compared with that induced in the women by infusions given before treatment (101).

It is clear that the mechanism is not fully understood, it may exert its effect on hot flushing through a reduction in peripheral vascular reactivity (102) and also through central mechanisms (103).

Serotonin

5-Hydroxytryptamine (5-HT), commonly known as serotonin, was identified in 1948 by Rapport (104), and was the name given to the a substance identified in serum (sero) that was a potent vasoconstrictor (tonin).

Synthesis

It is an intermediate product of tryptophan metabolism and is synthesised in the human body from the essential amino acid L-tryptophan (see Figure 13).

L-tryptophan is converted to serotonin via a series of reactions. The enzyme tryptophan hydroxylase (TPH) adds a hydroxide to tryptophan's benzene ring, thus forming the molecule 5-hydroxytryptophan. A further enzyme, aromatic amino acid decarboxylase (AAAD), removes a carbon dioxide molecule from 5-hydroxytryptophan, consequently forming 5HT. TPH is the rate-limiting step in the formation of 5HT and it exists in two isoforms; TPH1, found in several tissues (105) and TPH2, which is a neuron-specific isoform (106).

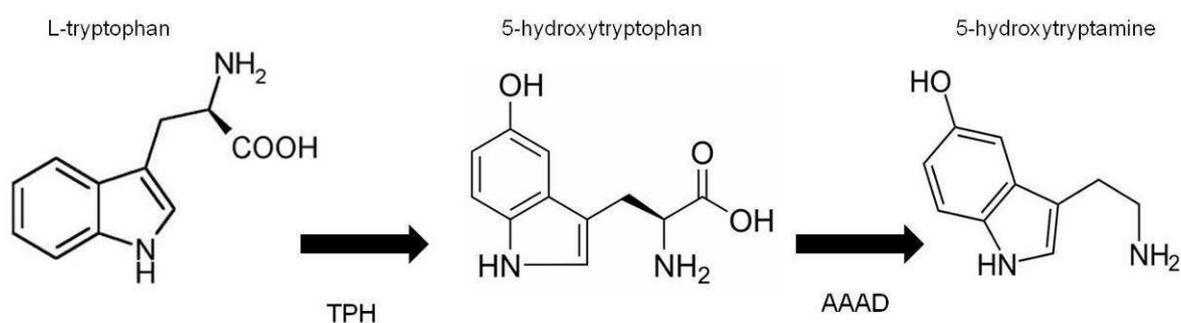


Figure 12: Synthesis of serotonin

Centrally produced 5-HT is dependent on the amount of tryptophan available to cross the blood brain barrier, as it is impermeable to 5-HT. Tryptophan is in competition with other large neutral and branched amino acids for the active transporter required for blood brain barrier carriage (107), in contrast to 5-hydroxytryptophan which moves freely across (108).

Over 95% of total body serotonin is produced and stored by the enterochromaffin cells of the duodenum (109), with only a small amount produced by the raphe nuclei of the brain (110), and neuroendothelial cells that line the lung.

Release and transportation

Serotonin released into the circulation by the enterochromaffin cells is rapidly taken up by platelets, which makes platelets the fundamental regulators of plasma 5-HT concentration. Platelet uptake is dependent on the serotonin transporter (SERT). After 5HT is transported by SERT across the platelet plasma membrane, it is either sequestered into dense granules by vesicular monamine transporters (VMAT) or degraded by monoamine oxidase (MAO) to 5-hydroxyindole acetic acid (5-HIAA). MAO is an intracellular enzyme, and therefore 5HT must be transported into the cell before it can be metabolised, SERT facilitates this.

Serotonin uptake into dense granules protects the organism from 5-HT induced uncontrolled, harmful vasoconstriction or vasodilation. So far, however, the reason for platelet 5-HT transport is unknown.

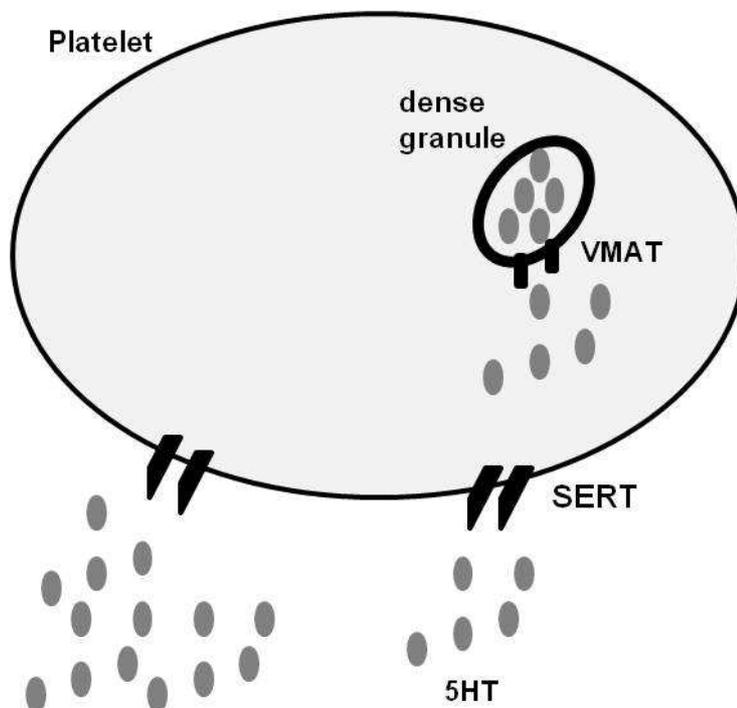


Figure 13: Platelet serotonin transport
VMAT (vesicular monamine transporters). SERT (serotonin reuptake transporter). 5-HT (serotonin).

SERT is a member of the Na^+Cl^- dependent solute carrier 6 family, which includes transporters of noradrenaline and dopamine, however the detailed mechanism by which it depends on transmembrane solute gradients is still not fully understood (111).

Function

The function of serotonin as a neurotransmitter in the central nervous system is well known, including its role in mood, anxiety, sleep, sexual behaviour and eating, but it is also important for platelet aggregation and regulation of vascular tone.

Sites of extra-pulmonary vascular synthesis have not yet been identified, so the current understanding of 5HT in the periphery is that the systemic vasculature is exposed to 5HT through its release from platelets via SERT or to freely circulating 5HT (112).

Receptors

The effects of serotonin are mediated by its interaction with one of its receptors. A recent review of serotonin receptor subtypes has identified 7 distinct families of 5-HT receptors, with 15 subpopulations (113).

With the exception of 5HT₃, which is a ligand-gated ion channel receptor, all other 5HT receptors are G-protein-coupled receptors that activate an intracellular second messenger cascade to produce an excitatory or inhibitory response.

Family	Type	Mechanism	Potential	Subgroup	Location
5HT ₁	G ₁ /G ₀ -protein coupled	Decreasing cellular levels of cAMP	Inhibitory	5HT _{1A}	Presynaptic serotonergic neurons Postsynaptic non-serotonergic neurons in corticolimbic areas
				5HT _{1B}	Heart and stomach fundus
5HT ₂	G _q /G ₁₁ -protein coupled	Increasing cellular levels of IP ₃ and DAG	Excitatory		
5HT ₃	Ligand-gated Na ⁺ and K ⁺ cation channel	Depolarising plasma membrane	Excitatory		GI system CNS (brainstem, higher cortical areas)
5HT ₄	G _s -protein coupled	Increasing cellular levels of cAMP	Excitatory		GI system
5HT ₅	G ₁ /G ₀ -protein coupled	Decreasing cellular levels of cAMP	Inhibitory	5HT _{5A}	Exclusively CNS; cortex, hippocampus, cerebellum
				5HT _{5B}	No functional protein encoded in humans
5HT ₆	G _s -protein coupled	Increasing cellular levels of cAMP	Excitatory		cognition, learning, appetite control, convulsive disorders
5HT ₇	G _s -protein coupled	Increasing cellular levels of cAMP	Excitatory		

Table 1: Serotonin receptors, type, mechanism, potential, subgroup and location.
Adapted from Jonnakuty, C. and Gagnoli, C. (114)

Role in hot flushes

In addition to the wide range of physiological functions already mentioned, serotonin is also thought to play a key role in thermoregulation (115).

Blood 5-HT levels have been shown to be lowered both in spontaneous and surgically menopausal women, i.e. after oestrogen withdrawal and at a time when hot flushes occur, and in both groups the 5-HT level was restored to normal values after treatment with oestradiol (116, 117).

In menopausal women, treatment with oestrogen has been found to augment serotonergic activity (118) and an increased urinary excretion of 5-HIAA, the main metabolite of 5-HT, was found after oestrogen treatment (119).

Furthermore, selective serotonin reuptake inhibitors (SSRI), designed to increase the available serotonin at the serotonergic synapse, have also been shown in placebo-controlled trials to be effective in reducing the number and severity of hot flushes (120), although the mechanism is poorly understood.

Fat

Previous studies have examined potential risk factors that may predispose certain women to hot flushes. Some of these studies have found that smoking (121, 122) increases the risk of flushes, whilst moderate alcohol use decreases the risk (123), and these were independent of sex hormone levels. It was also largely accepted for years that postmenopausal women with a high BMI were afforded some protection to flushing due to peripheral conversion of androgens to oestrogens in adipose tissue. This was borne of the theory that more adipose tissue equals more conversion of androgens and therefore higher levels of circulating oestrogen with a reduction in likelihood of flushing.

However, contrary to this, hot flushing is associated with high body mass index, which may occur due to relative hypoestrogenism (124) or to the insulating nature of fat. A change in frequency of flushes also follows an increase in blood glucose suggesting a possible role for carbohydrate metabolism (125), increased insulin resistance being commonly associated with obesity.

It is also been suggested that obesity is associated with hot flushes through a mechanism that involves alterations in cytokines, including leptin (126). Leptin is a highly conserved cytokine-like protein that is expressed by adipocytes in proportion to body fat mass and has been related to insulin resistance (127).

After menopause, adipose tissue becomes the main source of oestrogen. This can only occur after conversion of DHEA to androstenedione, elaborated from the adrenal cortex, followed by aromatisation to oestrogen (see Figure 9). This is catalysed by aromatase cytochrome P450. Aromatase is expressed in various sites in humans and is controlled by tissue-specific promoters. Aromatase is expressed in the stromal mesenchymal cells of adipose tissue. These stromal cells grow in culture into fibroblasts and are believed to be preadipocytes (75). When serum is present in culture, expression of aromatase is stimulated by glucocorticoids. This effect of serum stimulating aromatase expression has been mimicked by members of the Class I cytokine family, including IL-6. Tumour necrosis factor α (TNF α) also stimulates aromatase expression in adipose stromal cells in the presence of dexamethasone.

Obesity is also linked to impaired vasoreactivity when compared to lean populations. (128). Obesity leads to insulin resistance and endothelial dysfunction, mainly through fat-derived metabolic products, hormones, and cytokines (adipocytokines) (129). Sex steroid hormones also modulate vascular function through regulation of endothelium-derived factors (130). It is possible that poorly functioning endothelium and relatively lower circulating levels of oestrogen in obese women could be linked to worse vasomotor symptoms associated with the menopausal transition and post-menopause.

Men

Prostate cancer is the most common cancer diagnosed in men in the UK. It represents 24% of all new cancer cases and 6% of all cancer deaths. By the time a man reaches age 76, he will have a 1 in 6 chance of being diagnosed with prostate cancer. Between 1998 and 2007 new cases rose by 35% and it is estimated that the number of men with prostate cancer will increase by 50% over the next 20 years. This may be as a consequence of both an ageing population and a longer duration of survival as mortality rates for prostate cancer have been reducing since 1994 (whether as a result of improved screening, diagnosis or treatment).

Prostate cancer was described in 1853 and surgical castration has been used since around 1896, although it wasn't until 1941 that androgens were discovered to play a role in prostate cancer and that androgen ablation causes regression of primary and metastatic androgen dependent prostate cancer. In 1966, chemical castration won Huggins the Nobel Prize for medicine and this is now certainly the most typical method of androgen deprivation.

Prostate Cancer

Prostate cancer is the second leading tumour in males, with approximately 680,000 new cases annually worldwide and accounts for 11.7% of all male cancers. It is largely a disease of older men; the majority of patients are older than 65 years, and most of the deaths from prostate cancer are in this age group.

Staging of disease

Clinical and pathologic staging of prostate cancer involves determination of the anatomic extent and burden of tumour based on the best available data. The TNM system [primary tumour (T), regional lymph node (N), and metastases (M)] is the most widely used system for prostate cancer staging. It stratifies patients according to the method of tumour detection, separating nonpalpable 'incidental' prostate cancers detected during transurethral resection for clinically benign prostatic hyperplasia and palpable cancers detected by digital rectal examination.

Gleason Score

Gleason grading (131) is a strong predictor of survival among men with prostate cancer. The Gleason system, introduced in 1974, is an architectural grading system that ranges from 1 (well differentiated) to 5 (poorly differentiated). The Gleason score (GS) is the sum of the primary and secondary patterns with a range of 2 to 10. It has long been appreciated that patients with $GS \geq 7$ are at greater risk of extraprostatic extension and biochemical recurrence (132).

It is used together with the clinical stage of the disease and the prostate specific antigen (PSA) to categorise risk (see Table 2) and plan treatment.

	PSA (ng/ml)		Gleason Score		Clinical Stage
Low Risk	<10	and	≤6	and	T1-T2a
Intermediate Risk	10-20	or	7	or	T2b-T2c
High Risk	>20	or	8-10	or	T3-T4

Table 2: Risk stratification criteria for men with localised prostate cancer
Men with clinical stage T3-T4 cancers have locally advanced disease

The National Institute for Health and Clinical Excellence published guidelines in 2008 for the management of prostate cancer based on both the stage of the disease and risk stratification (133). Treatments range from watchful waiting and active surveillance to radical surgical treatments and adjuvant therapies in the form of radiotherapy, cryotherapy and brachytherapy.

Androgen Deprivation Therapy (ADT)

Huggins and Hodges first reported the dramatic clinical effects of suppressing serum testosterone levels in men with advanced prostate cancer in 1941 (134). ADT was achieved by surgical castration or suppression of GnRH production at the level of the hypothalamus with diethylstilbestrol (DES). Within a short period, hormonal therapy became widely accepted and Huggins and Hodges were awarded the Nobel Prize in 1967 for this pioneering work (135).

Today, ADT is the gold standard treatment for advanced prostate cancer. It is also sometimes used to treat patients with biochemical failure even without evidence of local or systemic recurrence, and as an adjunct in patients undergoing radiation for high-risk localised disease.

Approximately 90% of men will undergo medical castration therapy with gonadotrophin-releasing hormone (GnRH) agonist, while the other 10% have bilateral orchidectomy (136).

Gonadotrophin releasing hormone (GnRH) agonists

The Veterans Administrative Cooperative Urological Research Group (VACURG) was initiated in 1959 to investigate the role of ADT in the treatment of prostate cancer (137). Ultimately a daily dose of 3mg DES was thought best and became the accepted regimen. DES, however, was associated with cardiovascular toxicity.

It was not until 1971 that the decapeptide, luteinising-hormone-releasing-hormone (LHRH) was purified (138), and it was the availability of long-acting synthetic LHRH agonists in the 1980s which revolutionised the hormonal treatment of prostate cancer. Leuprolide acetate was the first LHRH agonist evaluated as a treatment for advanced prostate cancer and was found to be equivalent to 3mg of DES in reducing serum testosterone to castrate levels and had a lower incidence of cardiovascular toxicity (139).

Over the next 30 years, a number of other LHRH agonists were developed and all had similar abilities to lower serum testosterone to castrate levels and similar side effect profiles.

Chronic exposure to LHRH agonists causes downregulation of receptors in the anterior pituitary gland, leading to decreased luteinising hormone (LH), which is responsible for promoting testosterone production in the Leydig cells of the testes, thereby suppressing testosterone production. Injection of LHRH agonist initially triggers the secretion of LH, which results in a testosterone surge lasting 1 to 2 weeks. The desired castrate levels are reached within 3 weeks after initiation of therapy.

Microsurges of testosterone may occur, particularly at times of repeat injections. It has been suggested that repeated testosterone breakthroughs may be associated with inferior 5-year nonevidence of disease and, for this reason,

monitoring of testosterone concentrations throughout therapy has been recommended by some (140).

Recent research has focused on GnRH antagonists, which results in more rapid androgen deprivation without the testosterone surge (141). Degarelix binds to and subsequently blocks GnRH receptors in the anterior pituitary gland, leading to decreased secretion of LH and FSH and a reduction in testosterone concentrations to castrate levels in 1-3 days. Further trials are needed, however, to study long-term effects in comparison with LHRH agonists.

Side effects

An immediate and potentially life threatening effect of GnRH agonists in the treatment of, particularly high-volume metastatic, prostate cancer is the 'flare phenomenon'. This is attributed to a surge of testosterone levels due to the initial stimulation of GnRH receptors, and is prevented by pretreatment with anti-androgen, which inhibits the stimulatory effect of the testosterone surge at the level of the androgen receptor (142).

Other longer term side effects include increased fracture risk, maximal in the first year after initiation of therapy (143), gynaecomastia, memory loss, erectile dysfunction and loss of muscle mass with increases in adipose tissue. Current data also suggest that men who are receiving long-term ADT are at risk for developing insulin resistance (144), hyperglycaemia, and the occurrence of metabolic syndrome (145), thus leading to an increased risk of cardiovascular complications.

Total and free testosterone levels during treatment were 1.8-fold and 2.3-fold higher in obese men, which may contribute to the association between obesity and increased prostate cancer mortality (146).

Of particular interest to us, and in common with peri and postmenopausal women, is the presence of hot flushing, documented as early as the 19th century, and therefore, predating the link between androgens and the disease.

In some studies, following bilateral orchidectomy, as many as 50% of patients will experience hot flushes. With LHRH agonists, as many as 75% in some

studies, with symptoms continuing for months, years and even beyond discontinuation of therapy in some. In a study of 63 men, 48% still experienced flushes 5 years after treatment (147).

Alterations in skin blood flow have also been identified in men (148) and dependence on sex steroid levels suggest mechanisms may be similar to those found in women (149).

Hypothesis

The pathophysiology of vasomotor symptoms in postmenopausal women and hypogonadal men include alterations in subcutaneous microvascular reactivity.

Summary of Aims and Objectives

There are several aims which will be explored in this thesis:

1. Hot flushing is associated with vasodilation of skin blood vessels most particularly of the upper body and face, similar to that which occurs in an attempt to cool the body during increases in core body temperature. Whilst it has been suggested that flushing may be as a result of a narrowed thermoneutral zone, that is a centrally controlled mechanism, little consideration has been given to the potential role of a peripheral mechanism. Subcutaneous microvascular reactivity has been studied in postmenopausal women with flushing and compared to matched women with no flushing.
2. The alpha-adrenergic system is thought to play a key role in thermoregulation in humans, mainly thought to be as a result of central manipulation. Clonidine is an alpha-adrenergic agonist, thought to act principally in the vasomotor centre of the medulla, and has been shown in placebo-controlled trials to have a beneficial effect on flushing. What is the effect of successful treatment of flushes with clonidine on subcutaneous microvascular reactivity when compared to placebo?
3. The decline in prescribing HRT has led to an increased interest in non-hormonal alternatives and in recent years, serotonin has become a focus for study. The use of selective serotonin reuptake inhibitors (SSRI) has been demonstrated in placebo-controlled trials to be associated with the relief of flushing. It is possible to study its effect not only on peripheral microvasculature, but also to study the serotonin reuptake inhibitor (SERT) in vivo in the human brain.
4. Although there has been a notable decline in the prescription of HRT, this remains the single most effective treatment for flushing. There is increasing evidence of the effect of oestrogen on the endothelium and HRT has been shown to improve vascular reactivity in postmenopausal women with Type 2 diabetes, but it is unknown whether this contributes to its efficacy in treating flushing.

5. Obesity is linked to impaired vasoreactivity when compared to lean populations. Are vasomotor symptoms in obese peri- and post-menopausal women due to altered endothelial function as assessed by peripheral microvascular reactivity?

6. And, as already stated, alterations in skin blood flow have been identified in men who flush and a dependence on sex steroid levels suggest that mechanism underlying their flushing may be similar to those found in women.

Chapter 2

Materials and Methods

Introduction

This chapter describes the methods employed in recruiting volunteers for the study and the measurement of subcutaneous microvascular perfusion by LASER Doppler imaging and iontophoresis (LDI+Ion) together with endocrine and metabolic assessments. This chapter also discusses measurement of the density of the serotonin transporter with single photon emission computed tomography (SPECT) scanning.

Subjective and objective measurements of flushing were also recorded and these too will be discussed here.

This study was conducted with the ethical approval of Scotland A Research Ethics Committee (REC Reference 09/MRE00/40). Pilot data was collected with ethical approval (REC 07/50704/43) under the title; A study to investigate the value of LASER Doppler iontophoresis as a biomarker for vasomotor symptoms. This pilot study will be discussed separately.

There were 3 different groups of participants in this study and they will each be discussed in turn, prior to detailed methods descriptions.

All subjects were given an information leaflet at least 24 hours prior to participation and gave written informed consent.

Group 1

Forty postmenopausal women (defined as either last menstrual period greater than 12 months prior to participation in study or $FSH \geq 20IU/L$) who were between the age of 45 and 65 years old and who had at least 20 hot flushes per week. 20 of these women also had SPECT imaging, which was carried out at the Southern General Hospital. All participants in this group received venlafaxine as part of the study.

Exclusion criteria

Any participant with known hypertension, insulin-dependent diabetes, significant cardiovascular disease, bleeding disorders, or who were taking any antidepressants or drugs known to affect vascular reactivity were excluded.

Study design

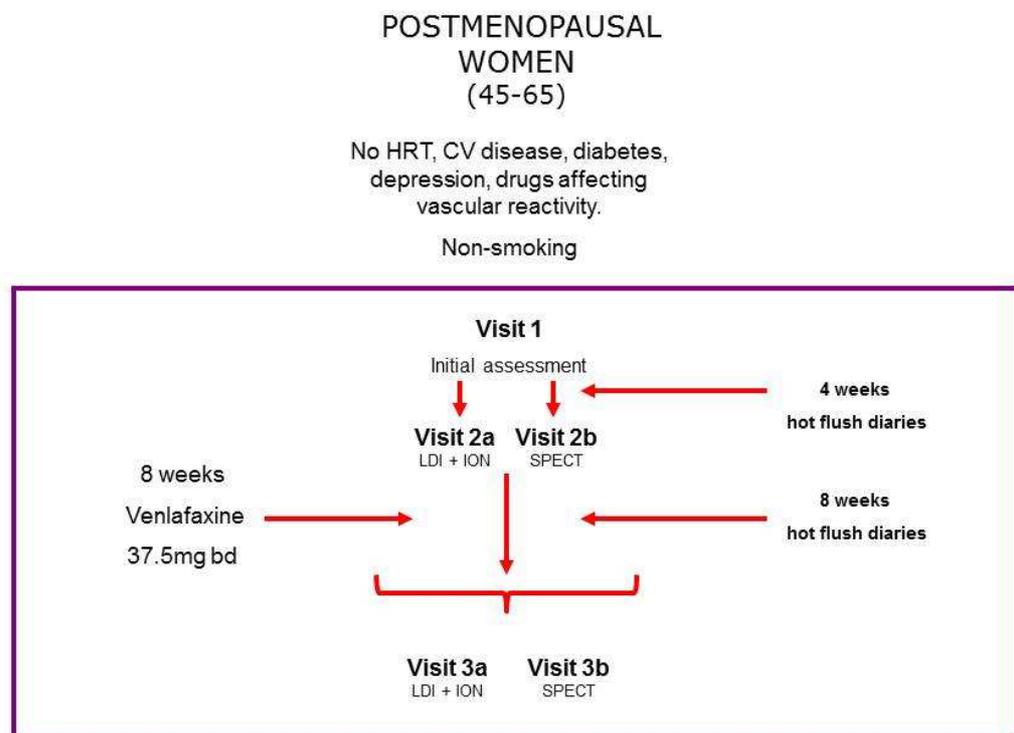


Figure 14: Group 1 study design

Methods

An initial visit (Visit 1) took place at least 24 hours following presentation of the appropriate study information leaflet. At this visit there was an opportunity to ask any remaining questions before signing a consent form. A full medical history was taken and hot flush diaries presented. These were completed for 4 weeks prior to baseline measurements of LDI + ION. At Visit 2, hot flush diaries were collected, and measurements of height, weight, blood pressure, and hip and waist circumference were taken. LDI + ION measurements were completed and a blood sample was collected before venlafaxine was prescribed and dispensed by clinical trials pharmacy. Clear instructions on the use of the tablets were given (one 37.5mg tablet in the morning and one at night, to be taken after food), a reminder of potential side effects (particularly nausea) and contact information was highlighted in the information leaflet. An 8 week return appointment was made.

At Visit 3 (after 8 weeks), hot flush diaries were collected, LDI + ION was repeated and a blood sample was collected. A 2 week supply of decreased dose of venlafaxine (37.5mg once daily) was prescribed and dispensed by the clinical trials pharmacy. A decreased dose was not required in those wishing to continue therapy, a letter was provided requesting that GP continue treatment.

Greene Climacteric Scale, Becks Depression Inventory and Hospital Anxiety and Depression Scale questionnaires were completed by all participants at V1 and V3.

Twenty of the participants in this group had also volunteered for SPECT imaging which was carried out at the Southern General Hospital (V2b and V3b). Detailed information on this will be provided later in this chapter. Venlafaxine was not commenced until the baseline scan had been completed, and a repeat scan was also carried out at 8 weeks.

Letters detailing participation in the study were sent to participants' General Practitioners with consent.

Group 2

Twenty postmenopausal women (definition as above) who were between the age of 45 and 65 years old and who had at least 20 hot flushes per day. These women were assessed by either their General Practitioner or at a local menopause clinic and were prescribed HRT, although had not commenced treatment yet.

Exclusion criteria

As for Group 1, but also including past history of DVT, stroke, breast cancer, and exposure to HRT within one month for transdermal preparations, three months for oral preparations and 6-9 months for an estradiol implant.

Study design

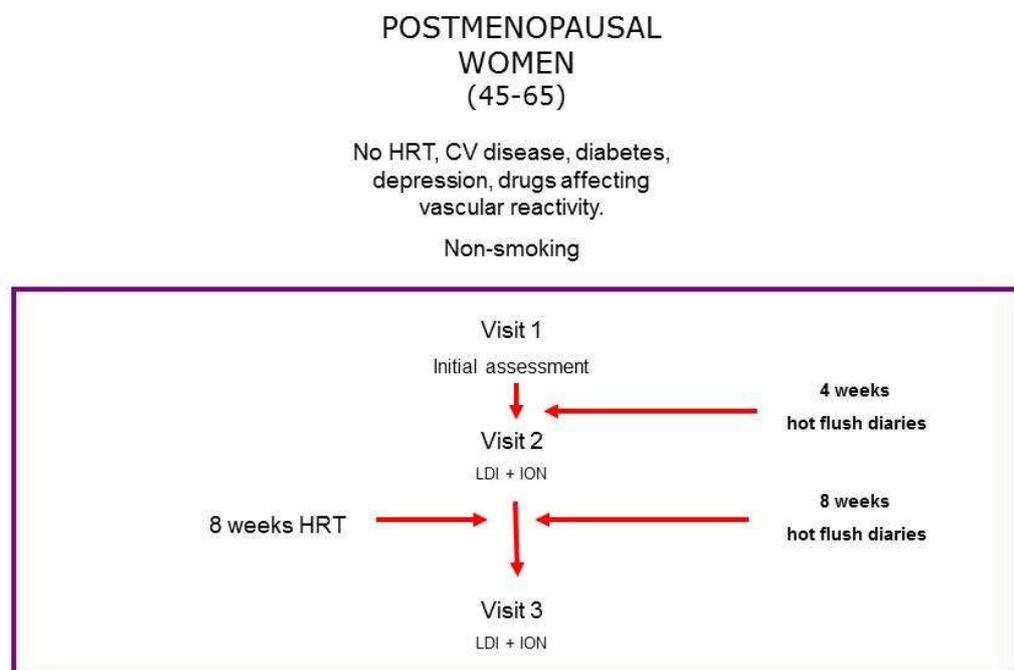


Figure 15: Group 2 study design

Methods

As with group 1, an initial visit (V1) took place at least 24 hours following presentation of the appropriate study information leaflet. At this visit there was an opportunity to ask any remaining questions before signing a consent form. A full medical history was taken and hot flush diaries presented. These were completed for 4 weeks prior to baseline measurements of LDI + ION. At Visit 2 (V2), hot flush diaries were collected, and measurements of height, weight, blood pressure, and hip and waist circumference were taken. LDI + ION measurements were completed and a blood sample was collected. An 8 week return appointment was made.

Participants then commenced HRT, as prescribed by GP or hospital practitioner, and hot flush diaries were recorded until the return appointment.

At visit 3 (V3) (after 8 weeks), hot flush diaries were collected, LDI + ION measurements were repeated and a blood sample was collected.

Subgroup from Group 1 and Group 2

It was the aim that at least 15 women from Group 1 and Group 2 should have a BMI greater than $30\text{kg}/\text{m}^2$; these women formed a subgroup to assess whether increased flushing seen in women who are obese is due to altered endothelial function as assessed by peripheral microvascular reactivity.

As these women were recruited to group 1 and group 2, inclusion and exclusion criteria were as above.

Group 3

Twenty men with prostate cancer for whom gonadotrophin-releasing hormone (GnRH) agonist therapy was to be prescribed, and who were between the age of 55 and 75 years old were to be recruited to this group.

Exclusion criteria

Any participant with known hypertension, insulin-dependent diabetes, significant cardiovascular, renal or liver disease, bleeding disorders, or taking any drugs known to affect vascular reactivity were excluded.

Study design

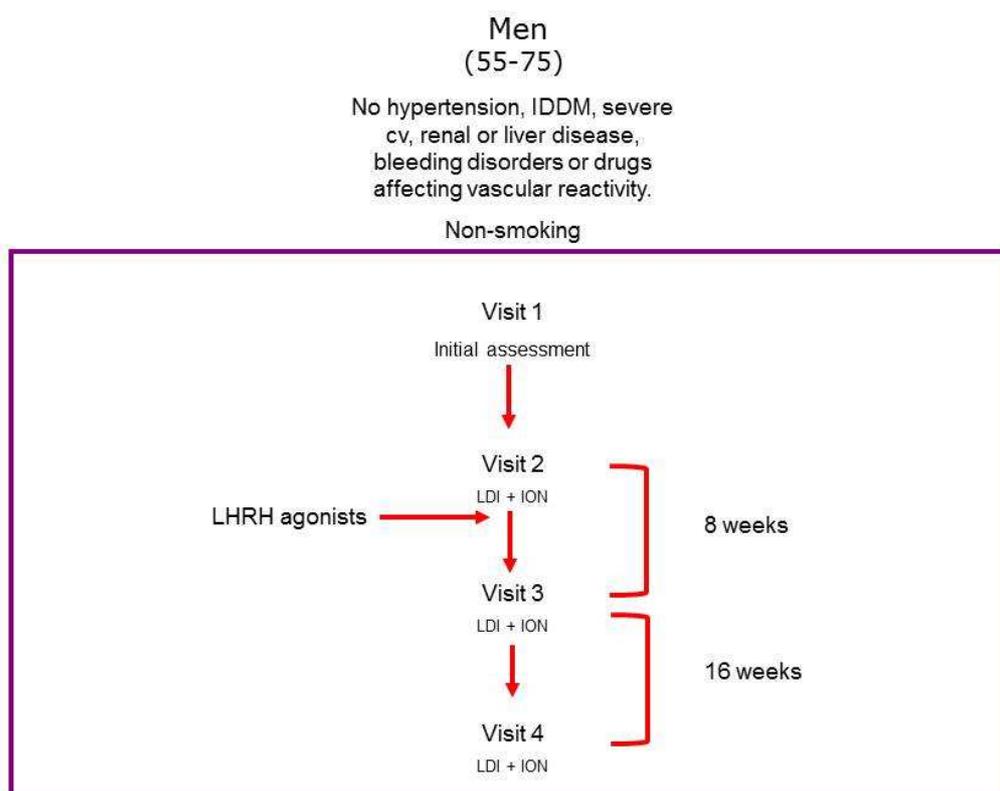


Figure 16: Group 3 study design

Methods

As with groups 1 and 2, an initial visit (V1) took place at least 24 hours following presentation of the appropriate study information leaflet. At this visit (V1) there was an opportunity to ask any remaining questions before signing a consent form. A full medical history was taken and measurements of height, weight, blood pressure, and hip and waist circumference were taken. At visit 2 (V2) LDI + ION measurements were completed and a blood sample was collected. An 8 week return appointment was made.

Participants then commenced GnRH agonist therapy as prescribed by their hospital practitioner. Hot flush diaries were presented to the participant, who was asked to complete these if flushing developed.

At V3 (after 8 weeks), hot flush diaries were collected, LDI + ION measurements were repeated and a blood sample was collected.

Flushes are most likely to coincide with achievement of castrate levels of testosterone a few weeks after commencement of therapy and increase in frequency at approximately three months. For this reason, participants were asked to return again at 24 weeks (V4).

Recruitment, screening and selection

Groups 1 and 2

Volunteers were recruited from the Greater Glasgow and Clyde area. A variety of media were utilised to publicise the study. Initial recruitment for Groups 1 and 2 was generated from a press release in the Glasgow Herald newspaper (see Appendix II) which coincided with an advertisement on the Glasgow University homepage. As a consequence of this, I was invited to speak about hot flushes and the study on BBC Radio Scotland (see Appendix II). Also being interviewed was Joan McFadden, an agony aunt who writes a column for the Daily Record, and a few months later there was further exposure in a piece written by her which included an interview of a study participant (see Appendix II).

Recruitment posters were displayed in Menopause clinics across the city, and a number of general practitioners agreed to display posters and leaflets in their surgeries. We also displayed posters and attended breast screening services in Glasgow as this was an age-appropriate group of women.

To further increase exposure, I spoke at a British Menopause Society Women's Health Meeting, which is designed as an informative day for those medical practitioners with a specific interest in women's health. Wellbeing of Women organised a Christmas Fair to which some 500 women were invited. A copy of our recruitment poster was inserted into the invitations. Unfortunately I was not able to attend the Fair as it was scheduled during a week of adverse winter weather and virtually all transport ceased. There was however still some interest generated from the invitations.

A notice was also put on the Menopause Matters website, which generated a number of enquiries, however most of these were from women in England who could not attend the Western Infirmary in Glasgow for 3 visits.

A number of interested volunteers were also identified at Professor Lumsden's menopause clinic, which I attended weekly.

Interested volunteers contacted us either by telephone or email to express an interest in the study. Basic information about the study was given and screening questions were asked, and if the volunteer was still interested and thought to be suitable, formal study information was sent either by email or post. Contact details were taken and a suitable time arranged for a call back. If the volunteer wished to participate, appointments for visit 1 (V1) were arranged.

Group 3

Men for group 3 were identified from the Greater Glasgow and Clyde Prostate cancer multidisciplinary team meeting, a weekly meeting attended by Urology surgeons, Oncologists, Pathologists, Radiologists and Biochemists. Prior to commencing recruitment for this group, the study protocol was presented to the group.

All new cases of prostate cancer (mean/week=13) were discussed at this meeting and potentially suitable gentlemen were identified. These gentlemen were then approached at their next hospital appointment. Basic information was given about the study, as they had usually had a significant volume of information regarding their diagnosis and treatment options at this visit, an information leaflet was given to them, and a contact number was taken and a suitable time to call arranged.

Posters were also displayed in urology and oncology clinics to alert clinicians to the study.

Body Composition

Height

Height was measured using a stadiometer. The participant was measured barefoot, with their back positioned against the stand and their arms relaxed in the lateral position. The head was also positioned against the stand, with the line of sight perpendicular to the stand. Measurement was performed when the volunteer was positioned and relaxed, and a movable headboard was lowered on to the top of the head with light pressure allowing hair compression. The measurement was made to the nearest 0.01 metre.

Body Mass

Body mass was measured using weighing scales (Seca, Germany). The same scales were used for all volunteers throughout the study. Subjects were dressed without shoes when weighed. Body mass was measured with both feet on the balance and with arms positioned in the lateral position. Body mass index (BMI, kg/m²) was calculated as:

$$\text{BMI} = \text{weight (kg)} / \text{height (m)}^2$$

Waist and hip circumference

Circumferences were measured using a non-elastic tape measure, and measurement was made with the abdominal muscles relaxed, at the end of normal expiration. Waist circumference was measured at the umbilical level. Hip circumference was measured around the point of maximal width around the hip region, at approximately the region of the pubic symphysis. The same investigator performed the measurements on each volunteer, on every occasion. Waist to hip ratio was determined by dividing the waist circumference by the hip circumference.

Blood pressure

Blood pressure was measured using an Omron automated monitor. Participants were seated with back supported and arm resting on the table. The cuff size and placement was correct and the participant was rested in a controlled environment for at least 5 minutes as recommended by the European Society of Hypertension guidelines on blood pressure measurement (150). Legs were uncrossed and the participant was relaxed and not talking.

Plasma Preparation and Analysis

Fasting blood sampling

A fasting blood sample was taken using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetra-acetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

Plasma preparation and storage

All blood samples were prepared in the laboratory within one hour of collection.

Haematocrit

All Vacuette® samples were turned on a roller for five minutes. Two glass capillary tubes were $\frac{3}{4}$ filled with whole blood from LiHep Vacuette®. The rest of the sample was kept to be spun with the others (see below). Capillary tubes were plugged with plasticine at marked end and placed in haematocrit centrifuge (*Thermo Scientific Heraeus Pico 21*) with the plugged end to the outside rim. The two capillary tubes were balanced in the centrifuge by placing at opposite ends to each other. Spin for 12 minutes at 13.3 xg. Haematocrit (Hct) was read by placing the capillary tubes on the reader one at a time and recording the values. If the values were different, a mean was taken and recorded.

Remaining samples

The remaining LiHep, EDTA and citrate samples were all spun in an underbench centrifuge (*Beckman GS-6KR*) at 3000rpm, 4°C for 10 mins. Using a fine tip pastette, plasma was aspirated and dispensed into labelled 2.0ml screwcap microtubes. Four 1 ml aliquots were closed with lilac lids (EDTA)(to correspond with Vacuette® top), two 1 ml aliquots closed with blue lids (Citrate) and two 1 ml aliquots closed with green lids (LiHep). All samples were placed into a small labelled (participant study number, date and Hct result) specimen bag and stored at -80°C.

Enzyme-linked immunoassays

All enzyme-linked immunoassay (ELISA) procedures were based on a 'sandwich' technique. Commercially produced plates were used for all ELISA. The wells of the plates were coated with a monoclonal antibody to the protein of interest. The addition of plasma to the wells bound protein to the antibody, with unbound molecules removed by washing. A second antibody specific to another area of protein was then added, with unbound molecules removed by a second washing. The second antibody was linked to an enzyme which would catalyse the conversion of a non-fluorescent substrate to a fluorescent product. The intensity of the subsequent fluorescence was directly proportional to the amount of protein in the initial sample.

Insulin was measured using a commercially available ELISA with <0.01% cross-reactivity with proinsulin (Mercodia, Uppsala, Sweden). Adiponectin, IL-6, VCAM-1, ICAM-1, and TNF α were analysed using commercially available kits (R&D Systems Europe, Abingdon, UK). High sensitivity kits were used for TNF α and IL-6. All samples for each participant were run in a single analyser run. The accuracy and precision of the assays was monitored using quality control sera (Mercodia AB, Uppsala, Sweden. R&D Systems Europe, Oxford, UK).

CRP, Cholesterol, Glucose, HDL, LDL, and Triglyceride were measured by standardised methods by vascular biochemistry at Glasgow University. All analysis was carried out on the ILAB 600 clinical chemistry analyzer, using Roche kits for HDL-cholesterol, triglyceride, and cholesterol. CRP, glucose, Apo A1 and APO B kits were from Randox Laboratories. All chemistries were calibrated using the assigned kit calibrators and checked against the relevant quality control. Vascular biochemistry is a member of the NEQAS UK wide external quality control scheme for all chemistries, with the exception of glucose.

LDL-cholesterol was calculated as follows;

Total cholesterol - HDL-cholesterol - (Trig/2.2).

Measuring vascular function

There are a number of methods that can be employed to measure vascular function.

Endothelial dysfunction and vascular injury have been described as precursors of atherosclerosis, which can result in coronary artery disease. In the normal coronary vasculature, acetylcholine releases nitric oxide (NO) and causes vasodilation, but in the presence of atherosclerosis and endothelial dysfunction (with decreased NO), acetylcholine causes vasoconstriction secondary to smooth muscle cell activation. Direct measurements of coronary flow responses are invasive and not readily repeatable, but brachial artery flow-mediated vasodilation (FMD) has been reported to be impaired in patients with abnormal coronary endothelial function, compared to those with normal coronary endothelial function, and so peripheral tests can be used as surrogates for coronary reactivity.

Whilst FMD is used to assess larger conduit arteries, peripheral vessels have traditionally been evaluated using venous occlusion plethysmography (VOP). This test involves placing a strain gauge around the forearm, and cuffs at the wrist and the upper arm. The wrist cuff is inflated to 200 mm Hg to prevent blood circulation in the hand and after the upper arm cuff is inflated to 40 mm Hg, venous occlusion causes forearm engorgement, which is then recorded on the plethysmograph to derive measurement of resting blood flow. The cuff is then inflated to suprasystolic pressures for between 4 and 10 minutes and then deflated, thereby measuring hyperaemic blood flow. Plethysmography is, however, technically difficult, and the recognition of a good waveform is somewhat subjective.

Microvascular function can of course also be assessed ex-vivo using wire myography.

However, none of these methods are specific to skin, and VOP measurements may include blood flow related to vessels in the muscle groups of the area under observation. LASER Doppler imaging is specific to the cutaneous microcirculation and as flushing is accompanied by warming of the skin as a

result of vasodilation of skin microvasculature, this method has advantages over the other methods. Iontophoresis of acetylcholine and sodium nitroprusside via perspex chambers on the skin allows assessment of vascular responses without the need for intravenous or intraarterial cannulation. As it is entirely non-invasive, this method is also completely painless, and may therefore be more acceptable to participants. It also has high temporal resolution as a result of continuous measurements.

LASER Doppler Imaging with Iontophoresis

Non-invasive skin perfusion can be measured using LASER Doppler Imaging with Iontophoresis (LDI+Ion) (see Figure 17) (151).

Iontophoresis is a technique which allows for transdermal delivery of vasodilator agents acetylcholine (ACh) and sodium nitroprusside (SNP) across the skin under the influence of an applied current. In the past iontophoresis has been used in conjunction with LASER Doppler flowmetry, a non-invasive method for assessing microvascular perfusion at a single point (152). More-recently, iontophoresis has been combined with LASER Doppler imaging, which reduces measurement variability (153, 154). This is because unlike LASER Doppler flowmetry, LASER Doppler imaging measures perfusion across many points (155), and an average measure of perfusion can be computed for any chosen area.

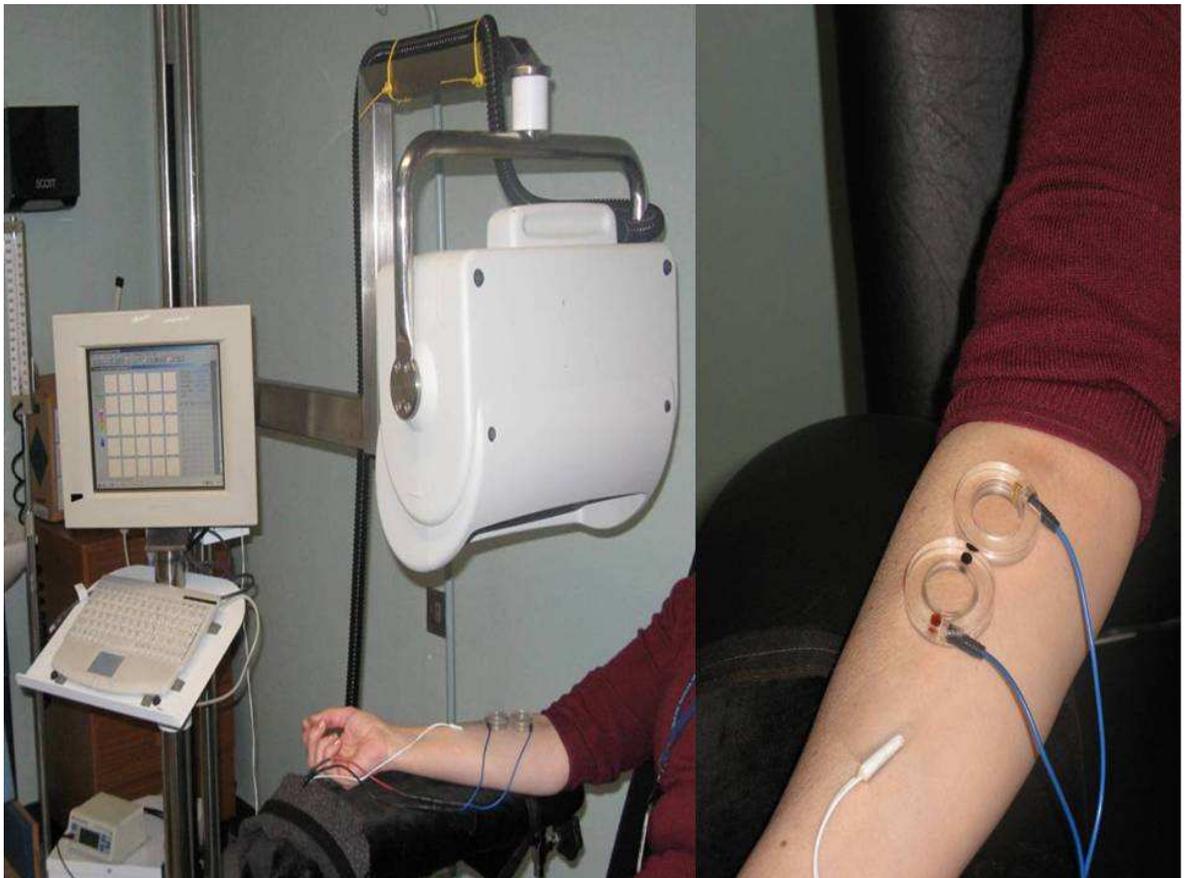


Figure 17: LASER Doppler Imaging with Iontophoresis (LDI+Ion)

Iontophoresis of acetylcholine (ACh) at the anode tests endothelial function since its vasodilator action involves binding to muscarinic receptors on endothelial cells, with subsequent generation of NO. It is therefore said to be 'endothelium dependent'. Vasodilatation is ultimately mediated by action of NO on vascular smooth muscle (via the cGMP pathway) and so iontophoresis of an NO donor, sodium nitroprusside (SNP), delivered at the cathode, is used as an 'endothelium-independent' control to test the integrity of vascular smooth muscle (See figure 18).

Drug delivery is achieved using a battery-powered constant-current iontophoresis controller (MIC-1e; Moor Instruments Ltd., Axminster, U.K.). The chambers used for iontophoresis (ION 6; Moor Instruments Ltd.) are constructed of Perspex (internal diameter 22mm; area 3.8cm²) with an internal platinum wire electrode. Two chambers are attached to the skin of the volar aspect of the forearm by means of double-sided adhesive discs (see Figure 18), avoiding hair, broken skin, and superficial veins. The chambers are connected to the anode and cathode connections on the iontophoresis controller and the voltage across the chambers is monitored. A thermometer is also attached to the arm in order to measure skin temperature.

2.5ml of 1% ACh (Sigma) is introduced to the anodal chamber and 2.5ml of 1% SNP (Sigma) is introduced to the cathodal chamber. The vehicle for these drugs is 0.5% sodium chloride (NaCl). Both of these agents are delivered simultaneously during each period of current administration. Fluid is prevented from escaping by placing circular 32mm coverslips over the chambers.

The iontophoresis protocol involves incremental current delivery with four scans at 5µA, four at 10µA, four at 15µA and two at 20µA, giving a total charge of 8mC.

The LASER doppler imager (Moor Instruments, UK) is equipped with a red LASER (wavelength 633nm, power 1mW, beam diameter 1mm). The LASER is scanned in a raster fashion over both chambers and through the coverslips. The backscattered light is collected by photodetectors and converted into a signal proportional to perfusion in arbitrary perfusion (flux) units (PU) that is displayed as a colour-coded image on a monitor (see figure 19). Perfusion measurements

are obtained using the imager manufacturer's image analysis software by outlining a region of interest (ROI) around the internal circumference of the chamber (see Figure 20). Statistical analysis of the ROI is subsequently performed to yield the median flux value (see Figure 21) across approximately 700 measurement points. Twenty repetitive scans are taken during each LDI assessment, the first being a control (before current administration), followed by the incremental current protocol as described above (fourteen scans), and followed by a further five scans with no current administration. An assessment of the overall response to the drugs is obtained by calculating the area under the curve (AUC).

This technique is reproducible with between-day and within-day coefficients of variation of $6.4 \pm 3.3\%$ and $8.9 \pm 5.3\%$ respectively (151). Variability being reduced by averaging perfusion over a large skin area (156). Good reproducibility has also been demonstrated between arms (157).

LASER Doppler imaging with Iontophoresis has been taught to me by Professor William Ferrell, who is a senior author on a number of publications validating this method (151, 156, 157). Whilst the published work from his group report good temporal and inter-arm reproducibility, I acknowledge that their data do not necessarily reflect my own practice.

All participants fasted for at least 5 hours prior to assessment (water only permitted). Prior to the procedure, patients were allowed to acclimatize for 15 minutes in a temperature-controlled room, to exclude temperature variation bias on skin perfusion responses to vasoactive agents. Participants all lie in a semi-recumbent position with the flexor aspect of the forearm exposed on an arm rest (see Figure 17).

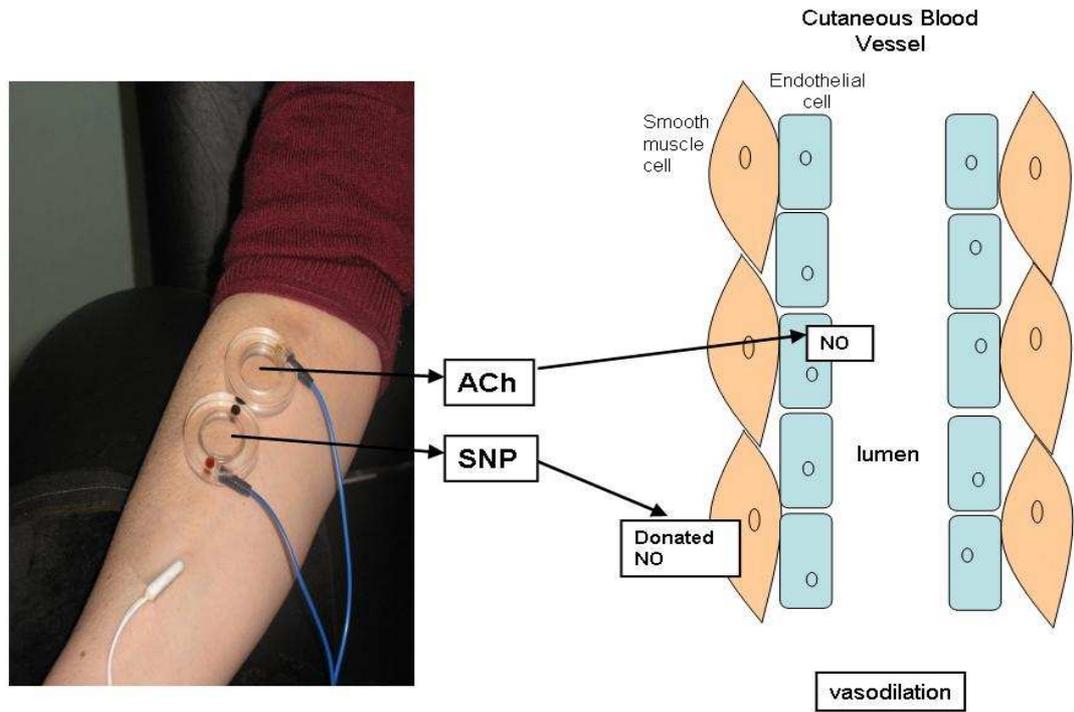


Figure 18: Iontophoresis of acetylcholine and sodium nitroprusside

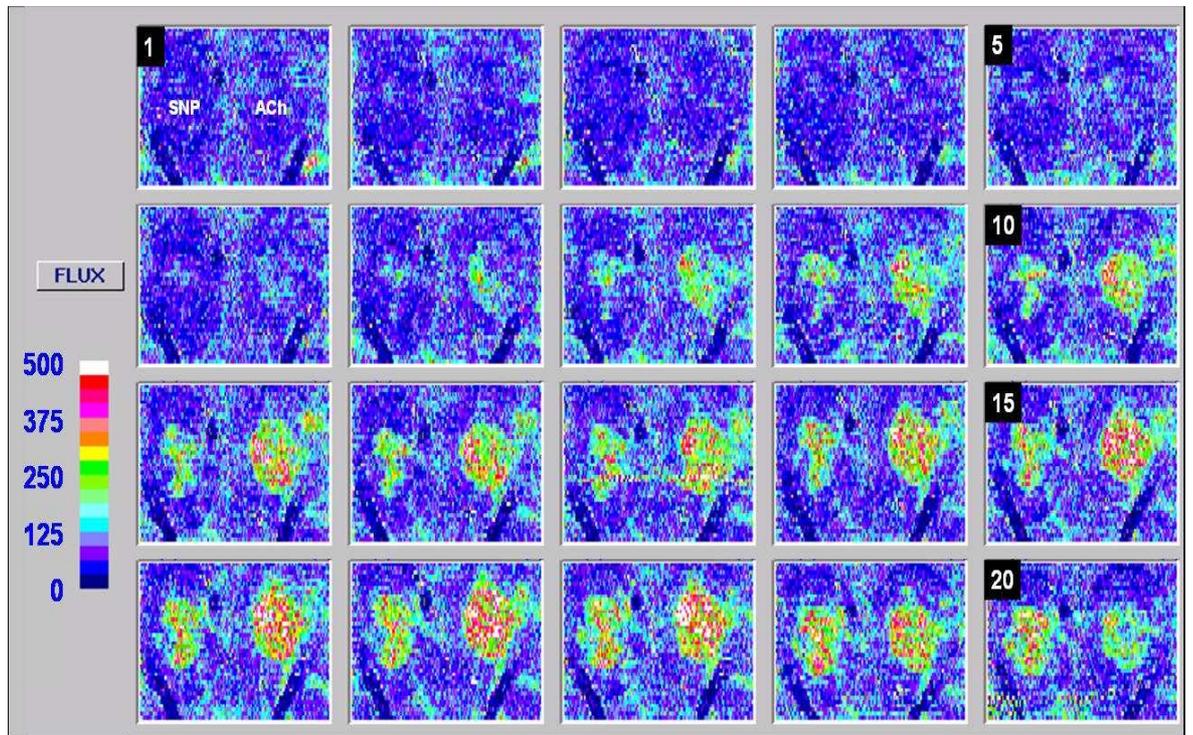


Figure 19: Colour-coded image proportional to perfusion

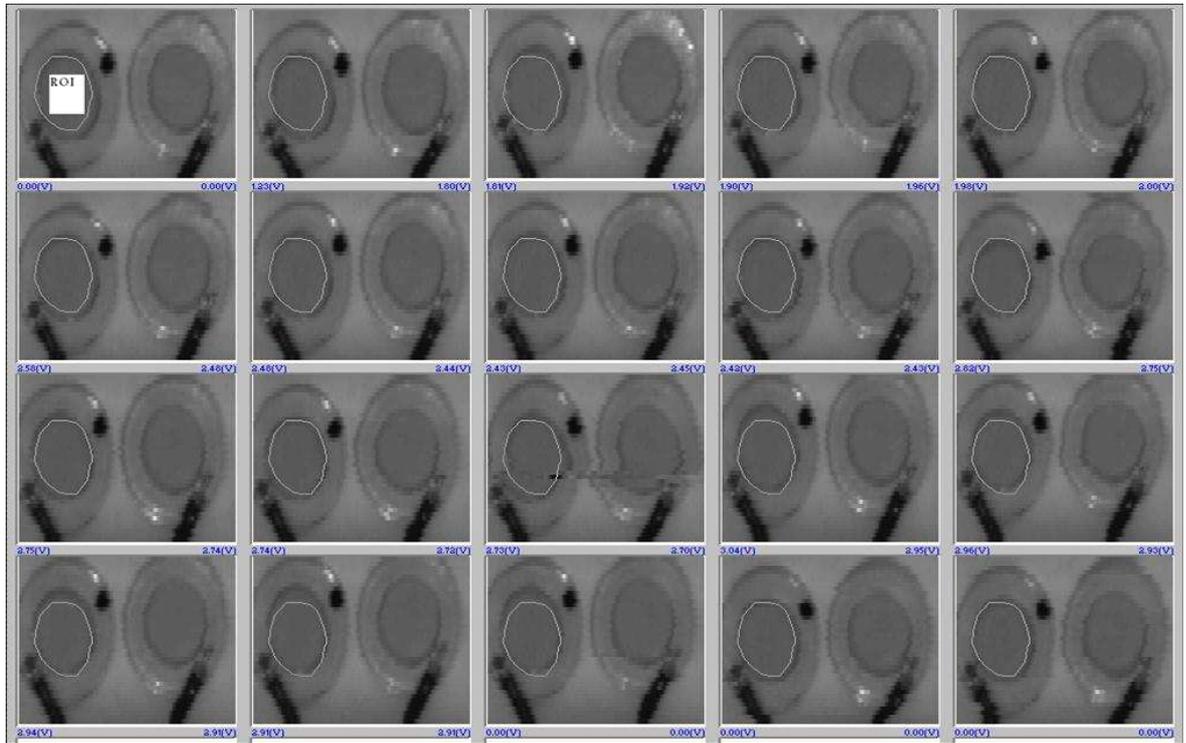


Figure 20: Define ROI

No: Time info.	FLUX (Perfusion Unit)					DC (Perfusion Unit)					Iontophoresis		
	Mean	Std	Min	Max	Median	Mean	Std	Min	Max	Median	I(uA)	V1(V)	V2(V)
1: 00:00:00	57.0	71.31	3	908	40	55.5	5.21	128	164	156	0	0.00	0.00
2: 00:00:50	47.9	20.88	6	155	45	53.7	6.68	129	165	154	5	4.11	4.31
3: 00:01:40	74.1	34.18	6	235	69	55.0	6.89	130	170	156	5	4.32	4.27
4: 00:02:30	80.6	38.53	9	231	74	54.6	7.66	127	185	156	5	4.28	4.14
5: 00:03:20	88.6	45.35	6	322	80	54.6	10.44	121	186	155	5	4.15	4.06
6: 00:04:10	110.3	58.21	13	389	101	55.7	9.74	121	185	156	10	6.13	5.33
7: 00:05:00	143.2	67.22	18	436	136	54.8	8.91	124	185	156	10	5.32	5.10
8: 00:05:50	181.4	81.22	12	431	178	54.8	8.31	126	183	156	10	5.09	4.92
9: 00:06:40	196.4	86.82	10	485	194	55.2	8.30	126	181	156	10	4.93	4.78
10: 00:07:30	209.9	91.69	23	522	209	55.0	8.64	124	182	156	15	6.14	5.60
11: 00:08:20	226.1	96.48	21	650	225	55.1	8.51	124	184	156	15	5.62	5.43
12: 00:09:10	253.6	105.64	24	691	259	54.9	8.52	125	181	156	15	5.41	5.27
13: 00:10:00	276.9	103.23	43	618	281	54.7	7.17	122	179	156	15	5.29	5.22
14: 00:10:50	270.2	119.73	18	647	270	53.9	9.44	125	185	156	20	6.26	5.90
15: 00:11:40	271.6	116.18	44	755	276	54.8	8.69	123	184	156	20	5.89	5.79
16: 00:12:30	286.7	115.16	37	692	281	54.6	8.50	125	183	156	20	5.80	5.71
17: 00:13:20	294.4	110.84	42	702	296	54.3	8.11	124	181	156	20	5.72	5.63
18: 00:14:10	286.9	114.92	47	644	284	54.4	7.96	125	180	156	0	0.00	0.00
19: 00:15:00	268.3	118.08	28	720	268	53.8	8.03	126	175	155	0	0.00	0.00
20: 00:15:50	249.9	117.23	21	736	255	53.2	8.77	120	180	155	0	0.00	0.00

Pixels in ROI = 986 ROI (Polygon) Coords.: 1(154,24), 2(166,26), 3(177,27), 4(192,25), 5(200,23), 6(205,18),
 7(202,14), 8(198,10), 9(189,8), 10(176,7), 11(163,7), 12(148,10), 13(143,13), 14(139,17), 15(143,21),
 16(149,23).

ROI Area: 2.88 (cmxcm)

Figure 21: Median values of perfusion

Power calculation

A change in vascular function of 25-30% is considered to be significant for the study in view of the observation that this order of change is clinically significant as indicated by studies of diabetic and non-diabetic women (158). A 25% change in vascular function at $\alpha = 0.05$, with 20 subjects per group would give a power of 0.86 (acetyl choline).

Data analysis

Measurement of vascular responses, in the whole group, was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis.

Statistical analysis was performed using Minitab ® 16.2.2.

SPECT scanning of SERT using [¹²³I]-beta-CIT

It is possible to study the serotonin transporter (SERT) in vivo in the human brain by using radioligands that bind to SERT in combination with single photon emission computed tomography (SPECT). The iodine-labelled radioligand [¹²³I]-beta-carbomethoxy-3-beta-(4 iodophenyl)tropane ([¹²³I]-beta-CIT) has a high affinity for both SERT and the dopamine transporter (DAT).

Radioligand Preparation

The radioligand ([¹²³I]-beta-CIT) was prepared by Dr. Sally Pimlott at the Western Infirmary, Glasgow.

[¹²³I]-beta-CIT was prepared via electrophilic iododestannylation of the corresponding tributylstannyl precursor (159). Briefly, reagents were added to carrier free Na¹²³I (370-740 MBq) in approximately 10-20 µl of 0.05 M NaOH, in the following order: 30 µg tributylstannyl precursor in 300 µl glacial acetic acid followed by 10% V/V peracetic acid. The reaction proceeded for 20 minutes at room temperature. 500 µl of NaOH was then added. The mixture was purified by reverse-phase HPLC and the solvent removed by rotary evaporation. The [¹²³I]-beta-CIT was formulated as 150 MBq in 5 ml ≤6% ethanol in isotonic citrate acetate buffer and filtered through a 0.22 µm filter. The [¹²³I]-beta-CIT was produced with an isolated radiochemical yield of 70.1 ± 8.6 % (n = 27), and had a radiochemical purity of between 96.8 to 100% (> 99 % for 25 synthesis). Pyrogenicity tests and sterility tests were performed.

SPECT Imaging

This protocol has been previously validated and described (160). [^{123}I] -beta-CIT binds with high affinity in vitro to both DAT and SERT (161). In vivo studies in both humans and nonhuman primates have shown that [^{123}I] -beta-CIT accumulates in two distinct brain regions. In the striatum, where the density of DAT is much higher than that of SERT, [^{123}I] -beta-CIT binding mainly reflects DAT density, whereas in the brainstem and diencephalon binding seems to be specific for SERT.

The kinetics of [^{123}I] -beta-CIT binding differ markedly between the DAT-rich and SERT-rich regions. The slow uptake in the striatum, which reaches a peak after 20-30 hours, is in contrast to the faster kinetics seen in the brainstem and the diencephalon, where peak activity is attained after 2-4 hours after administration. In this study, early imaging of SERT regions at 3 hours was performed for two reasons: first, to reduce the effects of radioactive decay, and second to minimize any possible effect of DAT on the SERT measurement because DAT uptake is proportionally lower at 3 hours.

Brain SPECT imaging was performed with a dedicated Neurofocus 900 SPECT scanner (spatial resolution 7mm full width at half maximum with a line source in air; NeuroPhysics, Shirley, Massachusetts), which acquires sequential single transaxial brain sections. Up to 25 axial sections 6 mm apart were scanned and the energy window (140-178 keV) was placed symmetrically around the ^{123}I gamma energy of 159 keV. A linear attenuation correction was applied, based on an automatically detected ellipse matching the outer head surface.

All scans were performed by Mrs Mary Hansen, and supervised by Dr Jim Patterson, until his retirement, and then by Dr Alice Nicol at the Neurological Institute, Southern General Hospital.

Subjects were scanned starting 2 - 3 hours after IV administration of [^{123}I] -beta-CIT. To minimize thyroid uptake of radioactive iodine, 120mg of potassium iodide was administered orally to each subject at least one hour prior to [^{123}I] -beta-CIT injection. Scanning time was approximately 50 min per scan.

Exclusion criteria included weight greater than 20 stones (127kg), difficulty lying flat, and short neck, for the purposes of lying on the scanning table for the length of time required to complete the scan, and fitting inside the scanner (see Figure 22). Other exclusion criteria specific to SPECT scanning can be seen in Figure 23.



Figure 22: Participant in SPECT scanner
Participants were made comfortable with pillows, and encouraged not to move for the duration of the scan. They could listen to music if they chose.

2. Exclusion Criteria

- | | |
|---|----------|
| • Significant heart disease, diabetes diabetes mellitus or malignancy | YES / NO |
| • Liver impairment or renal impairment | YES / NO |
| • History of bleeding disorder | YES / NO |
| • Use of drugs that affect vascular reactivity | YES / NO |
| • Antidepressant use in last 3 months or other long term use | YES / NO |
| • DSM IV Axis I diagnosis, other than untreated major depression
(incl. OCD and social phobia) | YES / NO |
| • Alcohol and/ or substance misuse | YES / NO |
| • Previous cerebrovascular accident | YES / NO |
| • Documented head trauma or epilepsy or other neurological disorder | YES / NO |
| • Weight >20 stones | YES / NO |
| • Thyroid disorder (relationship with mood disorders + might affect thyroid blocking) | YES / NO |
| • Severe Claustrophobia (may cause problems in SPECT) | YES / NO |
| • Difficulty in lying flat or very short neck (will cause difficulty with scans) | YES / NO |
| • Current or recent participant in another research project | YES / NO |

IF ANY YES → EXCLUDE OR DISCUSS

Figure 23: Exclusion criteria to participation in SPECT scanning

Image analysis

Region of interest (ROI) analysis, carried out by Dr. Alice Nicol, blinded to the subject's clinical history, was used to extract data from the [¹²³I]-beta-CIT scans. A standard ROI template was constructed with the aid of two image templates: (1) the standard magnetic resonance imaging template known as ICBM152, which is based on 152 normal MRI scans and is available from the Statistical Parametric Mapping web site of the Functional Imaging Laboratory (<http://www.fil.ion.ucl.uk/spm/>); and (2) and an in-house cerebral perfusion template based on 32 normal SPECT scans of cerebral perfusion.

The ROI template consisted of manually drawn regions representing the brain stem/diencephalon ROI (referred to as the brain stem ROI below for brevity), and a reference ROI. The striatum ROI (7.6 cm³ on each side) was drawn on three axial sections 5 mm apart and encompassed the head of caudate and putamen in both hemispheres. The brain stem ROI (23 cm³) was drawn on seven axial sections 5mm apart and comprised the thalamus-hypothalamus, mid-brain, and pons. The reference ROI (35 cm³) was drawn on three axial sections 5 mm apart in the medial and lateral occipital lobe bilaterally. The occipital region was chosen to represent nonspecific and nondisplaceable [¹²³I]-beta-CIT uptake (i.e., activity not associated with binding to transporters) because it has a negligible density of both dopamine and serotonin transporters, as does the cerebellum.

The [¹²³I]-beta-CIT uptake in each ROI was expressed as mean counts per pixel, and the specific uptake in each transporter ROI was calculated as:

$$\text{Beta CIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}$$

Transporter binding ratio for SERT was then defined as the ratio of specific uptake to uptake in the occipital reference region using the formula:

$$\frac{\text{Beta CIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}}{\text{BetaCIT uptake in the occipital ROI}}$$

Under equilibrium conditions the binding ratio is proportional to transporter binding potential, and provided transporter affinity and nonspecific binding are invariant across subjects, the ratio is then a measure of transporter availability (B_{max}).

Data analysis

Statistical analysis was performed by Dr. Rajeev Krishnadas using SPSS v19. A paired-samples t-test was conducted to compare the means of Beta-CIT uptake, BDI scores, Flush frequency, ACh - AUC, SNP - AUC, Flush score, HFRDIS and NTA, before and after treatment with Venlafaxine. Mean difference of scores, the 95% CI of the mean differences and effect sizes (Cohen's d) were also calculated (162).

Percentage change (reduction) in each of the above variables from baseline was calculated using the formula:

$$Reduction\ X = \frac{Pretreatment\ X - Posttreatment\ X}{Pretreatment\ X} \times 100$$

where X is the variable of interest ($[^{123}I]$ -beta-CIT binding ratio; BDI scores; Flushing frequency; Flushing score; ACh-AUC; SNP-AUC).

The relationship between the reduction in the $[^{123}I]$ -beta-CIT binding ratio (predictor variable) and other variables (dependent) of interest - namely BDI reduction; Flush frequency, and score, reduction; ACh reduction; SNP reduction - were examined using multivariate analysis of covariance (MANCOVA) with age as a nuisance covariate in the model. The p values were corrected for multiple testing using Holm-Bonferroni family wise error correction.

Power calculation

It was not possible to perform a power calculation for the SPECT study in this group, as there is no study from which to extract data. It is, however, generally accepted that a sample size of 15 is adequate, and studies with smaller groups have been published.

Qualitative Measurements

Hot flush diaries

Participants documented the number of, and the severity of flushes on a scale of 1 to 3. Participants also recorded the number of times that they were awake through the night, night time awakening (NTA), secondary to flushing (see Figure 24).

This 3-category diary defines severity as mild, moderate or severe, and although it has not been formally validated, it is considered valid by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) and is considered acceptable for use in clinical trials (163, 164).

Hot Flush Diary

Initials: _____

DOB: _____

Study Number: _____

Week beginning: _____

Please complete this information for each day of the week

Mild flush = heat, no sweating

Moderate flush = heat and sweating, not causing disruption to activity

Severe = heat and sweating, disrupting activity

Day and Date	No of flushes	Severity of flushes 1=mild 2=moderate 3=severe	No. of night time awakenings

Figure 24: Hot flush diary

Hot Flush Related Daily Interference Score

At the end of each 7 day diary period, participants completed a hot flush related daily interference scale (HFRDIS). This 10-item questionnaire (see Figure 25) aims to determine the impact of hot flashes on daily activities and quality of life (165). Women rated the degree of hot flush interference (during the past week) for each item on a scale from 0 (does not interfere) to 10 (completely interferes); a higher total score indicates greater interference (score range, 0-100).

Hot Flush Related Daily Interference Scale	
	Do Not Interfere Completely Interfere
Please circle one number to the right of each phrase to describe how much DURING THE PAST WEEK hot flushes have INTERFERED with each aspect of your life. Higher numbers indicate more interference with your life.	0 1 2 3 4 5 6 7 8 9 10
1. Work(outside the home and housework)	0 1 2 3 4 5 6 7 8 9 10
2. Social activities (time spent with family/friends etc)	0 1 2 3 4 5 6 7 8 9 10
3. Leisure activities (time spent relaxing, hobbies etc.)	0 1 2 3 4 5 6 7 8 9 10
4. Sleep	0 1 2 3 4 5 6 7 8 9 10
5. Mood	0 1 2 3 4 5 6 7 8 9 10
6. Concentration	0 1 2 3 4 5 6 7 8 9 10
7. Relations with others	0 1 2 3 4 5 6 7 8 9 10
8. Sexuality	0 1 2 3 4 5 6 7 8 9 10
9. Enjoyment of life	0 1 2 3 4 5 6 7 8 9 10
10. Overall quality of life	0 1 2 3 4 5 6 7 8 9 10

If you are not experiencing hot flushes or if they do not interfere with these aspects of your life, please mark the "0" to the right of each question.

Figure 25: Hot Flush Related Daily Interference Score

Greene Climacteric Scale

This scale published in 1998, by Greene (166) is intended to be a brief and standard measure of core climacteric symptoms or complaints to be used for comparative and replicative purposes across different types of studies (see Figure 26).

Symptoms were thought to fall into three major independent groups (see Figure 11); vasomotor (V), somatic (S), and psychological (P). The psychological group may be further sub-divided into an anxiety (A) and a depressed (D) mood component. An additional item on loss of sexual interest (S(21)) was added and intended as a 'probe' item to be followed up by more appropriate and sensitive evaluation of problems in that area.

This scale was completed at baseline (i.e. before treatment) and following treatment as appropriate to group.

The Greene Climacteric Scale		SYMPTOMS	Not at all	A little	Quite a bit	Extremely	Score 0-3
Date		1. Heart beating quickly or strongly					
Initials		2. Feeling tense or nervous					
Study Number		3. Difficulty in sleeping					
		4. Excitable					
		5. Attacks of panic					
		6. Difficulty in concentrating					
		7. Feeling tired or lacking in energy					
		8. Loss of interest in most things					
		9. Feeling unhappy or depressed					
		10. Crying spells					
		11. Irritability					
		12. Feeling dizzy or faint					
		13. Pressure or tightness in head or body					
		14. Parts of body feel numb or tingling					
		15. Headaches					
		16. Muscle and joint pains					
		17. Loss of feeling in hands or feet					
		18. Breathing difficulties					
		19. Hot flushes					
		20. Sweating at night					
		21. Loss of interest in sex					

Please indicate the extent to which you are bothered at the moment by any of these symptoms by placing a tick in the appropriate box

P (1-11) = A (1-6) =
 S (12-18) = D (7-11) =
 V (19-20) = S (21) =

Figure 26: Greene Climacteric Scale

Becks Depression Inventory

Becks Depression Inventory (BDI) is a 21-item self-report instrument (see Figure 27 and 28) for measuring the severity of, and not diagnosing, depression in adults and adolescents aged 13 and older. BDI II is a revision of the original BDI based on descriptive statements regarding symptoms that had been reported frequently by psychiatric patients with depression and only infrequently by non-depressed psychiatric patients (167). This assessment was administered at baseline (i.e. before any treatment) and following treatment as appropriate to group. A total score of 0-13 is considered to be minimal, 14-19 is mild, 20-28 is moderate, and 29-63 is severe.

BDI-II		Date:
Name: _____		Marital Status: _____
Occupation: _____		Age: _____
		Sex: _____
		Education: _____
<p>Instructions: This questionnaire consists of 21 groups of statements. Please read each group of statements carefully, and then pick out the one statement in each group that best describes the way you have been feeling during the past two weeks, including today. Circle the number beside the statement you have picked. If several statements in the group seem to apply equally well, circle the highest number for that group. Be sure that you do not choose more than one statement for any group, including Item 16 (Changes in Sleeping Pattern) or Item 18 (Changes in Appetite).</p>		
<p>1. Sadness</p> <p>0 I do not feel sad.</p> <p>1 I feel sad much of the time.</p> <p>2 I am sad all the time.</p> <p>3 I am so sad or unhappy that I can't stand it.</p> <p>2. Pessimism</p> <p>0 I am not discouraged about my future.</p> <p>1 I feel more discouraged about my future than I used to be.</p> <p>2 I do not expect things to work out for me.</p> <p>3 I feel my future is hopeless and will only get worse.</p> <p>3. Past Failure</p> <p>0 I do not feel like a failure.</p> <p>1 I have failed more than I should have.</p> <p>2 As I look back, I see a lot of failures.</p> <p>3 I feel I am a total failure as a person.</p> <p>4. Loss of Pleasure</p> <p>0 I get as much pleasure as I ever did from the things I enjoy.</p> <p>1 I don't enjoy things as much as I used to.</p> <p>2 I get very little pleasure from the things I used to enjoy.</p> <p>3 I can't get any pleasure from the things I used to enjoy.</p> <p>5. Guilty Feelings</p> <p>0 I don't feel particularly guilty.</p> <p>1 I feel guilty over many things I have done or should have done.</p> <p>2 I feel quite guilty most of the time.</p> <p>3 I feel guilty all of the time.</p>	<p>6. Punishment Feelings</p> <p>0 I don't feel I am being punished.</p> <p>1 I feel I may be punished.</p> <p>2 I expect to be punished.</p> <p>3 I feel I am being punished.</p> <p>7. Self-Dislike</p> <p>0 I feel the same about myself as ever.</p> <p>1 I have lost confidence in myself.</p> <p>2 I am disappointed in myself.</p> <p>3 I dislike myself.</p> <p>8. Self-Criticalness</p> <p>0 I don't criticize or blame myself more than usual.</p> <p>1 I am more critical of myself than I used to be.</p> <p>2 I criticize myself for all of my faults.</p> <p>3 I blame myself for everything bad that happens.</p> <p>9. Suicidal Thoughts or Wishes</p> <p>0 I don't have any thoughts of killing myself.</p> <p>1 I have thoughts of killing myself, but I would not carry them out.</p> <p>2 I would like to kill myself.</p> <p>3 I would kill myself if I had the chance.</p> <p>10. Crying</p> <p>0 I don't cry anymore than I used to.</p> <p>1 I cry more than I used to.</p> <p>2 I cry over every little thing.</p> <p>3 I feel like crying, but I can't.</p>	

Subtotal Page 1

Continued on Back

Figure 27: Beck's Depression Inventory (BDI) II Page 1

<p>11. Agitation</p> <p>0 I am no more restless or wound up than usual.</p> <p>1 I feel more restless or wound up than usual.</p> <p>2 I am so restless or agitated that it's hard to stay still.</p> <p>3 I am so restless or agitated that I have to keep moving or doing something.</p> <p>12. Loss of Interest</p> <p>0 I have not lost interest in other people or activities.</p> <p>1 I am less interested in other people or things than before.</p> <p>2 I have lost most of my interest in other people or things.</p> <p>3 It's hard to get interested in anything.</p> <p>13. Indecisiveness</p> <p>0 I make decisions about as well as ever.</p> <p>1 I find it more difficult to make decisions than usual.</p> <p>2 I have much greater difficulty in making decisions than I used to.</p> <p>3 I have trouble making any decisions.</p> <p>14. Worthlessness</p> <p>0 I do not feel I am worthless.</p> <p>1 I don't consider myself as worthwhile and useful as I used to.</p> <p>2 I feel more worthless as compared to other people.</p> <p>3 I feel utterly worthless.</p> <p>15. Loss of Energy</p> <p>0 I have as much energy as ever.</p> <p>1 I have less energy than I used to have.</p> <p>2 I don't have enough energy to do very much.</p> <p>3 I don't have enough energy to do anything.</p> <p>16. Changes in Sleeping Pattern</p> <p>0 I have not experienced any change in my sleeping pattern.</p> <hr/> <p>1a I sleep somewhat more than usual.</p> <hr/> <p>1b I sleep somewhat less than usual.</p> <hr/> <p>2a I sleep a lot more than usual.</p> <hr/> <p>2b I sleep a lot less than usual.</p> <hr/> <p>3a I sleep most of the day.</p> <hr/> <p>3b I wake up 1-2 hours early and can't get back to sleep.</p>	<p>17. Irritability</p> <p>0 I am no more irritable than usual.</p> <p>1 I am more irritable than usual.</p> <p>2 I am much more irritable than usual.</p> <p>3 I am irritable all the time.</p> <p>18. Changes in Appetite</p> <p>0 I have not experienced any change in my appetite.</p> <hr/> <p>1a My appetite is somewhat less than usual.</p> <hr/> <p>1b My appetite is somewhat greater than usual.</p> <hr/> <p>2a My appetite is much less than before.</p> <hr/> <p>2b My appetite is much greater than usual.</p> <hr/> <p>3a I have no appetite at all.</p> <hr/> <p>3b I crave food all the time.</p> <p>19. Concentration Difficulty</p> <p>0 I can concentrate as well as ever.</p> <p>1 I can't concentrate as well as usual.</p> <p>2 It's hard to keep my mind on anything for very long.</p> <p>3 I find I can't concentrate on anything.</p> <p>20. Tiredness or Fatigue</p> <p>0 I am no more tired or fatigued than usual.</p> <p>1 I get more tired or fatigued more easily than usual.</p> <p>2 I am too tired or fatigued to do a lot of the things I used to do.</p> <p>3 I am too tired or fatigued to do most of the things I used to do.</p> <p>21. Loss of Interest in Sex</p> <p>0 I have not noticed any recent change in my interest in sex.</p> <p>1 I am less interested in sex than I used to be.</p> <p>2 I am much less interested in sex now.</p> <p>3 I have lost interest in sex completely.</p>
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Figure 28: Becks Depression Inventory (BDI) II Page 2

Hospital Anxiety and Depression Scale

Low serotonin transporter availability is correlated with high anxiety (168). Venlafaxine is used as an approved drug treatment for generalised anxiety disorder. Becks Depression Inventory II is not designed to assess anxiety as a separate entity from depression. Therefore an amendment to ethical approval was sought and granted on 16 August 2010 to include Hospital Anxiety and Depression Scale (HADS) (see Figure 29). Each participant who participated in the study after this date completed this questionnaire at visit 2 and visit 3.

HOSPITAL ANXIETY AND DEPRESSION SCALE	
<p>A I feel tense or wound up</p> <p>3 () most of the time 2 () a lot of the time 1 () from time to time 0 () Not at all</p>	<p>A I get a sort of frightened feeling like butterflies in my stomach</p> <p>0 () not at all 1 () occasionally 2 () quite often 3 () very often</p>
<p>D I still enjoy the things I used to enjoy</p> <p>0 () definitely as much 1 () not quite as much 2 () only a little 3 () hardly at all</p>	<p>D I have lost interest in my appearance</p> <p>3 () definitely 2 () I don't take as much care as I should 1 () I may not take quite as much care 0 () I take just as much care as ever</p>
<p>A I get a sort of frightened feeling as if something awful is about to happen</p> <p>3 () very definitely and quite badly 2 () yes, but not too badly 1 () a little, but it doesn't worry me 0 () Not at all</p>	<p>A I feel restless, as if I had to be on the move</p> <p>3 () very much indeed 2 () quite a lot 1 () not very much 0 () not at all</p>
<p>D I can laugh and see the funny side of things</p> <p>0 () as much as I always could 1 () not quite as much now 2 () definitely not so much now 3 () not at all</p>	<p>D I look forward with enjoyment to things</p> <p>0 () as much as I ever did 1 () rather less than I used to 2 () definitely less than I used to 3 () hardly at all</p>
<p>A Worrying goes through my mind</p> <p>3 () a great deal of the time 2 () a lot of the time 1 () from time to time, but not too often 0 () only occasionally</p>	<p>A I get a sudden feeling of panic</p> <p>3 () very often indeed 2 () quite often 1 () not very often 0 () not at all</p>
<p>D I feel cheerful</p> <p>3 () not at all 2 () not often 1 () sometimes 0 () most of the time</p>	<p>D I can enjoy a good tv or radio Programme or book</p> <p>0 () often 1 () sometimes 2 () not often 3 () very seldom</p>
<p>A I can sit at ease and feel relaxed</p> <p>0 () definitely 1 () usually 2 () not often 3 () not at all</p>	<p>D I feel as though I am slowed down</p> <p>3 () nearly all the time 2 () very often 1 () sometimes 0 () not at all</p>

Figure 29: Hospital Anxiety and Depression Scale

Statistical Analysis

Hot flush diary data was calculated as follows; flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed.

Statistical comparison of all qualitative measurements was made by Wilcoxin Signed Rank test of paired non-parametric data using Minitab ® 16.2.2 and graphs drawn using GraphPad Prism 5.

Chapter 3

Flushing postmenopausal women and their non-flushing contemporaries – a study comparing cutaneous microvascular perfusion.

Acknowledgement

The work presented in this chapter, and chapter 4, is from a study which formed the basis of a BSc project by Dr. Helen Fox MBChB (see Preface). Initiation of the project as well as recruitment and some participant assessments were completed by Dr. Fox; however final participant assessments, full analysis of data, and subsequent publications have been completed as part of the work that I present in this thesis. Dr. Fox is a co-author in the publications (169, 170).

Introduction

A hot flush is a period of intense heat which resembles a heat dissipation response, in that both are characterised by sweating and peripheral vasodilation.

Physiological thermoregulation in humans comprises changes in heat dissipation (as above), and heat generation (shivering) in response to various internal and external stimuli. The central control of thermoregulation is in the medial preoptic area of the hypothalamus (MPOAH) and it has already been mentioned that hypotheses exist surrounding alterations to the thermoneutral zone within this area. However, what is not well studied is the effector mechanism of thermal control.

Information on internal (core) and surface (skin) temperatures is relayed to the MPOAH which then coordinates the appropriate response. This area is often related, conceptually, to a thermostat, which initiates heat generation when it is “too cold” and heat loss when it is “too hot”.

As a hot flush is a transient episode of “too hot”, focus of this chapter is on the mechanisms employed to dispense this heat.

Resting skin blood flow in thermoneutral environments is approximately 250ml/min. During exercise or heat exposure, increases in body temperature trigger cutaneous vasodilation, which increases the blood flow to the skin several fold (83).

The vasoconstrictor system in human skin is active in thermoneutral environments and withdrawal of this is responsible for 10-20% of the cutaneous vasodilation during heating. However, the large increases in skin blood flow are mediated primarily by activation of sympathetic vasodilator nerves in the skin. Studies from the 1950s have shown that the increase in skin blood flow with body heating was blocked by anaesthetic blockade of cutaneous nerves and that this blockade reduced skin blood flow to pre-heating levels (171).

The mechanism of active vasodilation also includes a moderate role for nitric oxide (NO).

Therefore alterations in thresholds for vasodilation in response to core body temperature changes could be as a result of changes in tonic vasoconstriction, active vasodilation thresholds or nitric oxide levels.

Co-transmission of an unknown neurotransmitter is also thought to be required for ACh mediated vasodilation, and vasoactive intestinal peptide (VIP) is also thought to play in role in activation of NO stimulated by histamine release from mast cells. See Figure 30.

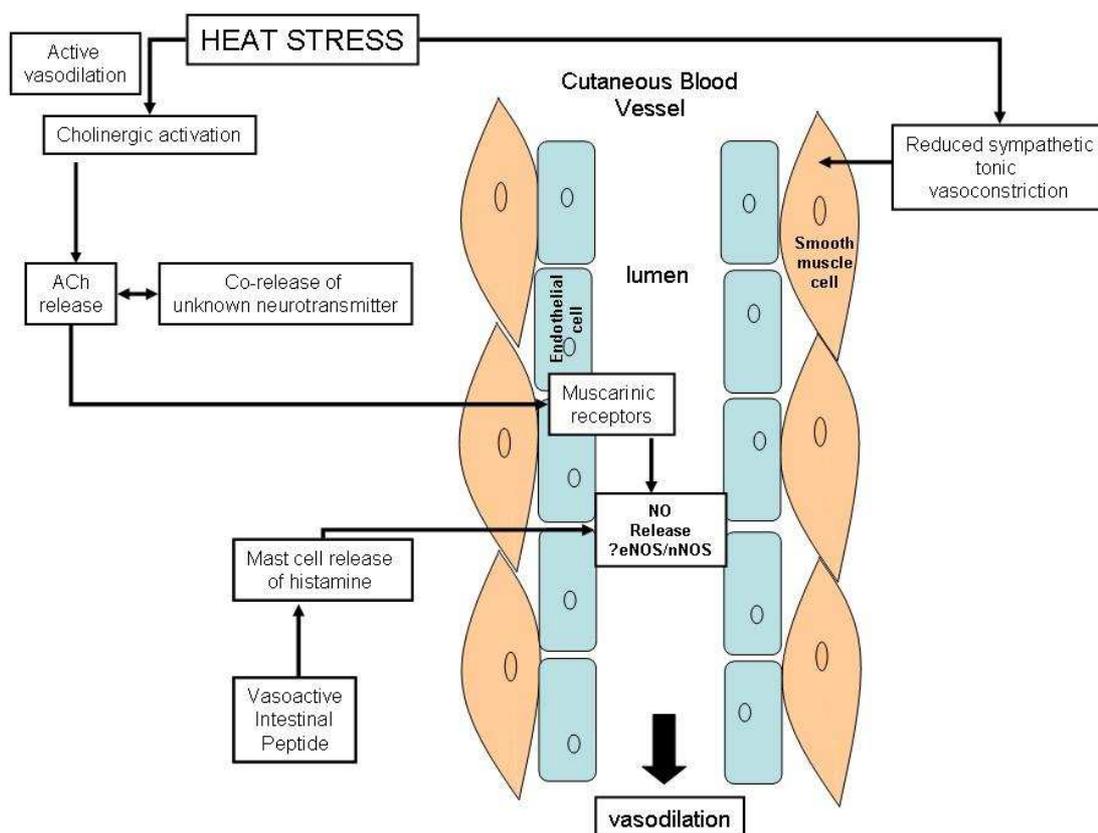


Figure 30: Mechanisms of cutaneous vasodilation

Derived from; In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. Kellogg, D.L. *J Appl Physiol* 100: 1709-1718.(172)

It has been shown that postmenopausal women who flush have a diminished vasoconstrictor response to cold (72) and that they have increased blood flow to the forearm and hand during a flushing episode (173). This altered (potentially heightened) peripheral vascular reactivity is another proposed mechanism responsible for the pathophysiology underlying hot flushes.

The objective of this study was to assess vascular function in postmenopausal women who flush and compare it with postmenopausal women who do not flush.

An additional aim was to study factors which might influence vascular reactivity, and to examine associations between hot flushes and endothelial function and several circulating cardiovascular disease (CVD) risk factors. These included lipids and apolipoproteins, inflammatory markers and intercellular adhesion

molecule-1 (ICAM-1), the latter being linked to vascular dysfunction and higher risk of CVD and especially diabetes in several studies (127).

Hypothesis

Subcutaneous microvascular reactivity is increased in postmenopausal women with severe flushing compared to post-menopausal women who do not flush.

Methods

Participants

A total of 32 postmenopausal women who each experience at least 20 flushes/day and 14 non-flushing women were recruited.

Flushing participants and controls formed part of a double-blind, cross-sectional, longitudinal study, whereby flushing participants were asked to take clonidine and placebo in a crossover design. These results will be discussed in chapter 4. Baseline results comparing flushing participants to controls will be discussed in this chapter.

Recruitment of volunteers followed Scottish media coverage, as well as from gynaecology and menopause clinics. A number of women were also recruited from the West of Scotland Breast Screening Centre in Glasgow.

Participating women were all aged 50-65 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function. Menopausal status was determined by either an FSH greater than 20 Units/Litre or amenorrhoea for 1 year or longer.

Study Design

All study participants were assessed at baseline using LASER Doppler Imaging with iontophoresis of vasoactive compounds (LDI + Ion). Control participants had this measurement repeated at 4 weeks. Blood was also obtained at the time of LDI assessment. Each participant in the flushing group was requested to keep a 'Hot Flush' diary for 3 weeks prior to assessment.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 01/50704/43). All patients gave written informed consent.

Body composition

Measurements of body mass, height, and blood pressure were made as described in chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made at baseline for flushing and control participants. Control participants had this measurement repeated at 4 weeks. This technique is described in detail in chapter 2.

Plasma analysis

A fasting blood sample was taken from each participant using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetraacetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

ICAM, VCAM, TNF- α , IL-6, Insulin and Adiponectin were measured by ELISA technique as described in Chapter 2.

CRP, Cholesterol, Glucose, HDL, LDL, and Trig were measured by standardised methods at The Institute of Cardiovascular and Medical Sciences at Glasgow University, as described in Chapter 2.

Statistical analysis

Measurement of vascular responses was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis.

Log_{10} transformation of the plasma data were also performed to allow for parametric data analysis with comparison by Student's t-test. In addition, plasma data were adjusted for BMI, Age, Years since last menstrual period (LMP) and Parity using general linear model.

Demographic data were analysed using Mann Whitney U test, and blood pressure analysis by Student's t-test.

Results

32 women with severe hot flushing and 14 women with no hot flushing, aged between 50 and 65, who were medically fit and were not taking drugs that might impact on vascular reactivity, were recruited and had LDI assessment of vascular reactivity of the subcutaneous vessels.

Demographic data and body composition

A full set of demographic characteristics were available for 29 flushing women and 13 control women.

	Flush	Control	P
n	29	13	
Age (years)	56 (53-61)	54 (52-57)	0.25
Years since last menstrual period (LMP)	8 (1-12)	5 (1-6)	0.15
Smoking	0	0	1
Body Mass Index (BMI)	25.1 (24.3-28.1)	23.0 (20.9-31.9)	0.28
Parity	2 (0-3)	2 (2-3)	0.74
*Systolic Blood Pressure	126.07 ± 2.67	120.25 ± 4.29	0.25
*Diastolic Blood Pressure	70.48 ± 2.11	72.66 ± 2.34	0.61

Table 3: Demographic characteristics of study subjects

Data are median (interquartile range). Statistical analysis was performed using the Mann-Whitney U test. All women are non-smokers and not known to be hypertensive. *Data are median ± SD

Responses to acetylcholine (ACh) and sodium nitroprusside (SNP)

Vascular reactivity for 32 flushers and 14 controls (non-flushers) was measured using LDI + Ion. Data was analysed as described above.

The response of the subcutaneous vessels was greater in women who flushed than in those who did not (see Figure 31). The enhanced vascular response occurred following administration of both the endothelium-dependent (ACh) and independent vasodilators (SNP), (ACh, $p < 0.001$, SNP, $p = 0.001$).

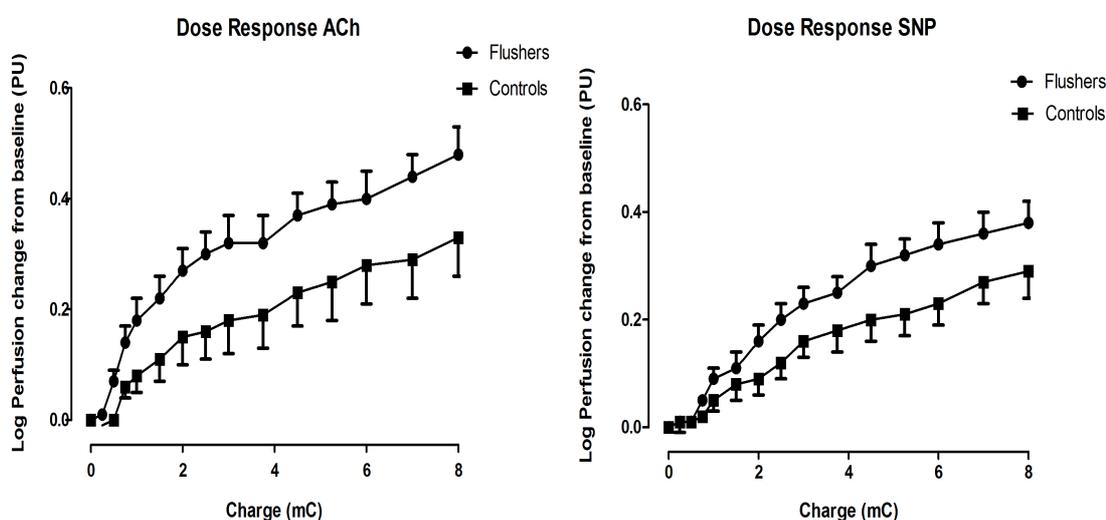


Figure 31: Dose Response Curves

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in control women compared with flushing women. Data are mean \pm SEM.

When comparing control participants at two time points 4 weeks apart, there is no difference in cutaneous microvascular perfusion for either endothelium dependent or endothelium independent vasodilator agent. $P=0.622$ and $P=0.597$ respectively (see Figure 32).

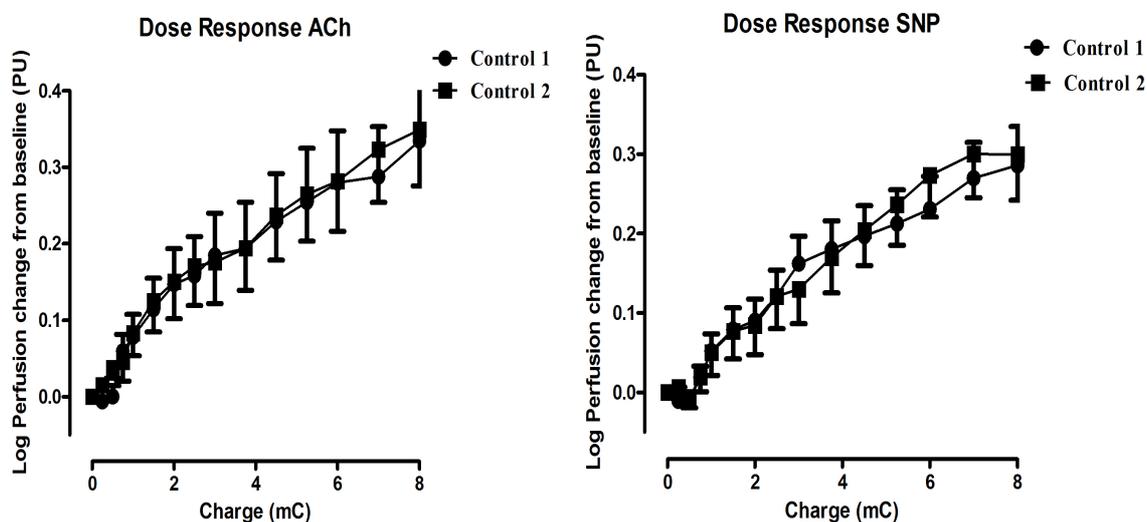


Figure 32: Dose Response Curves for control group on two occasions
Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in control women at baseline compared with 4 weeks later. Data are mean \pm SEM.

Plasma Analysis

Women with flushing had significantly lower HDL-cholesterol levels ($P=0.02$), lower apolipoprotein A1 ($P=0.002$), and higher ICAM-1 levels ($P=0.03$).

These results were not affected by adjustment for body mass index (BMI), age, years since last menstrual period (LMP) and parity: HDL-cholesterol ($P=0.007$), Apolipoprotein A1 ($P<0.001$), and I-CAM ($P=0.05$). See Table 4.

	Flushing	Control	P	Adjusted P	R-Sq (%)
Cholesterol (mmol/l)	5.77 (5.01-6.35)	5.93 (5.05-6.57)	0.91	0.97	6.86
Triglyceride (mmol/l)	1.17 (0.82-1.73)	1.09 (0.82-1.53)	0.83	0.97	11.33
Low Density Lipoprotein (LDL)-cholesterol (mmol/l)	3.51 (2.92-4.42)	3.17 (2.63-4.30)	0.29	0.29	14.14
High Density Lipoprotein (HDL)-cholesterol (mmol/l)	1.46 (1.21-1.76)	1.84 (1.51-2.10)	0.01	0.01	40.17
Total Cholesterol to HDL-cholesterol ratio	3.77 (3.08-4.74)	2.98 (2.59-3.73)	0.02	0.04	26.33
Apolipoprotein A1 (mg/dl)	155.30 (146.2-170.7)	194.80 (172.4-200.3)	<0.001	<0.001	94.81
Apolipoprotein B (mg/dl)	78.30 (71.0-104.7)	80.40 (63.0-100.5)	0.23	0.29	11.01
Insulin (mu/l)	5.69 (3.48-9.16)	6.17 (5.27-8.68)	0.59	0.67	22.99
Glucose (mmol/l)	5.30 (5.10-5.60)	5.60 (5.25-5.85)	0.68	0.69	29.14
Adiponectin (mg/ml)	11.6 (8.6-15.1)	11.7 (9.7-14.7)	0.38	0.48	11.51
C-Reactive Protein (CRP) (mg/l)	1.70 (0.64-5.85)	0.90 (0.35-1.42)	0.07	0.18	19.7
Inter-Cellular Adhesion Molecule 1 (ICAM-1) (ng/ml)	243 (202-263)	187 (164-228)	0.02	0.05	18.87

Table 4: Plasma analysis

Data are median (interquartile range). Statistical analysis was performed using Student's t-test on log transformed data. Adjusted P calculated using general linear model to adjust for BMI, Age, Years since last menstrual period (LMP) and Parity.

Correlations

In the control group HDL-cholesterol also correlated with vascular reactivity as measured by AUC for the ACh response ($P=0.01$, R-Sq 48.3%), but not in the flush group. Again, this relationship remained significant when adjusted for body mass index (BMI), age, and waist hip ratio (WHR), $P=0.035$, R-Sq 60.7%. Also, Apo B was inversely related to Ach mediated vascular reactivity in the control group ($P=0.02$, R-Sq 46%, and adjusted $P=0.004$, R-Sq 80.8%) (see Figure 33).

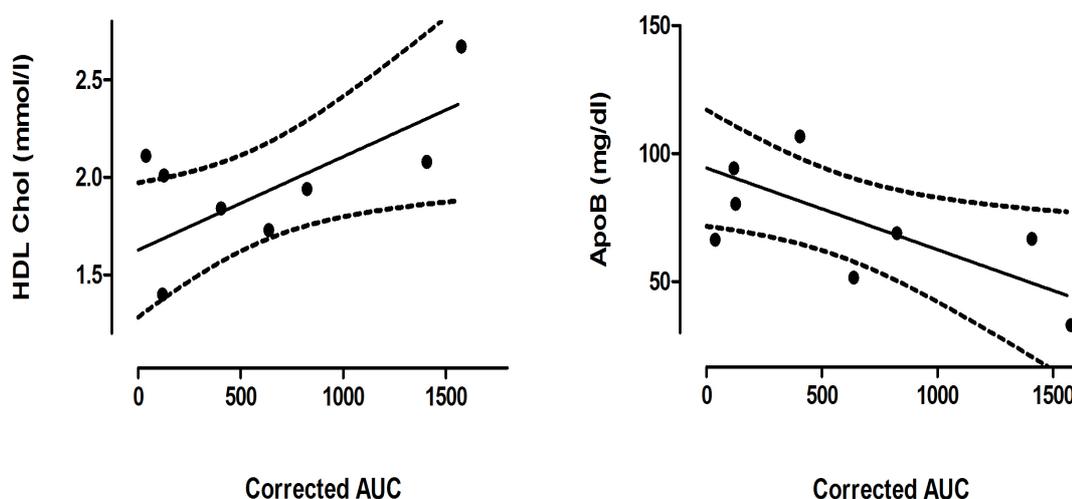


Figure 33: ACh corrected AUC correlation with HDL Cholesterol and Apolipoprotein B in control participants
Correlation plots and regression lines with 95% confidence intervals for ACh corrected AUC and HDL cholesterol and Apolipoprotein B in control participants.

	Flush ACh		Control ACh		Flush SNP		Control SNP	
	P	R sq	P	R sq	P	R sq	P	R sq
Cholesterol	0.69	0.6	0.10	4.7	0.8	0.2	0.76	1
Triglyceride	0.67	0.7	0.26	12.6	0.22	5.5	0.82	0.6
LDL	0.51	1.7	0.06	30.9	0.76	0.4	0.66	2
HDL	0.69	0.6	0.01	48.3	0.4	2.7	0.50	4.7
ApoA1	0.87	0.1	0.09	25.7	0.43	2.3	0.35	8.9
ApoB	0.52	15	0.02	46	0.94	0	0.43	6.4
Insulin	0.35	3.2	0.21	15.4	0.79	0.3	0.67	1.9
Glucose	0.58	1.1	0.86	0.3	0.98	0	0.80	0.7
Adiponectin	0.78	0.3	0.53	4.1	0.48	1.9	0.91	0.1
CRP	0.46	2.0	0.50	4.6	0.17	6.9	0.79	0.7
ICAM-1	0.15	7.6	0.75	1	0.08	11	0.47	5.3

Table 5: Correlations of vascular reactivity as determined by corrected area under the curve (AUC) and plasma results.

Summary of results

Responses to acetylcholine (ACh) and sodium nitroprusside (SNP)

The response of the subcutaneous vessels was greater in women who flushed than in those who did not for both endothelium dependent and independent measures.

There was no difference in endothelium dependent or independent responses in control participants at two time points 4 weeks apart.

Plasma analysis

Women with flushing had significantly lower HDL-cholesterol, lower apolipoprotein A1 and higher I-CAM levels when compared to controls.

In the control group HDL-cholesterol correlated with endothelium dependent vascular reactivity and Apolipoprotein B inversely correlated with the same.

Discussion

In this group of postmenopausal women, those with severe flushing have a greater vasodilator response than those women with no flushing. This is in keeping with previous studies showing a diminished vasoconstrictor response, and increased blood flow during a hot flush (72, 173). The alteration to their vascular function seems to be an increased vasodilatory response in comparison with asymptomatic women

This increase has been demonstrated to be present in both the endothelium dependent (ACh) and independent (SNP) responses. It is therefore possible to suggest that there is an increased response of the vascular smooth muscle to the NO donated by SNP compared to asymptomatic postmenopausal women. Sodium nitroprusside dissociates in the circulation to release NO, which activates guanylate cyclase in vascular smooth muscle and increases intracellular production of cGMP. cGMP stimulates calcium movement from the cytoplasm to the endoplasmic reticulum and reduces calcium available to bind with calmodulin. Vascular smooth muscle then relaxes and vessels dilate.

Endothelium dependent responses are also increased this group of flushing women compared with non-flushing women as a result of administration of acetylcholine which acts to stimulate NO production by endothelial cells, by binding to muscarinic receptors. However, the endothelial produced NO must still act upon vascular smooth muscle to have a response. Therefore, in order to examine the endothelial response in isolation, the vascular smooth muscle response can be removed from the equation by analysing the difference in the ACh and SNP response, no variation was found. It is possible, then, that the enhanced response in the flushing group, is due purely to an increased vasomotor smooth muscle response.

It is also possible that there are other endothelial derived factors activated that may cause vasodilation. Prostacyclins may be involved, since administration of aspirin prior to LDI, leads to reduced endothelial dependent responses following both oral (174) and intravenous (175) aspirin, although this has not been demonstrated by all studies (151, 154).

Electrically induced vasodilation has also been implicated, however, the vehicle 0.5%NaCl and the chamber size used have been shown to minimise an electrically induced hyperaemic response. Furthermore, application of a local anaesthetic agent in an attempt to eliminate the 'axon reflex' thought to be a possible cause of electrically induced hyperaemia did diminish this response, but also caused vasoconstriction (156), therefore any vasodilatory effects would be superimposed upon already vasoconstricted vessels.

This apparently 'better' vascular response in women who flushed excessively may be anticipated to suggest a protective phenotype against vascular risk since impaired skin microvascular function has been linked to several conditions associated with greater CVD risk to include diabetes (176), maternal obesity (177), and hypercholesterolaemia (178) as well CAD itself (179). However, by contrast, results of recent studies suggest the presence of menopausal flushing may be a marker of cardiovascular risk, as marked by reduced flow-mediated dilation (brachial artery) and greater aortic calcification in flushers in the Study of Women's Health Across the Nation Heart Study (180). To examine these issues in more details, CVD risk factors in these women were examined.

Women who flushed in the present study had lower levels of HDL-cholesterol and ApoA1, and higher levels of ICAM-1 than asymptomatic women. Recent work from the Emerging Risk Factor Collaboration, which represents the most complete lipid-CVD risk analyses performed anywhere, has shown that greater non-HDL-cholesterol (or total cholesterol) but lower HDL-cholesterol are strong and independent risk factors for vascular disease in both men and women (181) thereby in keeping with greater vascular risk over the long term.

The blood and vascular data therefore appear contradictory, but can they be explained?

It could be said that these women have an imbalance between endothelium-derived vasodilators that have anti-thrombotic and antimitogenic properties and vasoconstrictors with proatherogenic activity (182). However, greater vasodilatory response is usually considered a sign of vascular health.

Endothelium-derived NO is now recognised to be an anti-inflammatory and anti-arteriosclerotic molecule; mice lacking the endothelial-type NO synthase gene exhibit hypertension and enhanced vascular remodelling in response to injury (183). One possibility is that these postmenopausal flushing women are at increased risk of cardiovascular disease compared with their non-flushing counterparts and have reduced synthesis of NO as a result of endothelial dysfunction, and as a result of this have increased sensitivity to NO and therefore when exposed to NO, have a greater vasodilatory response.

The study by Bechlioulis and colleagues (184) found that women in the early stages of menopause had endothelial dysfunction, but that this was not associated with a change in plaque size, as the carotid intima-media thickness was comparable to that in the premenopausal controls. Perhaps the results of the present study indicate an increased response to NO due to increased sensitivity as a result of longer term underlying endothelial dysfunction leading to decreased NO synthesis.

Although there appeared to be no endothelial dependent variation between groups when the difference in independent and dependent responses were examined, it is possible that the increased response which was smooth muscle driven, is superimposed on a background of early endothelial dysfunction that will become more apparent with time.

As with Findings from the Study of Women's Health Across the Nation Heart Study (180), Gambacciani (185) found arterial blood flow was altered only in women with hot flushes. This too, is in support of Bechlioulis (184) as they also demonstrated that severity of flushes was the most important independent predictor of endothelial dysfunction.

However, flow mediated dilation (FMD) is expressed as percentage change meaning that a larger percentage change will occur with a small diameter increase in a narrow vessel, than the same increase in a vessel with a higher baseline lumen diameter. It is possible then that flushers overall have more dilated brachial arteries to begin with, which would be consistent with the results of the present investigation.

Also, it is possible that smaller diameter cutaneous vessels and the vasodilation that has been found to be associated with hypooestrogenism (186), cannot be extrapolated to larger vessels such as those studied using the flow mediated dilation (FMD) technique.

While measures of peripheral vascular function are used as surrogate markers for coronary vascular function, there is a paucity of data showing any clear correlation. This may be as a result of the difference in size of vessels studied. However there is evidence demonstrating that peripheral vascular function acts a prognostic tool. Impaired brachial artery FMD predicts cardiac events in hypertensive postmenopausal women(187). We have demonstrated in our group, a positive correlation between HDL and ACh-mediated vasodilation in the control group, as well as a negative correlation for ApoB. This may give further credence to the use of peripheral vascular function as a surrogate for coronary vascular function, in women without flushing. Further, larger, studies would, of course, be required.

Women with climacteric symptoms have a lower level of plasma antioxidant activity (188), and oxidative stress is associated with cardiovascular risk. Leal also demonstrated that HRT decreases oxidative stress in addition to decreasing number of flushes. A recent review (189) suggested that a possible reason for the opposite effects of the beneficial effect of HRT in younger women within the first year of use (190) and detrimental effects in women distant to the menopause may be as a result of genuinely altered vascular reactivity making them vulnerable to the effects of HRT.

Although total cholesterol, elevated LDL-C and low HDL-C are well established risk factors for cardiovascular disease (CVD), an association with endothelial dysfunction has not been consistently demonstrated. It is also possible that there is an uncoupling of central and peripheral control, with impulses from the hypothalamus overshadowing peripheral responses. Whatever, the mechanisms for this discrepancy between microvascular function and CVD risk factors, in particular lipids, the link between CVD risk factors and outcomes is of course far better established and thus the results more informative about future risk.

Chapter 4

Alpha-adrenergic system

Acknowledgement

The work presented in this chapter is from a study which formed the basis of a BSc project by Dr. Helen Fox MBChB (see Preface). Initiation of the project as well as recruitment and some participant assessments were completed by Dr. Fox; however final participant assessments, full analysis of data, and subsequent publications have been completed as part of the work that I present in this thesis. Dr. Fox is a co-author in the publications (169, 170).

Introduction

As already demonstrated in Chapter 3, postmenopausal women with severe flushing have shown an increased vasodilator response when compared to those women with no flushing.

Physiological thermoregulation in humans, described in great detail by Charkoudian (83), comprises changes in heat dissipation (cutaneous vasodilatation and sweating) and heat generation (shivering) in response to various internal and external stimuli.

During exercise or heat exposure, increases in body temperature trigger cutaneous vasodilatation and sweating, concurrently the evaporation of sweat decreases skin temperature thereby cooling the blood in dilated skin vessels before it returns to the core.

In the human, cutaneous circulation is controlled by sympathetic adrenergic vasoconstrictor nerves and sympathetic vasodilator nerves. Sympathetic vasoconstrictor nerves release noradrenaline, which interacts with postsynaptic α_1 - and α_2 -receptors on cutaneous arterioles. In addition, these nerves release one or more co-transmitters that also cause vasoconstriction. Within a thermoneutral environment, there is tonic activation of the vasoconstrictor system. Withdrawal of the activity of this system during hyperthermia is responsible for 10% to 20% of the cutaneous vasodilatation, whilst large increases in skin blood flow are mediated primarily (80-90%) by activation of sympathetic vasodilator nerves in the skin (83).

Local cooling stimulates the mobilisation of α_2 -receptors from the Golgi apparatus to the vascular smooth muscle plasma membrane (191) and human studies have demonstrated inhibition of cold-induced vasoconstriction during iontophoresis of α_2 -antagonist to the dorsal aspect of the finger (192).

Whilst peripheral vasculature may be amongst the mediators of thermoregulation, the control centre is the preoptic area of the hypothalamus and noradrenaline is thought to be the primary neurotransmitter responsible for lowering the thermoregulatory set point here, triggering hot flushes (55, 64).

Animal studies have shown that intrahypothalamic injection of noradrenaline acts to narrow the thermoregulatory zone (86). It has also been shown that plasma levels of the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) are significantly increased both before and during hot flush episodes in postmenopausal women (59). Hot flushes can be provoked in symptomatic postmenopausal women with the α_2 -adrenergic antagonist yohimbine, and ameliorated with clonidine, an α_2 -adrenergic agonist (93). It has also been demonstrated that clonidine acts to widen the thermoregulatory zone in humans (91, 92).

Clonidine is an α_2 -adrenergic agonist which is licensed for the treatment of hypertension, migraines and postmenopausal vasomotor symptoms. Clonidine is thought to exert its hypotensive effect through stimulation of α -adrenergic receptors in the vasomotor centre of the medulla (193). It has also been shown to have a beneficial effect on menopausal flushing (194-196), in particular by decreasing the intensity of the flushes. The mechanism is not fully understood, however it may exert its effect on hot flushing through a reduction in peripheral vascular reactivity (102) and also through central mechanisms.

The aim is to study the role of the alpha-adrenergic system at a peripheral level utilising clonidine and its effect on flushing.

Hypothesis

Subcutaneous microvascular reactivity is altered in flushing postmenopausal women on successful treatment of their flushes with clonidine when compared to placebo.

Methods

Participants

A total of 32 postmenopausal women who each experienced at least 20 flushes/day were recruited to participate in this study. Recruitment of volunteers followed Scottish media coverage, as well as from gynaecology and menopause clinics. A number of women were also recruited from the West of Scotland Breast Screening Centre in Glasgow.

Participating women were all aged 50-65 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function. Menopausal status was determined by either an FSH greater than 20 Units/Litre or amenorrhoea for 1 year or longer.

Study Design

This was a double-blind, cross-sectional, longitudinal study of crossover design. Study participants were all seen at baseline when skin blood flow was assessed using LASER Doppler Imaging with Iontophoresis (LDI + Ion) of vasoactive compounds. Participants were randomised in a double-blind manner to receive either Clonidine 0.1mg/day or placebo and after 4 weeks, LDI + Ion was assessed as before. The participants were then crossed over to receive the alternate treatment for 4 weeks and LDI + Ion assessment performed for the final time (see Figure 34).

A washout period between treatments was not required as Clonidine has a short half life and any effects upon peripheral vascular reactivity are likely to be rapid. Qualitative measures were obtained at the same time points.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 01/50704/43). All patients gave written informed consent.

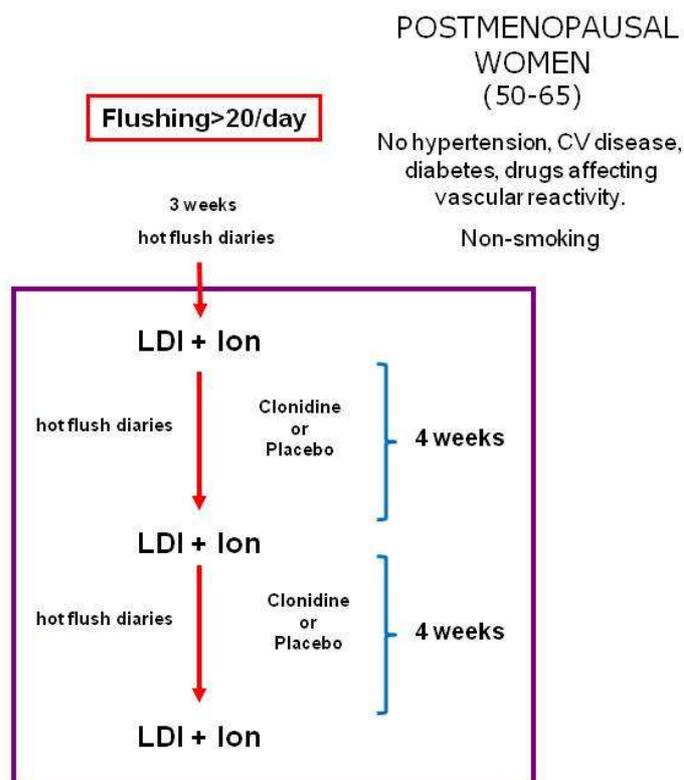


Figure 34: Study design

Body composition

Measurements of body mass, height, and blood pressure were made as described in Chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made at baseline and after 4 weeks of treatment with each of clonidine and placebo in a cross-over design. This technique is described in detail in chapter 2.

Hot flush diary

Each participant was asked to keep a 'Hot Flush' diary for 3 weeks prior to initial assessment and throughout the study, as described in detail in Chapter 2.

Qualitative Measurements

Greene Climacteric Scale

Each participant completed this questionnaire at each visit as described in Chapter 2.

Statistical analysis

Measurement of vascular responses was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis

Demographic data were analysed using Mann Whitney U test, and blood pressure analysis by Student's t-test.

Diary data, HFRDIS and qualitative data were compared using Wilcoxin Signed Rank test of paired non-parametric data. Diary data included analysis of mean number of flushes per day, hot flush score and night time awakening.

For comparison of clonidine and placebo treatment, neither log nor square root transformation resulted in a Gaussian distribution. Therefore graphs are presented as raw - baseline perfusion values and analysis of the data was by Mann Whitney analysis of the corrected area under the curve (AUC); that is the AUC calculated from raw values with the baseline data subtracted.

Results

32 women with severe hot flushing, aged between 50 and 65, who were medically fit and were not taking drugs that might impact on vascular reactivity, were recruited and had LDI assessment of vascular reactivity of the subcutaneous vessels. Following initial assessment, 7 women declined continued participation in the study; therefore there is data for 25 women comparing clonidine to placebo.

Demographic characteristics

Full demographic criteria can be seen below in Table 6.

	Flush
n	25
Age (years)	59 (55-63)[51-65]
Years since last menstrual period (LMP)	8 (2.3-10.8)[0.3-23]
Smoking	0
Body Mass Index (BMI)	25.5 (24.2-28.4)[22.3-37.2]
Parity	2 (0-3)[0-5]
*Systolic Blood Pressure	126.8 ± 14.4
*Diastolic Blood Pressure	72.3 ± 11.23

Table 6: Demographic characteristics
Data are median (interquartile range)[range]. *Data are mean ± SD

Responses to acetylcholine (ACh) and sodium nitroprusside (SNP)

There was no difference in the response of subcutaneous vessels when comparing treatment with clonidine to treatment with placebo in either the endothelium dependent or independent response ($P=0.98$ and $P=0.50$ respectively, Mann Whitney) (see Figure 35).

On examination of the baseline LDI assessment (i.e. no treatment), there was a statistically significant increase in AUC with both clonidine and placebo during administration of the endothelium dependent vasodilator ($P=0.04$ and 0.01 respectively, Mann Whitney). This did not appear to be present during administration of endothelium independent vasodilator ($P=0.29$ and 0.06 for clonidine and placebo respectively, Mann Whitney) (see Figure 35).

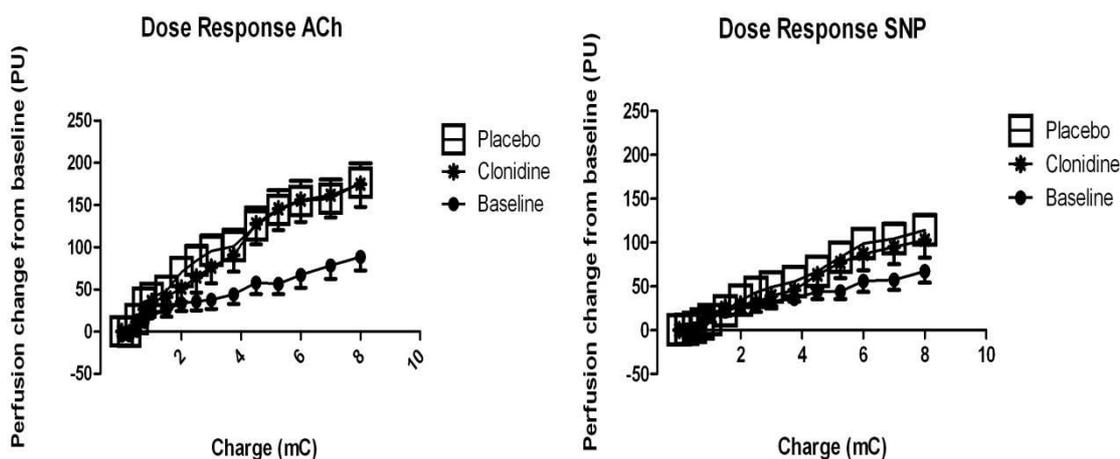


Figure 35: Dose Response Curve

Flux raw – baseline values (perfusion units) with increasing charge for acetylcholine and sodium nitroprusside in flushing women when treated with clonidine and compared to treatment with placebo and to baseline. Data are mean \pm SEM. As described in text, data are analysed as AUC and compared by Mann Whitney. P values for clonidine vs placebo are 0.98 and 0.50 for Ach and SNP respectively. P values for clonidine vs baseline are 0.04 and 0.29 for Ach and SNP respectively. P values for placebo vs baseline are 0.01 and 0.06 for Ach and SNP respectively.

As there appeared to be a consistent difference between groups the difference was calculated and the analysis repeated. There was no significant difference detected. (clonidine vs placebo: $p=0.85$, clonidine vs baseline: $p=0.095$, and placebo vs baseline; $p=0.22$) (see Figure 36).

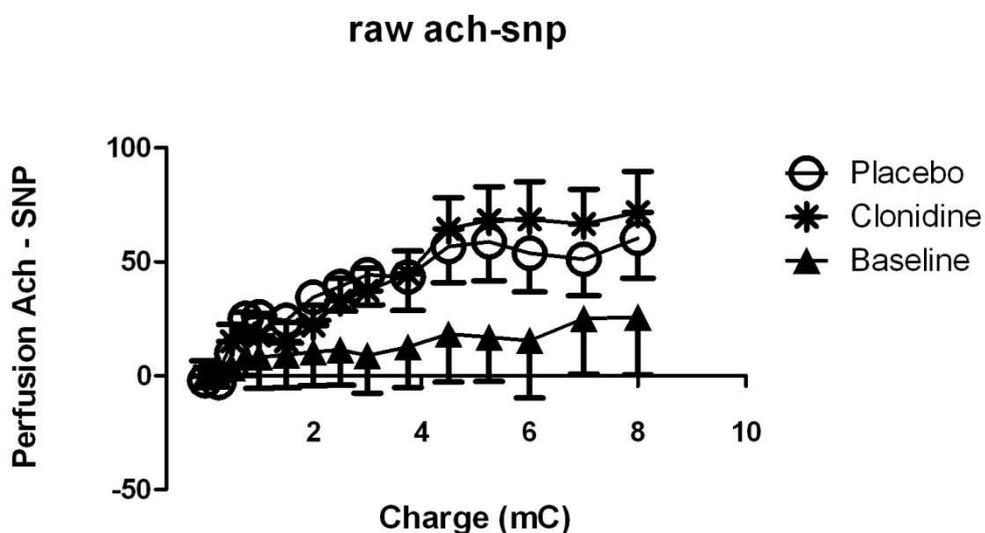


Figure 36: Dose Response Curve
Endothelial response (difference between endothelium dependent and independent responses). Clonidine vs Placebo; $p=0.85$. Clonidine vs Baseline; $p=0.095$. Placebo vs Baseline; $p=0.22$. Data are mean \pm SEM.

Hot Flush Diaries

In this group of women, for the duration of this study, there was no statistically significant improvement in either the number or severity of flushes when comparing clonidine to placebo (P=0.21). However, when examining clonidine against baseline (no treatment) there was a reduction in the number of flushes experienced (P=0.02) and the severity of the flushes also appeared to be reduced (P=0.01). There was no improvement in night time awakening or hot flush related daily interference (see Table 7).

	Placebo	Clonidine	Baseline	P values		
				P vs C	C vs B	P vs B
Flush frequency(mean flush/day)	5.2 (2.6,18.8)	4.4 (0.7, 19.4)	6.1 (3.1, 17.6)	0.21	0.02	0.25
Night time awakening (NTA)	2.7 (0.4, 5.3)	2.5 (0.6, 4.31)	2.5 (0.63, 4.7)	0.45	0.79	0.73
Hot flush related daily interference score (HFRDIS)	37.3 (2.7, 89.3)	33.8 (5.0, 100.0)	44.2 (8.5, 89.0)	0.50	0.43	0.70
Hot flush score	11.9 (4.4, 56.3)	8.9 (0.6, 58.2)	12.1 (5.7, 52.9)	0.13	0.01	0.40

Table 7: Statistical comparison of placebo, clonidine and baseline
 Flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed. Placebo and Clonidine weeks, diaries were completed for 4 weeks each, and baseline diaries were completed for 3 weeks.

Data are P values determined by Mann Whitney analysis of mean values as demonstrated in table 2.

Qualitative Data

Greene Climacteric Scale

GCS Scores were significantly lower following treatment with clonidine than at baseline ($P=0.001$), and were significantly lower following treatment with clonidine than following treatment with placebo ($P=0.01$). However, there was no difference when comparing placebo to baseline scores ($P=0.48$). This held true when examining only the scores relating to vasomotor symptoms ($P=0.002$, 0.004 , and 1 respectively) (see Figure 37).

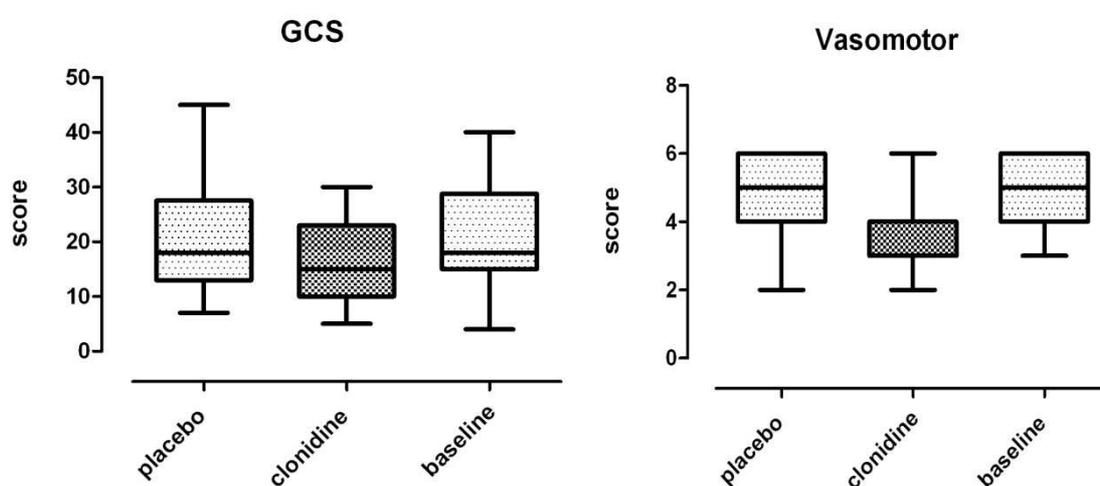


Figure 37: GCS in flushing women comparing clonidine, placebo and baseline
 Total GCS scores; Clonidine vs Baseline $P=0.001$, Clonidine vs Placebo $P=0.01$, and Placebo vs Baseline $P=0.48$. Vasomotor scores; Clonidine vs Baseline $P=0.002$, Clonidine vs Placebo $P=0.004$, and Placebo vs Baseline $P=1$. Wilcoxin Signed Rank test of paired data.

Summary of Results

Responses to acetylcholine (ACh) and sodium nitroprusside (SNP)

There was no difference in the response of subcutaneous vessels when comparing treatment with clonidine to treatment with placebo in either the endothelium dependent or independent response.

There was a statistically significant increase in AUC with both clonidine and placebo from baseline during administration of the endothelium dependent vasodilator but not with the endothelium independent vasodilator.

Diary data

Clonidine vs. Placebo

There was no improvement in either the number or severity of flushes.

Clonidine vs. Baseline

There was a reduction in the number and severity of flushes.

Placebo vs. Baseline

There was no improvement in number or severity of flushes.

Qualitative Data

Green Climacteric Scores were reduced from baseline with clonidine. There was no difference with placebo from baseline, and clonidine was better than placebo.

Discussion

Dilation of cutaneous vessels, with resultant increase to blood flow, is a hallmark of flushing. This resembles a heat dissipation response, whereby increases in core temperature trigger nonevaporative heat loss through vasodilation of subcutaneous vessels. In the rat, cutaneous vasodilation of the tail is a primary mechanism of thermoregulation and it has been shown that after ovariectomy, tail skin temperature increases, and that this effect can be abolished by treatment with oestrogens (197). Furthermore, castration in rats is associated with increased hypothalamic noradrenaline levels (198) and oestrogen replacement decreases turnover (95).

Monoamines, especially noradrenaline (NA), have been shown to play an important role in the control of thermoregulation. Noradrenergic stimulation of the pre-optic area of the hypothalamus in monkeys (87) and baboons (88) by microiontophoretic application of NA causes peripheral vasodilation, heat loss and a drop in core temperature, similar to changes which occur in women during hot flushes.

This is consistent with NA involvement in hot flushes, which is further supported by clinical data showing that clonidine, an α_2 -adrenergic agonist that reduces brain NA, reduces hot flush frequency. Yohimbine, an α_2 -adrenergic antagonist has also been shown to provoke hot flushes (93).

Whilst clonidine did decrease the number and intensity of flushes in our group of women, this was not significant when compared to placebo. This is perhaps not surprising as a recent meta-analysis examining ten trials comparing clonidine to placebo found only 4 showed an improvement in number and severity of flushing and 2 out of 4 were of poor quality (120).

What is surprising, perhaps, is the increase in vascular reactivity seen with both clonidine and placebo when compared to baseline measurements, with no difference between the two treatments.

The placebo effect is well documented, and in randomised controlled trials (RCTs) is designed to assess the efficacy of the potential treatment. The

magnitude of the placebo response is found to be partly dependent on the condition. In studies of anti-depressant medications, placebo response rates average approximately 30%, ranging from 12% to more than 50% and in RCTs of hormone therapy for vasomotor symptoms in menopause, the placebo response rate averages 51% (199). This may explain the subjective diary results and it has been suggested that if harnessed reliably, the placebo response, may enhance treatment outcomes (200).

The increase in cutaneous microvascular perfusion seen after 4 weeks of treatment with placebo is more difficult to explain. The placebo effect is well recognised and long studied in analgesia and it was in 1978 that endogenous opioids were first shown to be involved in placebo analgesia. Levine et al demonstrated that the opiate antagonist naloxone was able to reduce the placebo response in dental postoperative pain (201). Since then, placebo and placebo-related effects have been analysed and specific mechanisms at both the biochemical and cellular level have been uncovered. Benedetti has conducted an extensive review of a number of these mechanisms, and amongst them is a reduction of β -adrenergic activity of the heart, changes of metabolic responses in different brain regions (possibly inhibition of serotonin reuptake), as well as modulation of immune factors including IL-2 and IFN- γ (202). It is therefore possible that placebo treatment could affect peripheral vascular reactivity, but this would clearly require further study.

Equally, the peripheral effects of clonidine are not clear. Ginsburg et al, found that an increase in forearm blood flow induced by intravenous infusion of adrenaline, angiotensin and noradrenaline was significantly less in women treated for at least 6 weeks with clonidine compared with that induced in the women by infusions given before treatment. (101). However, these results would seem to contradict these. One possible explanation is that the treatment duration in this study (4 weeks) was insufficient to elicit peripheral vasoconstriction. However, a more likely explanation may relate to differences in methodology. Ginsburg used venous occlusion plethysmography to measure hand and forearm perfusion but, for the forearm, overall change in perfusion will be much more strongly influenced by change in blood flow to the forearm muscles, rather than the skin. However, the LDI + ION methodology only assesses skin perfusion, and is therefore more appropriate for studies of flushing.

Ginsburg et al found that constrictor responses in the hand were unchanged. Hand perfusion would be less affected by muscle blood flow as there are fewer muscles, and may therefore be more comparable with these results.

One could argue that a 4 week duration following commencement of clonidine was too long to see the peripheral vasoconstriction that might be elicited by the treatment as this anti-hypertensive agent has a dual action. When first administered, clonidine stimulates peripheral α_1 -adrenoceptors (ARs) resulting in vasoconstriction, but subsequently acts on the central ARs to inhibit sympathetic drive resulting in vasodilation (203). The central action predominates over the peripheral, therefore perhaps any initial decrease in cutaneous microvascular perfusion that there may have been is now overridden by the centrally mediated and predominant vasodilatory effect.

This may in fact, explain the increase in perfusion responses seen in our group of women after 4 weeks of clonidine, and may explain why anecdotally there often appears to be an initial improvement in symptoms followed by a return of vasomotor symptoms.

However, clonidine is used for postoperative shivering because it is thought that, like general anaesthetic agents and sedatives, it decreases shivering thresholds by a generalised impairment of central thermoregulatory control (91). These same authors also demonstrated that clonidine administration increased the sweating threshold. They went further to suggest, that as the thermoregulatory effects of clonidine resembled those of volatile anaesthetics, it was likely that the alteration in the shivering and sweating thresholds were as a result of central thermoregulatory inhibition (92).

Chapter 5

Serotonin

Introduction

We have already demonstrated that postmenopausal women with severe flushing have a greater vasodilator response than matched women with no flushing (see Chapter 3). This seems logical, as one of the hallmarks of hot flushes is the dilatation of cutaneous vessels, which results in increased blood flow (flushing, increased skin temperature and nonevaporative heat loss).

However, it is generally accepted that oestrogens promote cutaneous vasodilation and therefore heat dissipation (83), and in this study, in an environment that is likely to be relatively oestrogen deficient for all, there is an increase in the vasodilator response in only the flushing women. This is difficult to quantify accurately as postmenopausal oestradiol levels are too low to measure with routine laboratory tests, but it has previously been shown that circulating levels of oestrogen do not differ significantly between symptomatic and asymptomatic postmenopausal women (54), therefore whilst it is likely that oestrogen plays a part, it is certainly not alone.

Serotonin, in addition to its extensive role as a neurotransmitter in the central nervous system has been implicated in thermoregulation.

Animal studies in the 1960s suggested that increases in hypothalamic release of serotonin resulted in an increase in body temperature, and that control of core temperature was as a result of finely balanced 5-HT, adrenaline and noradrenaline (204, 205).

In addition, elevated levels of serotonin with severe hyperthermia are associated with the drug 3,4-methylenedioxymethamphetamine (MDMA), which causes release of monoamines in the central nervous system, specifically elevating extracellular levels of serotonin.

The 2 subtypes believed to be most closely associated with thermoregulation are the 5-HT_{1A} receptor and the 5-HT_{2A} receptor (206, 207). Administration of 5-HT_{2A} antagonists prevents hyperthermia in animal models of the serotonin syndrome, and direct stimulation of 5-HT_{2A} receptor induces hyperthermia in rodents (207, 208). In contrast, peripheral administration of 5-HT_{1A} agonists to humans and

rodents results in a reduction in core body temperature, which can be blocked by 5-HT_{1A} antagonists (209, 210). This information suggests that a balance between the 5-HT_{2A} and 5-HT_{1A} receptors might be important for thermoregulation in mammals.

Sympathetic control of the cutaneous vascular bed during a febrile reaction, often accompanied by sweating, occurs via a pathway descending from the hypothalamus and basal forebrain to the spinal cord (211). Dysfunction here would be in keeping with Freedman's hypothesis. Animal studies have also demonstrated that activation of inhibitory 5-HT_{1A} receptors have been shown to cause a fall in body temperature associated with dilation of the cutaneous vascular bed (212).

Vasodilatation, as a response to increases in core temperature, occur firstly as a result of loss of sympathetic tone in the subcutaneous vessels, followed by active vasodilatation (83). In animal studies, 5-HT_{1A} receptor activation causes dilation of the cutaneous vascular bed associated with marked inhibition of cutaneous sympathetic nerve activity (213), and hyperthermia has been elicited by activation of 5-HT_{2A} (214).

A hot flush resembles a heat loss mechanism, with peripheral vasodilatation and sweating, and we have shown that peripheral cutaneous vascular responses to vasoactive substances are increased in postmenopausal women with severe flushing (169).

A low blood oestrogen level (as one might expect to see in postmenopausal women) has been shown to correlate with a high concentration of the 5-HT_{2A} receptor subtype on blood platelets (215) and an upregulation of central 5-HT_{2A} receptors (216).

Selective serotonin reuptake inhibitors (SSRI) are designed to increase the available serotonin at the serotonergic synapse and have been shown in placebo-controlled trials to be effective in reducing the number and severity of hot flushes (120).

Serotonin exhibits strong vasoactive properties (217), possibly through stimulation of 5-HT receptors on endothelial cells (110). Venlafaxine, too, has been found to be effective in reducing flushing (218) and although it is a serotonin and noradrenaline reuptake inhibitor, at low doses it probably acts as an SSRI (219).

In an attempt to explain the involvement of 5HT and its receptors in the pathophysiology of a hot flush, focus has largely been on the effect that mild stress has on the central control of thermoregulation. It has been suggested that anxiety, coffee, alcohol and small increases in ambient temperature increase 5-HT moduline in the cortex, hippocampus and hypothalamus leading to increased release of 5-HT by blockade of inhibitory 5-HT_{1A} receptors, and increased activation of the upregulated 5-HT_{2A} receptors, leading to an imbalance of thermoregulation and activation of heat dissipation functions (see Chapter 1, Figure 7.) (220).

However, the role of serotonin in central regulatory pathways is complex because binding at some serotonin receptors can exert a negative feedback on other serotonin receptor subtypes. Thus the effect of a change in serotonin activity varies depending on the type of receptor activated.

Further study is needed on the role of 5-HT and its receptors in subcutaneous microvascular responses, central receptor availability and their relationships with symptoms of the menopause (specifically vasomotor).

This study aimed to assess the role of serotonin in flushing and the mechanism whereby venlafaxine improves vasomotor symptoms both peripherally, by examining cutaneous microvascular perfusion, and centrally by studying the serotonin transporter (SERT) in vivo in the human brain. Examination of central SERT is made possible by using the iodine-labelled radioligand [¹²³I]-beta-CIT, which binds with a high affinity to SERT (161), in combination with single photon emission computed tomography (SPECT).

Hypothesis

The altered endothelial function of women who flush is reversed by successful treatment of the flushes by venlafaxine. This is reflected in altered skin blood flow and vascular response to vasoactive agents, acetylcholine and sodium nitroprusside. This will be correlated with alterations in central SERT availability.

Methods

Participants

A total of 46 women were recruited to receive venlafaxine treatment. 22 of these women were recruited to undergo SPECT scanning in addition to LDI + ION.

Recruitment of volunteers followed Scottish media coverage, as well as from gynaecology and menopause clinics. A number of women were also recruited from the West of Scotland Breast Screening Centre in Glasgow.

Participating women were all aged 45-65 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function. Menopausal status was determined by either an FSH greater than 20 Units/Litre or amenorrhoea for 1 year or longer.

Study Design

The study had a longitudinal design (see Figure 38). At visit 1 (V1), consent was obtained and baseline questionnaires were completed. At visit 2 (V2), baseline cutaneous microvascular perfusion was assessed using LASER Doppler imaging with iontophoresis (LDI + ION), and participants returned for repeat assessment at V3 following 8 weeks of treatment with venlafaxine 37.5mg twice daily. Qualitative measures were obtained at the same time points.

Participation in the SPECT subgroup was offered to each participant, and SPECT scans were carried out, in addition to LDI + ION, before treatment (V2) and after 8 weeks of treatment (V3)

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 09/MRE00/40). All patients gave written informed consent.

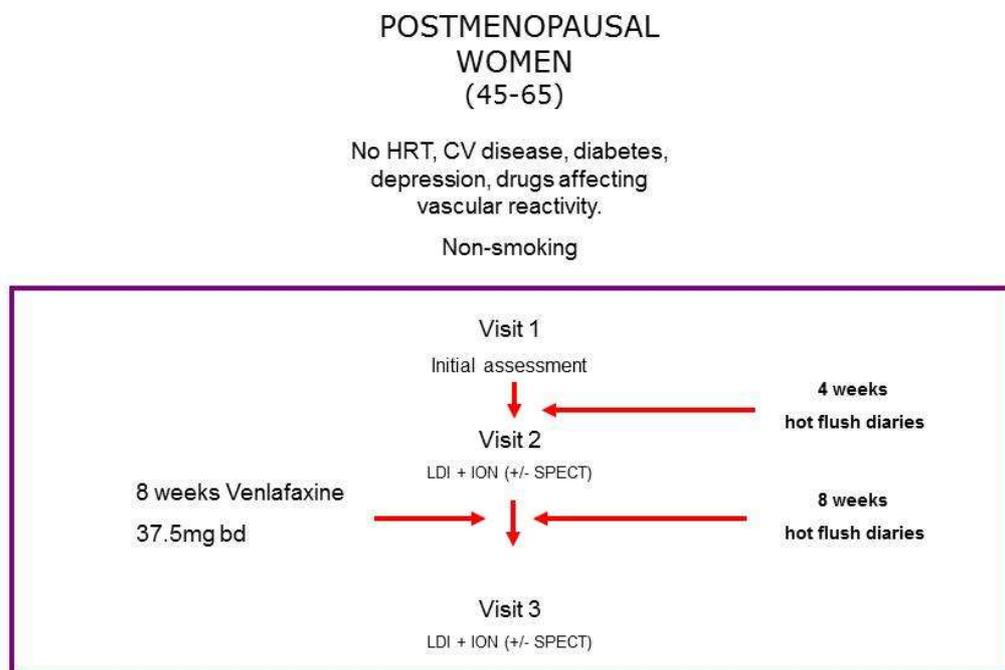


Figure 38: Study design

Body Composition

Measurements of body mass, height, waist and hip circumference, and blood pressure were made as described in Chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made at baseline and after 8 weeks of treatment with venlafaxine 37.5mg bd. This technique is described in detail in Chapter 2.

Hot flush diary

Each participant was asked to keep a 'Hot Flush' diary for 4 weeks prior to initial assessment and throughout the study, as described in detail in Chapter 2.

[¹²³I] -beta-CIT Preparation and SPECT Imaging

[¹²³I] -beta-CIT was prepared via electrophilic iododestannylation of the corresponding tributylstannyl precursor (159) by Dr Sally Pimlot and is described in detail, together with the SPECT imaging protocol, in Chapter 2.

SPECT Image Analysis

Region of interest (ROI) analysis of the [¹²³I] -beta-CIT scans, was carried out by an investigator blinded to the subject's clinical history. The ROI template consisted of manually drawn regions representing the brain stem/diencephalon ROI (referred to as the brain stem ROI below for brevity), and a reference ROI - the occipital region. The occipital region was chosen as the reference ROI that represented the nonspecific and nondisplaceable [¹²³I] -beta-CIT uptake (i.e., activity not associated with binding to transporters) because it has a negligible density of both dopamine and serotonin transporters.

The [¹²³I] -beta-CIT uptake in each ROI was expressed as mean counts per pixel, and the specific uptake of [¹²³I] -beta-CIT was calculated as:

$$\text{Beta CIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}$$

Transporter binding ratio for SERT was then defined as the ratio of specific uptake to uptake in the occipital reference region using the formula:

$$\frac{\text{Beta CIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}}{\text{BetaCIT uptake in the occipital ROI}}$$

Under equilibrium conditions the binding ratio is proportional to transporter binding potential, and provided transporter affinity and nonspecific binding are invariant across subjects, the ratio is then a measure of transporter availability (B_{\max}).

Qualitative Measurements

Greene Climacteric Scale

Each participant completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Becks Depression Inventory

Each participant completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Hospital Anxiety and Depression Scale

Each participant who participated in the study after 16 August 2010 completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Plasma analysis

A fasting blood sample was taken from each participant using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetra-acetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

A small volume was removed from the 6ml lithium heparin sample before centrifuge and storage as described in Chapter 2.

ICAM, VCAM, TNF - α , IL-6, Insulin and Adiponectin were measured by ELISA technique as described in Chapter 2.

CRP, Cholesterol, Glucose, HDL, LDL, and Trig were measured by standardised methods at The Institute of Cardiovascular and Medical Sciences at Glasgow University, as described in Chapter 2.

Statistical analysis

Measurement of vascular responses, in the whole group, was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis.

Diary data, HFRDIS and qualitative data, in the whole group, were compared using Wilcoxin Signed Rank test of paired non-parametric data. Diary data included analysis of mean number of flushes per day, hot flush score and night time awakening.

Statistical analysis pertaining to the SPECT group was performed using SPSS v19 by Dr Rajeev Krishnadas. A paired-samples t-test was conducted to compare the means of Beta-CIT uptake, BDI scores, Flush frequency, ACh - AUC, SNP - AUC, Flush score, HFRDIS and NTA, before and after treatment with Venlafaxine. Mean difference of scores, the 95% CI of the mean differences and effect sizes (Cohen's d) were also calculated (162).

Percentage change (reduction) in each of the above variables from baseline was calculated using the formula:

$$Reduction\ X = \frac{Pretreatment\ X - Posttreatment\ X}{Pretreatment\ X} \times 100$$

where X is the variable of interest ($[^{123}I]$ -beta-CIT binding ratio; BDI scores; Flushing frequency; Flushing score; ACh-AUC; SNP-AUC).

The relationship between the reduction in the $[^{123}I]$ -beta-CIT binding ratio (predictor variable) and other variables (dependent) of interest - namely BDI reduction; Flush frequency, and score, reduction; ACh reduction; SNP reduction - were examined using multivariate analysis of covariance (MANCOVA) with age as a nuisance covariate in the model. The p values were corrected for multiple testing using Holm-Bonferroni family wise error correction.

Results

46 women with severe hot flushing were recruited to receive venlafaxine 37.5mg bd and 22 of these women were recruited to undergo SPECT in addition to LDI + ION.

Participation in the SPECT subgroup was offered to each participant. Reasons for not wishing to take part included the additional time required to participate; the procedure required an additional 2 full days at a separate unit. The space inside the scanner is small; therefore claustrophobia as well as a short neck, and/or weight greater than 130kg were also exclusion criteria.

Following recruitment, three participants declined continued participation prior to initial assessment. A further 2 women were excluded following V1, but before baseline measurements; one participant was commenced on therapy by her GP, which excluded her from the study, the other attended for Visit 2b (SPECT imaging), however there was no radioisotope available and she was unable to return for a rescheduled appointment as a result of personal time constraints.

Following V1, 1 participant experienced a syncopal episode during baseline LDI + Ion measurements (V2), likely as a result of fasting, and had to withdraw from the study. A further 10 withdrew during the treatment period and prior to treatment assessment (V3). Seven as a result of nausea, one as a result of improved vasomotor symptoms prior to commencement of therapy, and therefore declined to start therapy, and one complained of muscle spasms whilst on treatment and therefore discontinued (see Figure 38). 31 women completed LDI + Ion assessments for baseline and treatment measurements, and 14 of these women also completed both baseline and treatment SPECT assessments (see Figure 39).

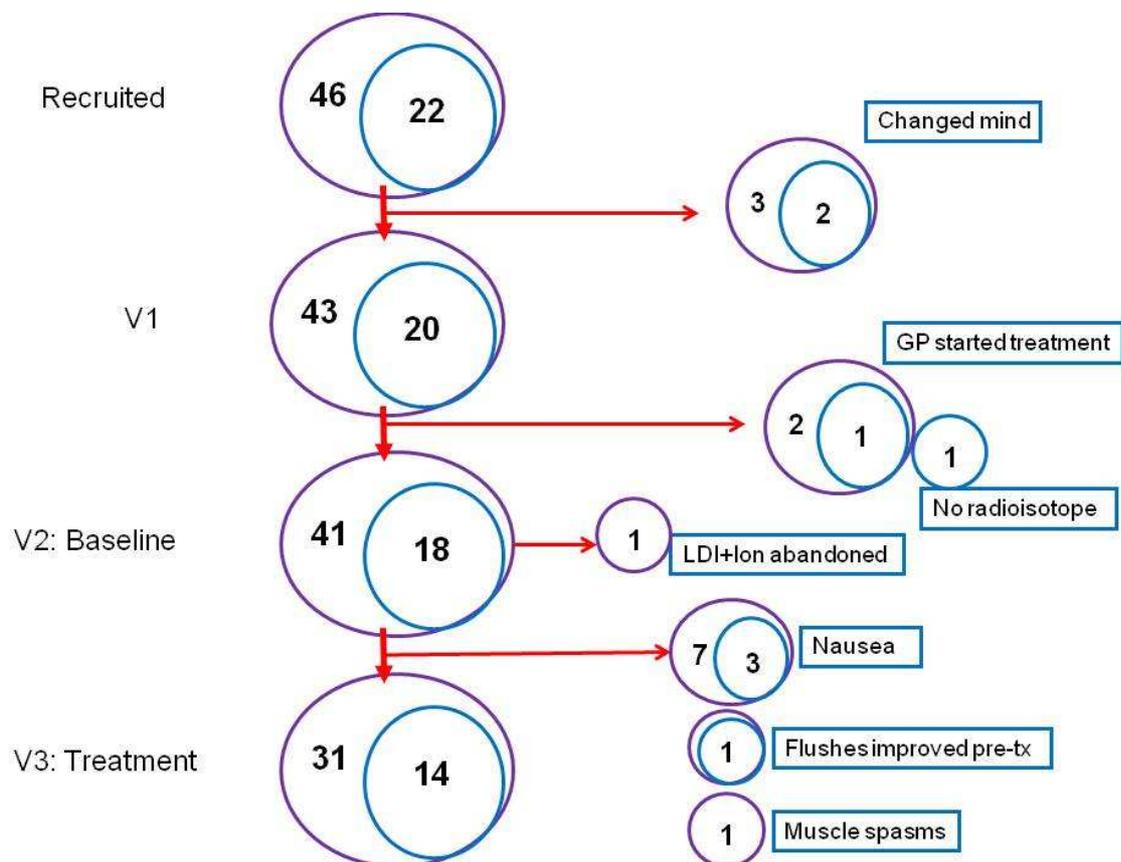


Figure 39: Recruitment and Participant numbers

Figure illustrating longitudinal study design with numbers of participants in LDI + ION (purple circle) group and SPECT (blue circle) subgroup. Numbers of withdrawals with reasons (same colour-code) illustrated.

Demographic Characteristics

	Baseline LDI+ION	Treatment LDI+ION	SPECT subgroup
n	40	31	14
Age (years)	58 (54-63) [46-65]	58 (54-62) [46-65]	58 (54-60) [46-64]
Years since LMP	7 (1.1-10.8) [0.3-20]	7 (1-10) [0.3-16]	6.5 (1.4-8.5) [0.3-15]
BMI	25.8 (24.2-29.7) [20.7-37.1]	26.1 (24.5-30.3) [21.4-37.1]	26.9 (24.8-29.7) [22.1-34.6]
WHR	0.81 (0.76-0.83) [0.70-0.96]	0.80 (0.75-0.83) [0.70-0.96]	0.80 (0.76-0.82) [0.70-0.96]
Parity	2 (1-3) [0-4]	2 (1-3) [0-4]	2 (1-3) [0-4]
Smoking	0	0	0
*SBP	128.5 ± 16.1	128.2 ± 15.1	122.8 ± 12.0
*DBP	80.1 ± 8.9	79.6 ± 8.8	77.0 ± 7.9

Table 8: Demographic characteristics

Data are median (interquartile range)[range]. *Data are mean ± SD

Responses to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Following 8 weeks of venlafaxine therapy, enhanced vascular responses are seen to be decreased following administration of ACh ($p = 0.005$), but not SNP ($p = 0.400$). See figure 40.

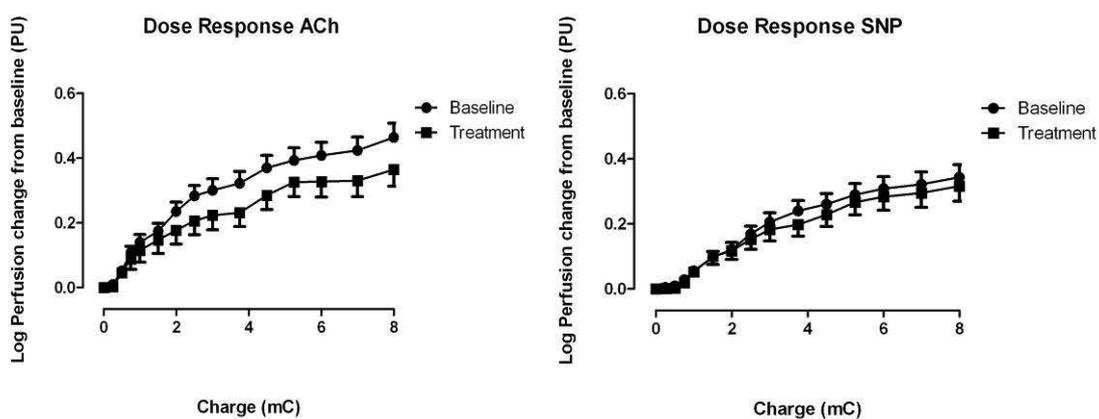


Figure 40: Dose Response Curve

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in flushing women when treated with venlafaxine and compared to baseline (i.e. no treatment). Data are mean \pm SEM.

Hot Flush Diaries

One participant did not return either baseline or treatment diary data, and one further participant did not return treatment diary data.

Diary data was calculated as follows; flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed.

Data are presented as mean \pm standard deviation to illustrate the results as determined by above calculations. It is also presented as median (interquartile range)[range] as the data were not normally distributed. Statistical comparisons were therefore made by Wilcoxon Signed Rank Analysis of paired data.

Treatment with venlafaxine significantly reduced the number of ($p < 0.001$) and severity of flushes ($p < 0.001$), frequency of night time awakenings ($p < 0.001$), and perception of daily hot flush interference ($p < 0.001$) (see Table 9 and Figure 41). Flushes were decreased by a mean of 4.9 ± 3.1 per day.

	Baseline n=39	Treatment n=29	P
Flush frequency (flush/day)	8.2 ± 3.9 7.7 (5.8-9.5) [2.1-21.2]	4.4 ± 3.92 3.5 (2.5-6.1) [0.1-13.8]	<0.001
Night time awakening (NTA)	2.4 ± 1.1 2.4 (1.8-3.4) [0.3-4.6]	1.2 ± 0.9 1.0 (0.4-1.8) [0.04-3.6]	<0.001
Hot flush related daily interference score (HFRDIS)	40.7 ± 22.1 37.3 (23.3-58.7) [5.3-84.7]	19.3 ± 17.7 14.4 (7.8-21.8) [1.5-72.6]	<0.001
Hot flush score	16.2 ± 7.9 15.8 (10.3-20.4) [1.9-42.4]	8.0 ± 8.1 6.4 (3.2-10.8) [0.03-41.3]	<0.001

Table 9: Hot flush diary data

Data are mean ± SD, and median (interquartile range)[range]. P values are determined by Wilcoxon Signed Rank analysis of paired data.

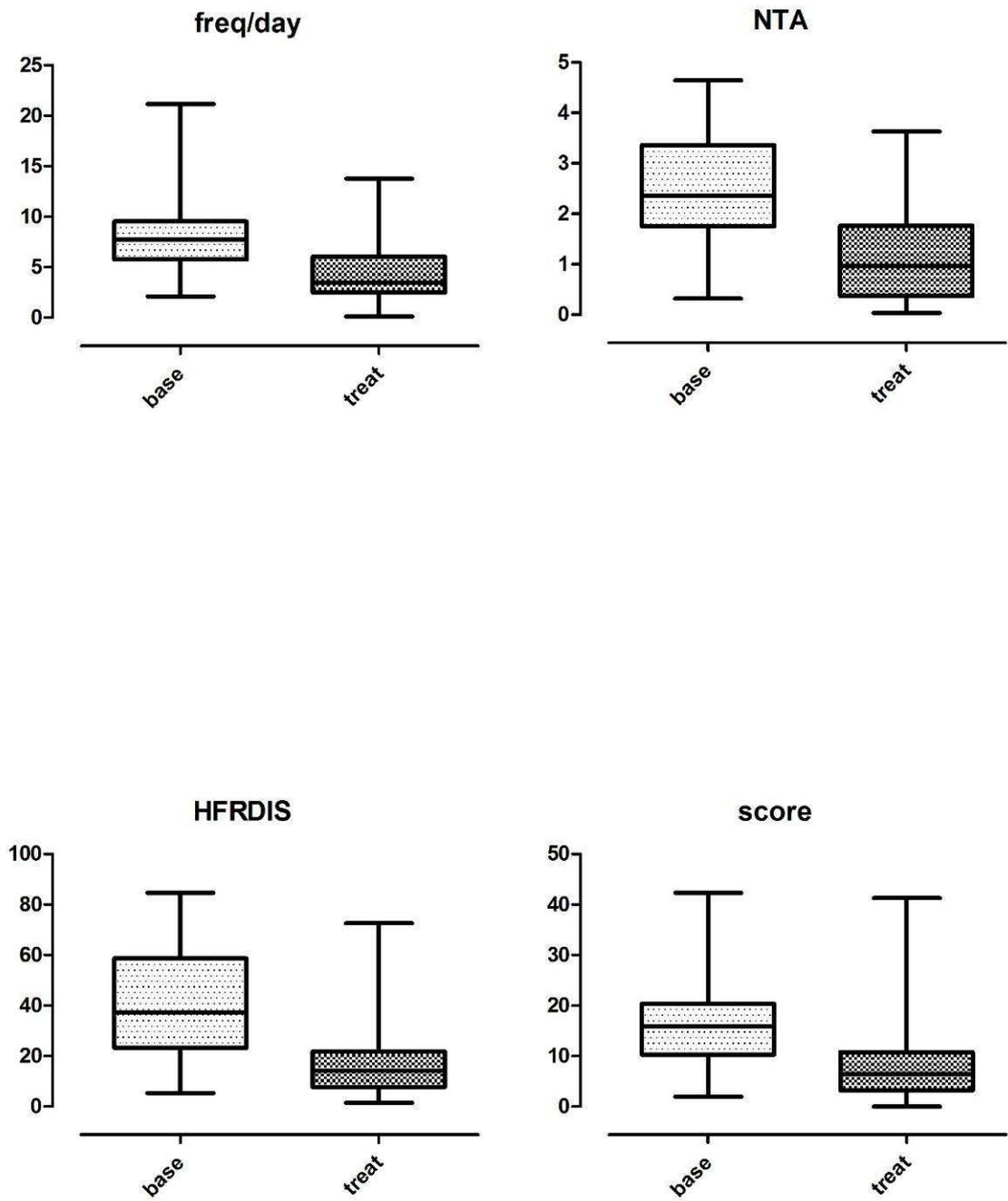


Figure 41: Diary data
Data are shown as box and whisker plots with median, IQR and range.

Perfusion responses and their correlation with demographic and diary data

To evaluate the association between perfusion responses and diary data, including flush frequency, flush score, hot flush related daily interference scores (HFRDIS) and night time awakening (NTA); the correlation between each of these was examined.

Perfusion responses were assessed as area under the curve (AUC) values for raw data minus baseline values. Diary data were assessed as mean values as described in detail in chapter 2.

There were no correlations demonstrated for any relationship (see Figure 42 and Figure 43 and Table 10).

In addition there was no relationship demonstrated between the reduction in perfusion responses and the reduction in flush frequency or flush score (see Figure 44 and Table 11).

We did not demonstrate any significant association between perfusion response, for either ACh or SNP, and age, body mass index (BMI) or waist hip ratio (WHR). Although there may be a trend towards increasing perfusion responses with increasing WHR (see Figure 45 and Table 10).

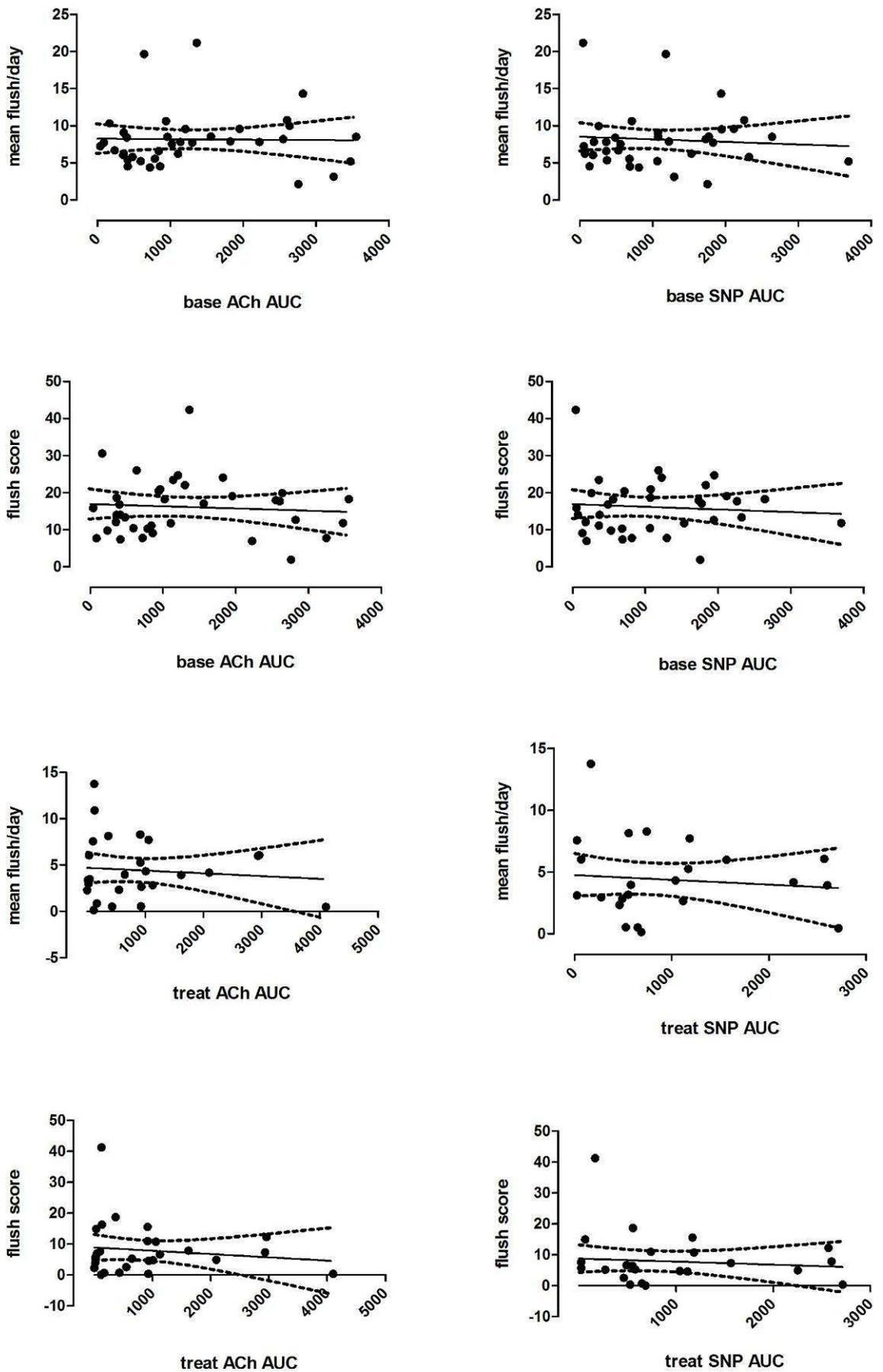


Figure 42: Correlation of perfusion responses with flush frequency and flush score
Correlation plots and regression lines with 95% confidence intervals for perfusion responses, (ACh and SNP) and flush frequency and score. Baseline and treatment measurements.

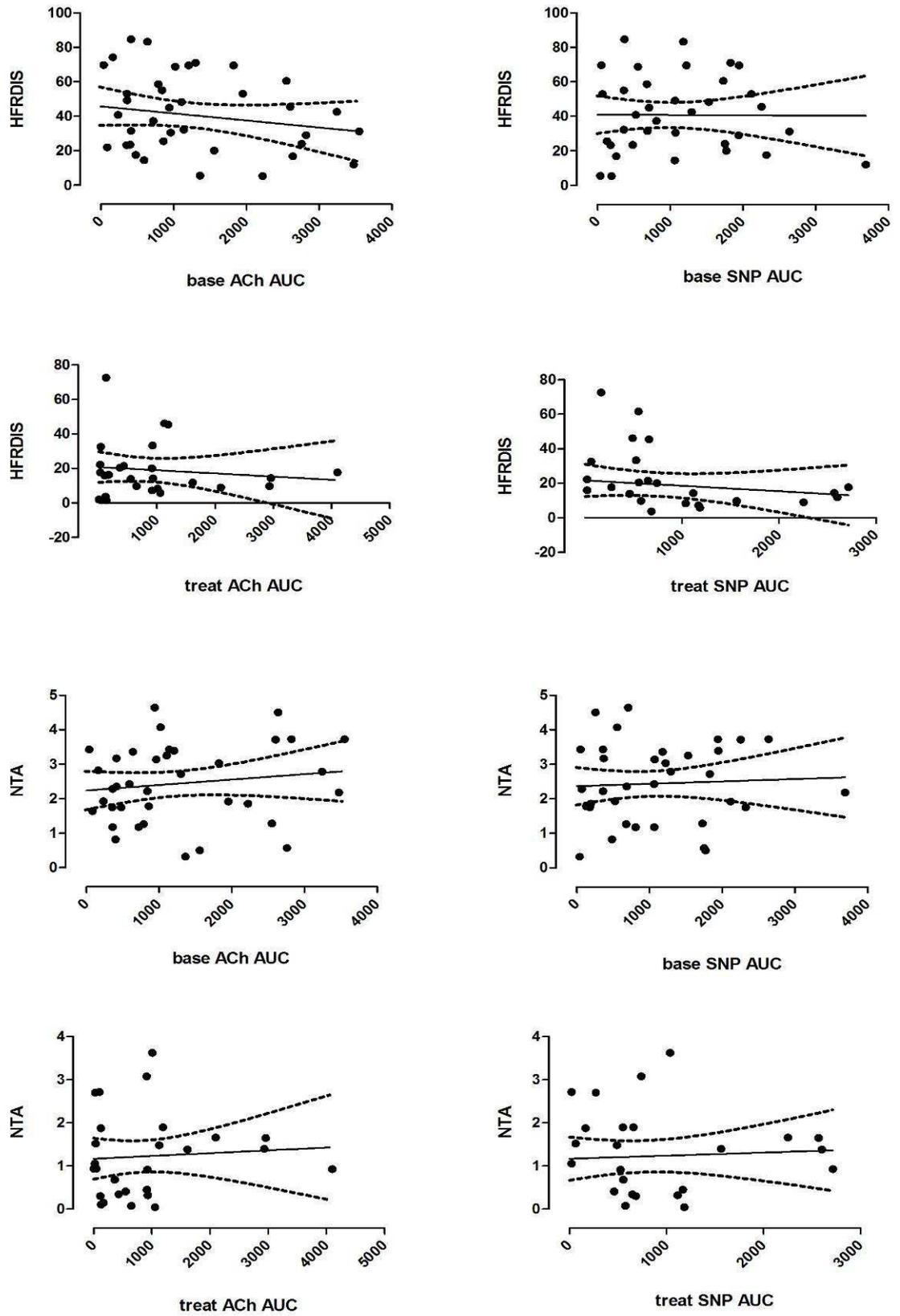


Figure 43: Correlation of perfusion responses with HFRDIS and NTA
 Correlation plots and regression lines with 95% confidence intervals for perfusion responses, (ACh and SNP) and hot flush related daily interference scores (HFRDIS) and night time awakening (NTA). Baseline and treatment measurements.

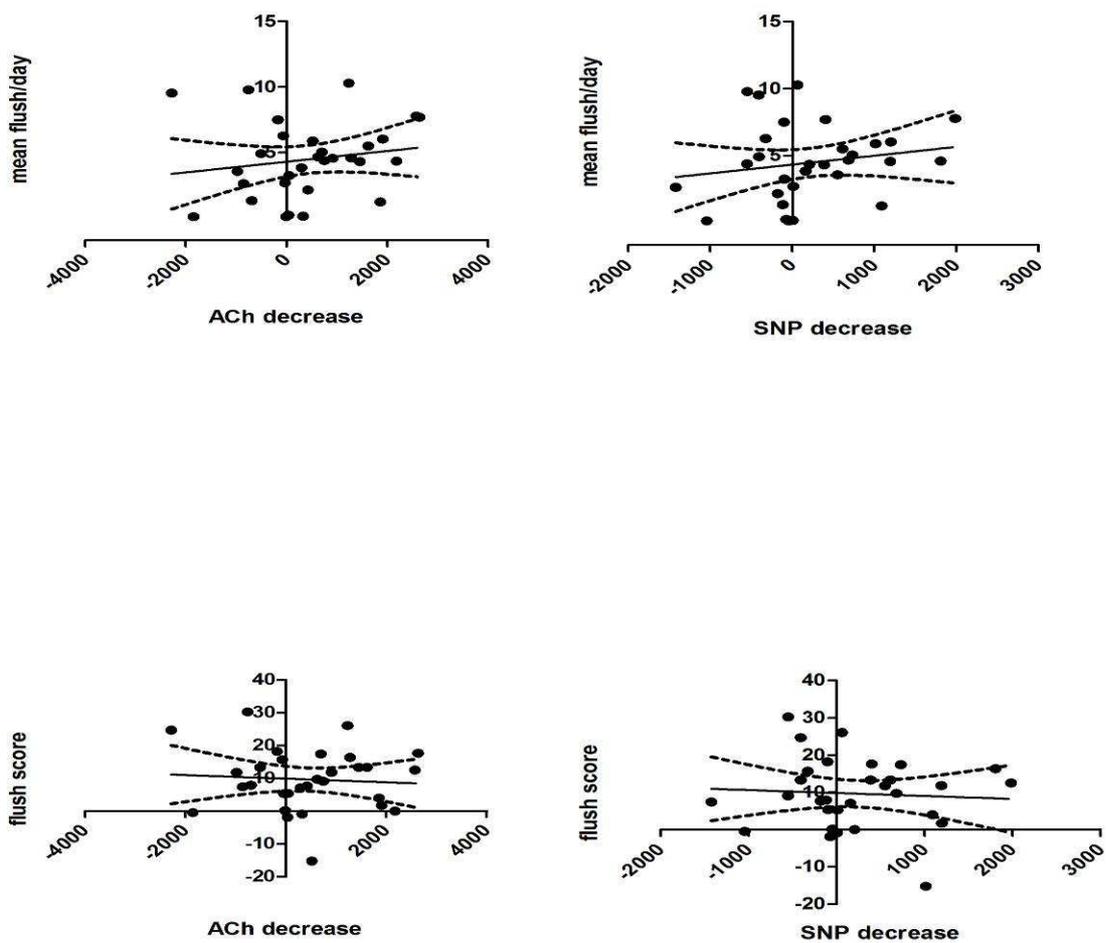


Figure 44: Correlation of decrease in perfusion response with decrease in flush frequency and score

Correlation plots and regression lines with 95% confidence intervals for reduction in perfusion response (baseline-treatment) for ACh and SNP and their association with the reduction in flush frequency and flush score.

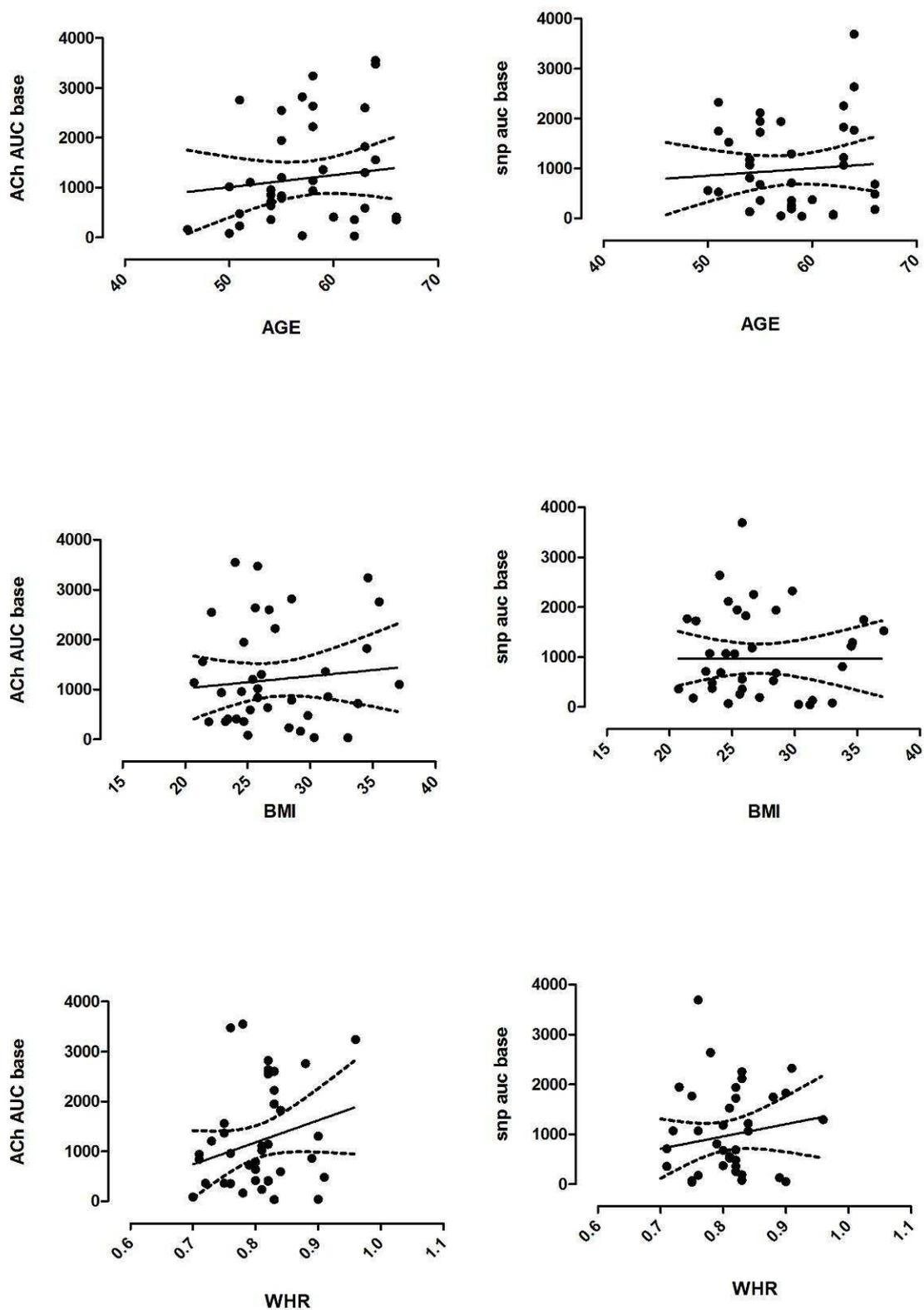


Figure 45: Correlation of perfusion responses with age, BMI and WHR
 Correlation plots and regression lines with 95% confidence intervals for perfusion response at baseline (ACh and SNP) and their association with age, body mass index (BMI) and waist hip ratio (WHR).

		ACh AUC				SNP AUC			
		Baseline		Treat		Baseline		Treatment	
		r	p	r	p	r	p	r	p
flush freq/day	Base	0.114	0.491			0.013	0.937		
	Treat			0.077	0.698			0.049	0.806
score/day	Base	0.062	0.707			0.057	0.728		
	Treat			0.034	0.864			0.039	0.842
HFRDIS	Base	-0.122	0.458			0.104	0.530		
	Treat			0.018	0.926			0.018	0.924
NTA	Base	0.190	0.248			0.122	0.458		
	Treat			0.047	0.808			-0.014	0.942
Age		0.121	0.456			0.085	0.604		
BMI		0.097	0.553			-0.001	0.997		
WHR		0.247	0.124			0.160	0.325		

Table 10: Correlation of perfusion responses (ACh and SNP) with flush frequency, flush score, HFRDIS, NTA, age, BMI and WHR.

Table illustrating correlation of perfusion responses (ACh and SNP) with flush frequency, flush score, HFRDIS, NTA, age, BMI and WHR. Baseline and treatment measurements. Analysis was performed using Spearman's correlation of non-parametric data.

	ACh decrease		SNP decrease	
	r-value	p-value	r-value	p-value
Flush frequency decrease	0.239	0.204	0.200	0.289
Flush score decrease	0.005	0.979	-0.028	0.881

Table 11: Correlation of decrease in perfusion response with decrease in flush frequency and score

Table illustrating correlation of decrease in perfusion responses (ACh and SNP) with decrease in flush frequency and flush score. Analysis was performed using Spearman's correlation of non-parametric data.

Qualitative Data

Greene Climacteric Scale

Assessment of climacteric symptoms was made at baseline and following 8 weeks of treatment and total scores were found to be significantly reduced ($p < 0.001$).

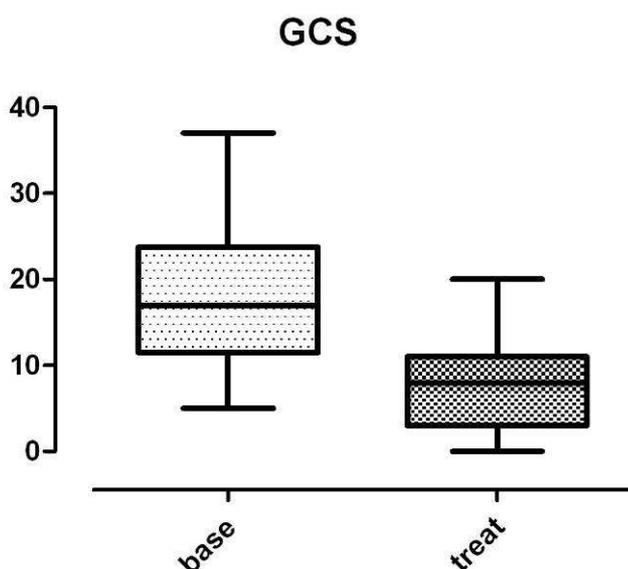


Figure 46: Greene Climacteric Scale
Data are shown as box and whisker plots with median, IQR and range.

Symptom scoring can be divided into three major independent groups (as described in detail in Chapter 2), vasomotor, somatic and psychological. Psychological symptoms can be further subdivided into symptoms relating to anxiety and those relating to depression. There is an additional item on loss of sexual interest, which is intended as a 'probe' item to be followed up by more appropriate and sensitive evaluation of problems in that area. When these independent groups were individually analysed before and after treatment, there was a significant reduction in all groups of symptoms ($p < 0.001$ and $p = 0.04$ in loss of sexual interest). However, the greatest reduction was seen in depression (70% decrease), with anxiety and total psychological scoring decreased by 66% and 68% respectively. Vasomotor symptoms decreased by 57%,

somatic scores by 52% and loss of interest in sex scores decreased by 30%. See figure 47.

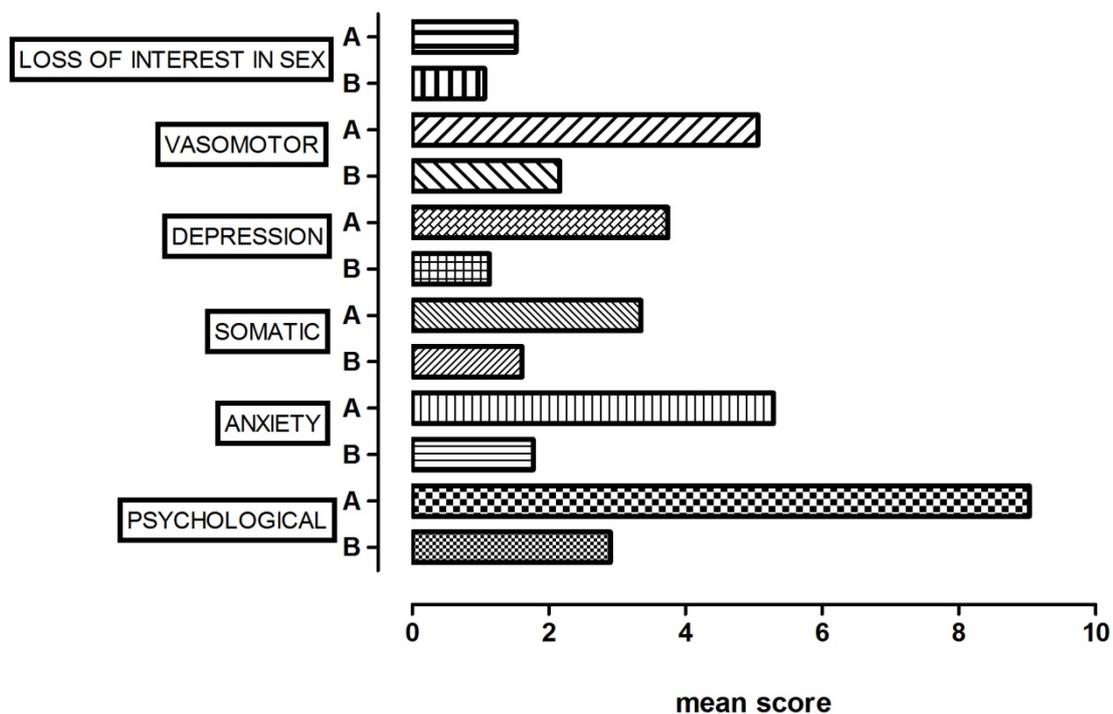


Figure 47: Deconstructed GCS

Data shown are scores for independent groups within Greene Climacteric Scale (psychological, anxiety, somatic, depression, vasomotor, loss of interest in sex), before treatment (A), and after treatment (B).

Becks Depression Inventory

Becks Depression Inventory assessments were also made at baseline and following treatment, total scores were reduced ($p < 0.001$) (See Figure 48). When deconstructed into the individual elements, highest scores were given to 'changes to sleep pattern' and 'loss of interest in sex' (see Figure 49). These symptoms have been described in detail in the climacteric and could be as a consequence of vasomotor symptoms, therefore these elements were removed and the analysis repeated. Scores were still significantly lower following 8 weeks of treatment ($p < 0.001$).

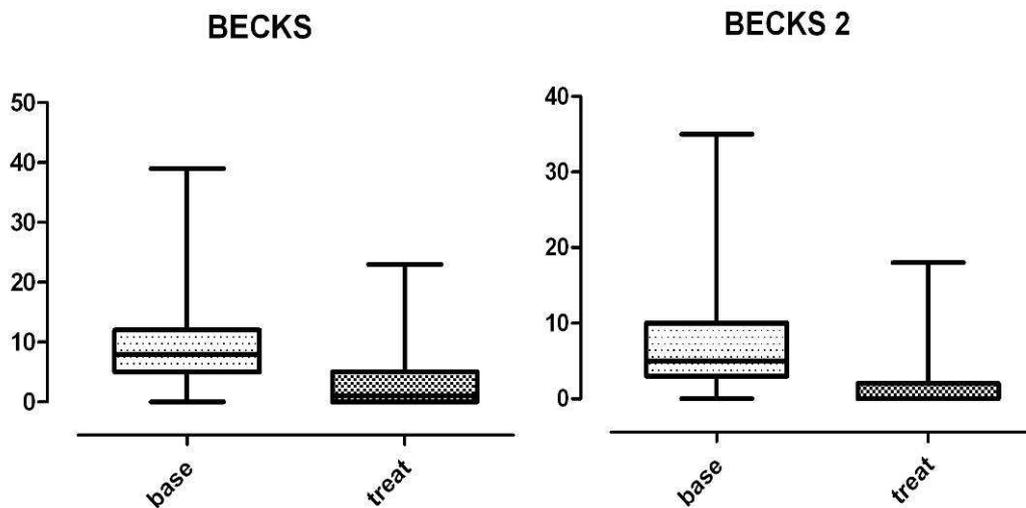


Figure 48: Becks Depression Inventory II. Becks 2 = Becks analysis with removal of 'changes to sleep pattern' and 'loss of interest in sex'. Data are shown as box and whisker plots with median, IQR and range.

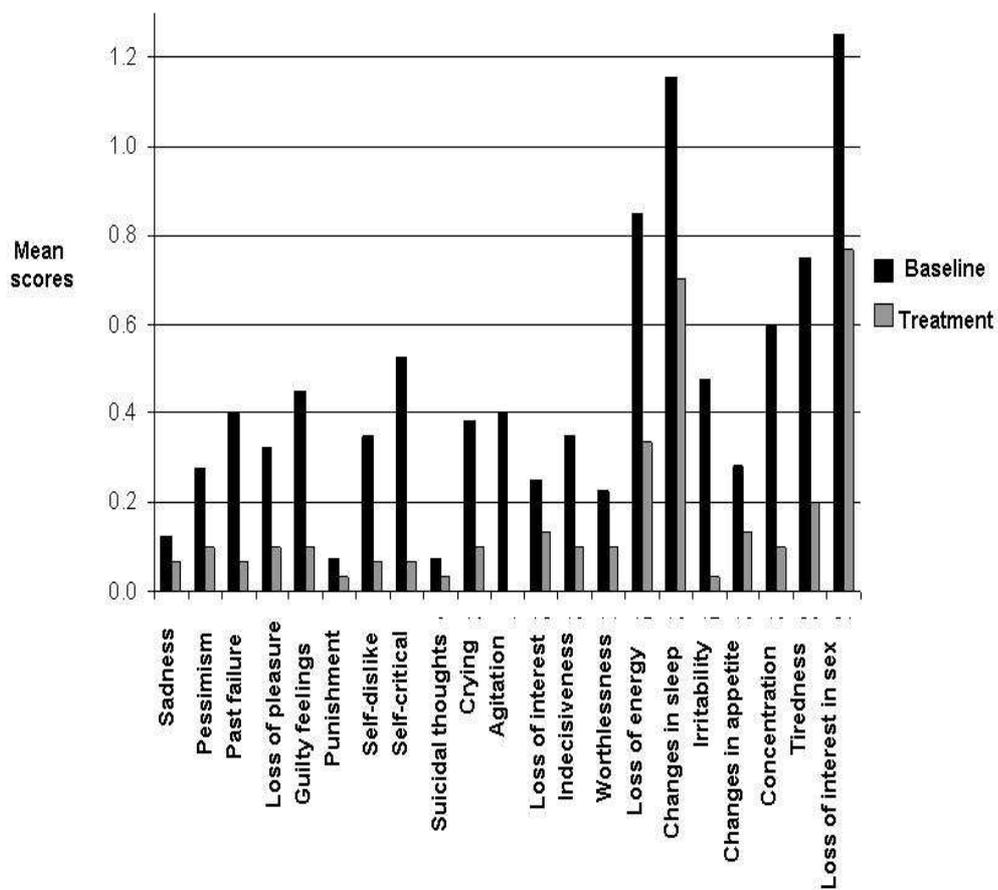


Figure 49: Deconstructed Becks Depression Inventory II

Hospital Anxiety and Depression Scale

An amendment to add the Hospital Anxiety and Depression Scale questionnaire to the protocol was submitted 22 June 2010 and a favourable opinion was granted 16 August 2010. There is evidence that low serotonin transporter availability is correlated with high anxiety (168) and Becks Depression Inventory II is not designed to assess anxiety as a separate entity from depression. In addition venlafaxine is used as an approved drug treatment for generalised anxiety disorder. HADS was first used in this study on 20 August 2010 and continued to be used for the remainder of the study. 15 participants completed this questionnaire.

Total scores were reduced following eight weeks of treatment ($p=0.01$).

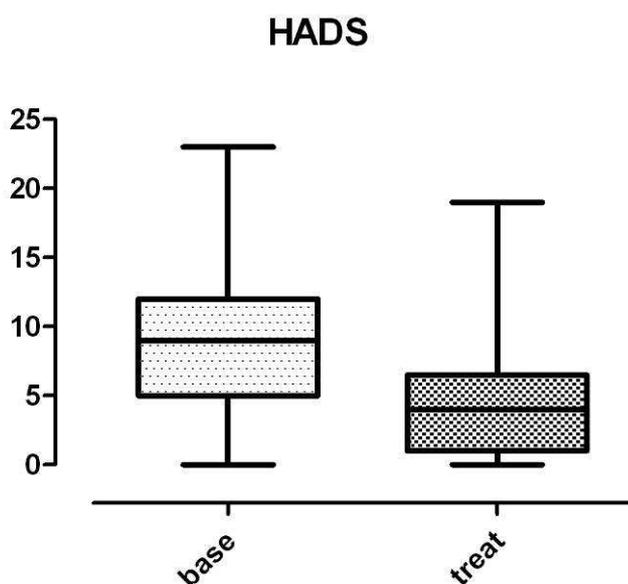


Figure 50: Hospital Anxiety and Depression Scale
Data are shown as box and whisker plots with median, IQR and range.

SPECT Imaging

The results illustrated in this section pertain only to those 14 individuals who underwent SPECT scanning.

Following 8 weeks of treatment with venlafaxine, there was a significant reduction in [^{123}I]-beta-CIT binding (see Figure 51 and 52), BDI scores, flush frequency and score, HFRDIS, NTA and ACh-AUC (see Table 12). The difference in SNP failed to reach statistical significance.

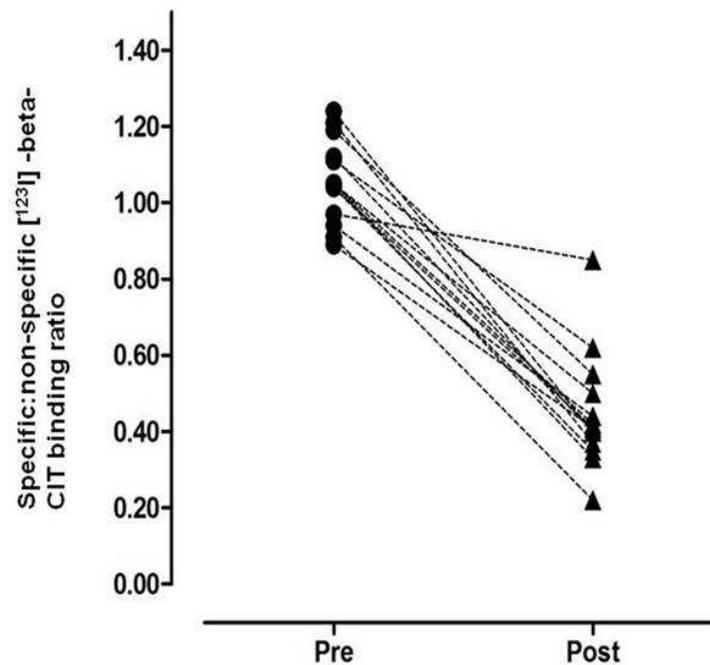


Figure 51: Specific:non-specific [^{123}I]-beta-CIT binding ratio in the brain Pretreatment (Pre) and following 8 weeks of treatment with venlafaxine 37.5mg bd (Post).

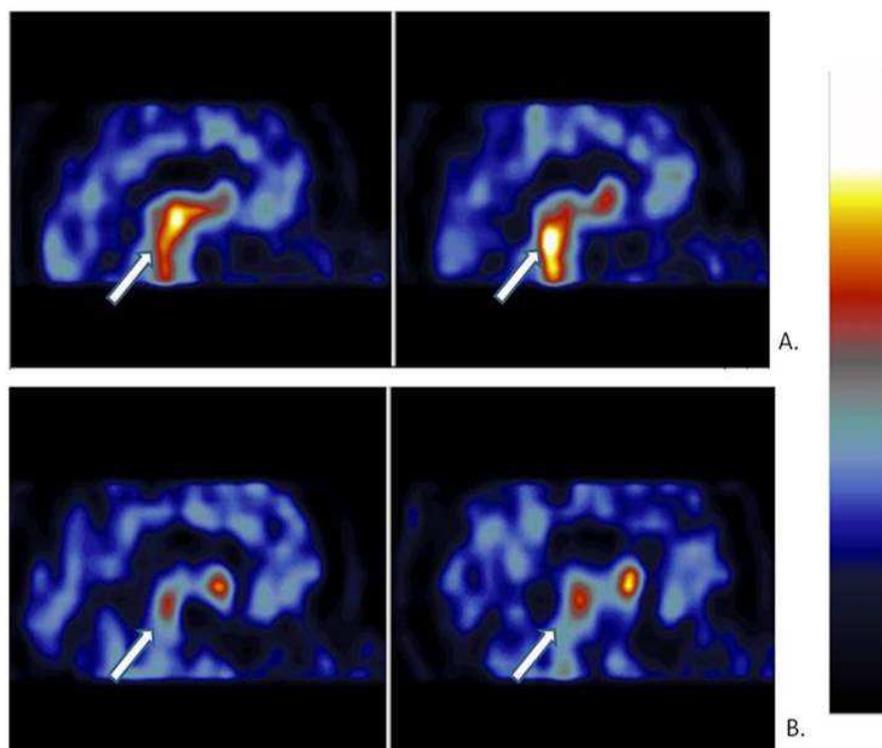


Figure 52: Sagittal brain [123I]-beta-CIT SPECT scan images at (A) baseline and (B) following treatment with venlafaxine. Images from 2 study participants, demonstrating a reduction in [123I]-beta-CIT binding ratio in the brainstem in the post-treatment scans (B) from the baseline scans (A). The arrows point to the brainstem and [123I]-beta-CIT uptake is depicted by the colour scale shown, where white is high uptake.

	Pre treatment (Mean; SD)	Post treatment (Mean; SD)	Mean difference (95% CI)	t	p	Cohen's d (effect size)
[¹²³I] beta-CIT	1.06 (0.11)	0.45 (0.16)	0.61 (0.50-0.71)	12.371	<0.001	3.45
BDI scores	9.21 (9.29)	4.14 (6.38)	5.07 (1.92-8.21)	3.487	0.004	1.09
Flushing frequency	9.88 (5.17)	4.92 (4.06)	4.96 (3.28-6.64)	6.388	<0.001	1.85
ACh AUC	5.86 (3.22)	3.56 (3.32)	2.30 (0.27-4.32)	2.459	0.029	0.66
SNP-AUC	3.63 (2.9)	2.95 (3.15)	0.67 (-0.78- 2.13)	1.004	0.334	0.27
Flush Score	18.20 (10.05)	8.6 (8.6)	9.55 (2.8-16.25)	3.080	0.009	0.82
HFRDIS	39.9 (26.32)	19.15 (19.77)	20.22 (11.28- 30.37)	4.714	<0.001	1.36
NTA	2.41 (1.2)	1.17 (0.93)	1.22 (0.27-0.62)	4.390	0.001	1.21

Table 12: Effect of treatment with venlafaxine on variables
t= paired t test. Cohen's d= corrected for dependence between means using Morris and DeShon's correction. BDI= Becks depression inventory. ACh-AUC= acetylcholine-area under the curve. SNP-AUC= sodium nitroprusside-area under the curve. HFRDIS= hot flush related daily interference score. NTA= night time awakening.

Next, a MANCOVA was performed to explore if the reduction in the [¹²³I]-beta-CIT binding ratio was associated with variance in other variables of interest, namely BDI reduction; flush frequency reduction; flush score reduction; Ach reduction; SNP reduction). Since age showed a negative correlation with BDI score reduction ($r = -0.38$); ACh-AUC reduction ($r=0.62$) and SNP reduction ($r=-0.65$), we included age as a nuisance covariate in the model. On the multivariate analysis, [¹²³I]-beta CIT reduction was associated with significant variance in BDI reduction ($r^2 = 0.54$; $p=0.004$), but not flush frequency reduction, flush score reduction, ACh-AUC reduction or SNP reduction (See Table 13).

Dependent variables	Parameter estimate (Beta)	t	p
BDI reduction	0.69	3.66	0.004 (0.02)
Flushing reduction	0.12	0.41	0.68 (0.68)
Ach-AUC reduction	-0.29	-1.35	0.203 (0.61)
SNP-AUC reduction	0.11	0.50	0.62 (0.68)
Flush score reduction	0.26	0.29	0.77 (0.78)

Table 13: Relationship between [¹²³I]-beta-CIT reduction and other dependent variables
Parameter estimates represent the strength of the relationship between the predictor and dependent variables. A positive beta value represents a positive association. Predictor variable: [¹²³I]-beta-CIT reduction; Nuisance covariate: Age; values in brackets corresponds to the p values corrected using Holm-bonferroni family wise error procedure. BDI= Becks depression inventory. ACh-AUC= acetylcholine-area under the curve; SNP-AUC= sodium nitroprusside-area under the curve.

Analysis of blood samples

Whole group analysis of blood samples was carried out, i.e. 39 participants at baseline and 29 participants following 8 weeks of venlafaxine.

Haematocrit

As the technique of LASER Doppler imaging is dependent on scattering of light by red blood cells, there is a theoretical confounder in haematocrit values if found to be significantly different in individuals. Therefore before samples of blood were spun, separated and stored, a sample of whole blood was taken and assessed for haematocrit as described in detail in Chapter 2. There was no difference detected in haematocrit in individuals between visit 2 (baseline) and visit 3 (treatment) (see figure 53).

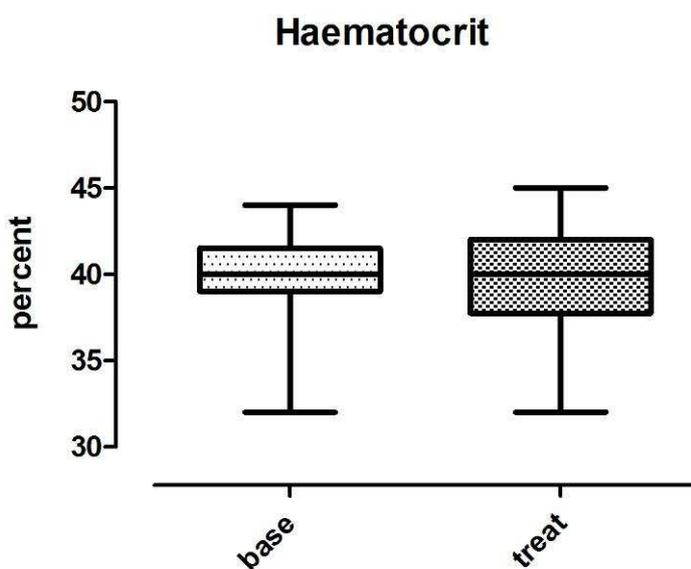


Figure 53: Haematocrit
Data are shown as box and whisker plots with median, IQR and range.

Plasma analysis

Plasma was analysed for HDL, LDL, Triglycerides, Glucose, total Cholesterol, Apolipoprotein A1 and B, ICAM and VCAM, TNF α , IL6, Adiponectin and CRP. Samples were taken at visit 2 before treatment was commenced (base) and following 8 weeks of treatment with venlafaxine (treat). The methods are described in detail in Chapter 2.

CRP was found to be significantly higher when measured after 8 weeks of treatment, when compared to samples taken at baseline. No other measures were found to be significantly different.

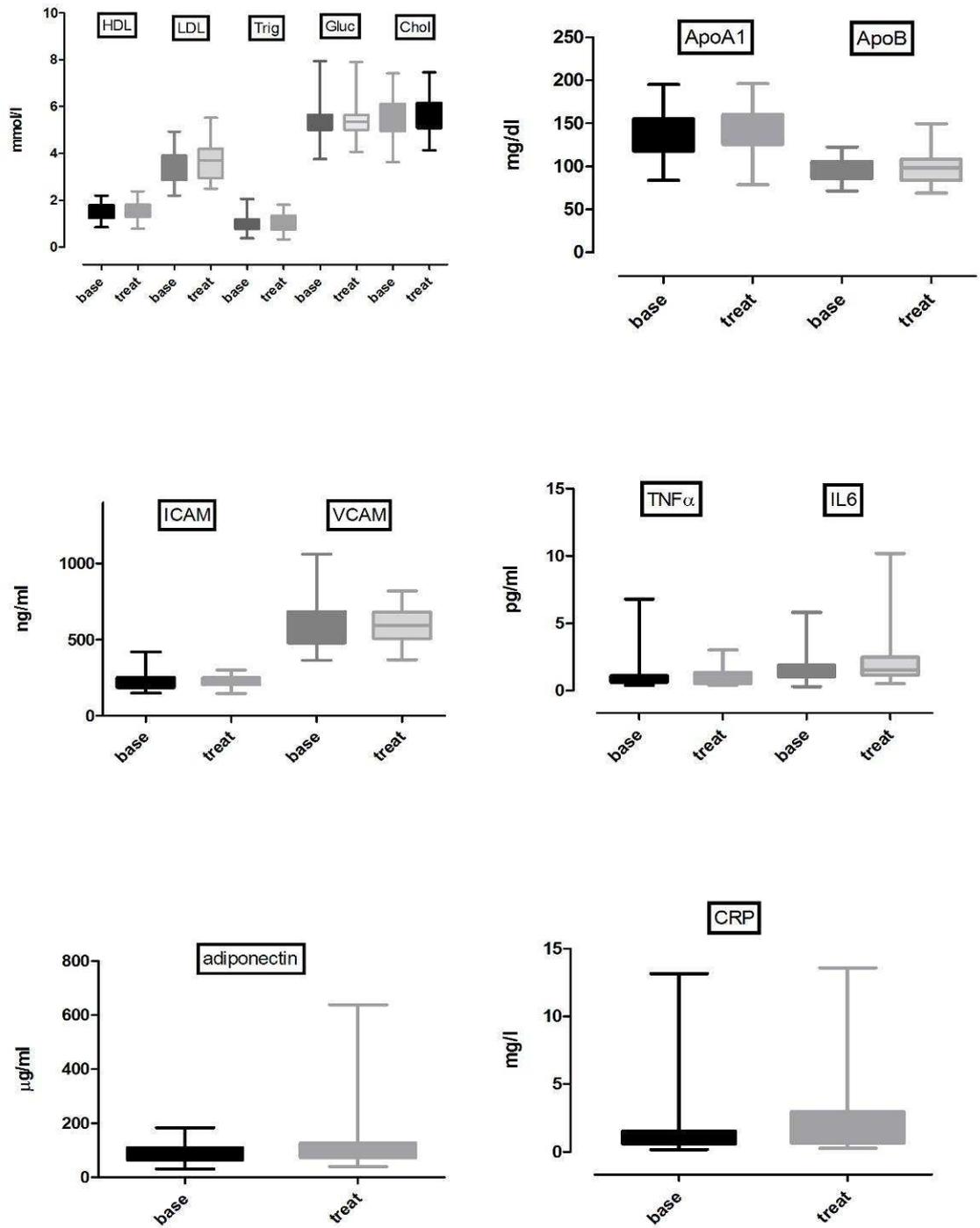


Figure 54: Plasma analysis
 Data are shown as box and whisker plots with median, IQR and range.

	Baseline (n=39)	Treatment (n=29)	P
HDL (mmol/l)	1.57 (1.25-1.80) [0.85-2.20]	1.52 (1.29-1.81) [0.79-2.39]	0.99
LDL (mmol/l)	3.30 (2.87-3.91) [2.20-4.94]	3.72 (2.95-4.20) [2.50-5.53]	0.08
Triglycerides (mmol/l)	0.96 (0.78-1.18) [0.38-2.06]	0.99 (0.75-1.35) [0.33-1.81]	0.72
Glucose (mmol/l)	5.36 (5.01-5.65) [3.76-7.95]	5.34 (5.01-5.63) [4.07-7.91]	0.85
Cholesterol (mmol/l)	5.4 (4.96-6.12) [3.62-7.42]	5.65 (5.09-6.16) [4.14-7.46]	0.18
ApoA1 (md/dl)	137.6 (117.6-155.5) [83.9-195.3]	142.7 (125.8-160.4) [78.7-196.1]	0.10
ApoB (mg/dl)	92.6 (85.9-105.7) [71.3-122.9]	98.3 (83.9-108.6) [68.9-149.7]	0.29
ICAM (ng/ml)	233.7 (186.6-251.6) [149.1-418.9]	223.0 (205.3-250.1) [146.0-300.4]	0.67
VCAM (ng/ml)	590.6 (479.2-684.5) [362.9-1060]	592.9 (506.8-679.6) [368.6-819.2]	0.67
TNF α (pg/ml)	0.8 (0.6-1.1) [0.4-6.8]	0.8 (0.5-1.3) [0.4-3.0]	0.81
IL6 (pg/ml)	1.3 (1.0-1.9) [0.3-5.8]	1.55 (1.1-2.5) [0.5-10.2]	0.16

Adiponectin (µg/ml)	79.04 (64.05-109.3) [30.96-183.6]	91.60 (72.05-127.2) [39.65-639.2]	0.05
CRP (mg/l)	1.02 (0.60-1.55) [0.17-13.16]	1.12 (0.66-2.99) [0.28-13.59]	0.008*

Table 14: Analysis of plasma at baseline and following treatment
Data shown as median (IQR)[range]. P values are determined by Wilcoxin Signed Rank analysis of paired data.

* Statistically significant result

Summary of Results

Perfusion response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Venlafaxine altered skin blood flow and vascular response to vasoactive agents, acetylcholine and sodium nitroprusside.

Venlafaxine and vasomotor symptoms

Venlafaxine improved both the number and severity of flushes, and also the number of times women are awakened at night as a result of flushes. Hot flush related daily interference scores were also improved following 8 weeks of treatment with venlafaxine.

Perfusion responses and their correlation with demographic and diary data

Diary data

No relationship was demonstrated in this group between perfusion responses to vasoactive agents and flush frequency, severity, night time awakening or HFRDIS. This was true for both baseline assessment and assessment following 8 weeks of treatment.

Demographic data

No correlation has been shown between perfusion responses to vasoactive agents and age, BMI or WHR.

Qualitative Data

Green Climacteric Scores, Becks Depression Inventory Scores, and Hospital Anxiety and Depression Scores were all reduced following 8 weeks of treatment with venlafaxine.

SPECT Scanning

Following 8 weeks of treatment with venlafaxine, there was a significant reduction in [¹²³I] -beta-CIT binding, BDI scores, flush frequency and score, HFRDIS, NTA and ACh-AUC.

[¹²³I] -beta CIT reduction was associated with BDI reduction.

Blood analysis

Haematocrit

No differences were seen within the group between baseline and treatment assessments.

Plasma analysis

CRP was found to be significantly higher when measured following treatment as compared to measurement at baseline. No other significant differences were determined.

Discussion

As already stated, oestrogen withdrawal is associated with decreased blood serotonin levels, which is returned to normal with oestrogen therapy (216) and it is well documented that serotonin has a role to play in thermoregulation.

We have shown that treatment with venlafaxine, designed to increase available serotonin at the serotonergic synapse, has altered the increased perfusion responses seen in flushing women.

Serotonin is widely accepted to be a potent vasoconstrictor. Serotonin receptors 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄, and 5-HT₇ are present on vascular smooth muscle cells and endothelial cells (221). These receptors regulate vascular tone by modulating contraction and relaxation of blood vessels, depending on the species, type of blood vessel and status of the endothelial cell layer (112).

It has been postulated that flushing may be more than a symptom of the climacteric and that it may indeed be a sign of underlying cardiovascular disease (180, 185). Our group has additionally shown that peri- and postmenopausal women have markers of an adverse cardiovascular risk profile when compared to matched non-flushing women (see Chapter 4) (169).

5-HT_{1A} receptors have been shown to be increased in atherosclerotic vessels, and activation of these in diseased vessels has been shown to cause vasoconstriction. Therefore, if those with flushing do have underlying cardiovascular disease, with which atherosclerosis and endothelial dysfunction is closely linked, perhaps the activation of 5-HT_{1A} receptors with increasing levels of available 5-HT, following treatment with venlafaxine, may play a role in the decreased vasodilatory response seen in endothelial measurements.

However, we may simply be demonstrating vasoconstriction in subcutaneous microvascular vessels as a result of increased activation of constrictor 5-HT receptors in healthy endothelium.

Subcontractile concentrations of 5-HT have also been shown to potentiate arteriolar contractile response to other vasoconstrictor agents, such as angiotensin II, noradrenaline and endothelin-1 (222, 223).

In addition to alterations in the perfusion responses, we demonstrated significant improvements in flush frequency, flush severity, night time awakening and hot flush related daily interference. However, this was not correlated with perfusion responses.

SSRIs have been shown in placebo controlled trials to be effective in improving flush frequency and severity. In a meta-analysis (120), paroxetine performed well, but two studies examining venlafaxine were conflicting. Loprinzi (218) demonstrated a significant reduction in flush score and flush frequency when compared with placebo, whereas Evans (224) reported no improvement in flush frequency but did report an improvement in quality of life. 51% of venlafaxine users had a reduction in a measure of how hot flushes interfere with daily living, compared with 15% of placebo users ($P < 0.001$).

We were also able to demonstrate significant improvements in hot flush related daily interference scores, as well as improvements in the Greene Climacteric Scale (GCS).

Interestingly the greatest reduction in GCS was in the depression scores, and we also found a significant reduction in BDI II and HADS scores. Our participants, however, were not depressed, and indeed a diagnosis of depression was amongst the exclusion criteria for the study.

There are many studies evaluating the risk of depression and low mood during the menopausal transition (225-229) and its relationship to changes in reproductive hormones, however data are not consistent. Depressed mood certainly appears amongst commonly reported menopausal symptoms along with hot flashes and disturbances in sleep pattern. Some believe that alterations in mood are the end result of endless flushes and night sweats, leading to poor sleep, exhaustion and ultimately low mood. Amongst our participants, some of the highest scores in BDI II were given to changes in sleep, tiredness and loss of energy (Figure 48). A recent study, however, found that amongst women who

reported symptoms of both hot flushes and depression, it was symptoms of depression that were more likely to present first (230). It might be suggested, however, that irrespective of which presented first, an improvement in flushing resulting in an improvement in sleep and energy levels would result in an improvement in depression scores.

Interestingly, however, there was no relationship demonstrated between decreases in BDI II scores and decreases in flush frequency or severity.

There was, however, a dramatic reduction in [^{123}I] -beta-CIT binding ratio post treatment with venlafaxine and this was associated with the reduction in BDI II scores.

Whilst the antidepressant efficacy of SSRIs has been reasonably well established, the relationship between SERT binding and antidepressant efficacy is less clear. There is also a substantial body of molecular imaging data demonstrating the effects of serotonergic antidepressants on SERT but the significance of this has not been determined. In this group, a percentage reduction in SERT was associated with a percentage reduction in BDI. While pharmacokinetic studies specify that 80% occupancy of SERT is a necessary minimum for SSRI treatment of depression, most studies have failed to show a direct relationship between antidepressant occupancy and treatment response (231). However more recent studies have found that treatment response in patients with Major Depressive disorder (MDD) treated with SSRIs was predicted by pre-treatment SERT binding in a number of key regions of depression including bilateral habenula, amygdala-hippocampus complex and subgenual cingulate cortex in relation to SERT binding in the median but not dorsal raphe nucleus (232). It should also be noted that the mean pre-treatment scores on the BDI were in the normal range. This was not expected since antidepressants have been shown to be most efficacious in severe depression (233) and the women in our study were not considered depressed on recruitment.

Whether the relationship we demonstrate are related physiologically (mechanistically) is unclear. It could be argued that the reduction in BDI scores was secondary to a reduction in flushing. However we found no association

between BDI reduction and flushing frequency reduction or flush score reduction and so this explanation seems less likely.

This might suggest that mood and vasomotor symptoms are discrete entities with some overlap, and altered as such with treatment with venlafaxine. It also suggests that venlafaxine may be important in mood alteration beyond its use as a treatment for moderate to severe depression.

This study has focused on central SERT as an index of the central serotonergic contribution to postmenopausal flushing and the peripheral vascular response to vasoactive agents following treatment to block SERT. Although we have not measured either peripheral (platelet) SERT or serum serotonin, these data appear to demonstrate a separation of effects of venlafaxine in the periphery and the centre.

Chapter 6

HRT

Introduction

In this study, it has been demonstrated that postmenopausal women with severe flushing have increased cutaneous microvascular perfusion responses to vasoactive agents when compared to their non-flushing contemporaries. It has also been shown that successful treatment with venlafaxine decreases this increased response.

However, the most commonly used treatment for vasomotor symptoms in the climacteric and post-menopause is hormone replacement therapy (HRT). We know that the overwhelming change occurring at the time of the menopause and during the climacteric is reduction in production of oestrogen by the ovaries as a result of follicle depletion, and we know that replacing oestrogen is a most effective treatment for flushes.

As already stated, it is thought that a hot flush is the result of a narrowed thermoregulatory zone within the hypothalamus, resulting in heat loss mechanisms being triggered by only small increases in core temperature (61). There is uncertainty surrounding the trigger for this narrowing, however it has been suggested that imbalances in various neurotransmitters might be responsible and this has been discussed in chapter 1 and chapter 4. It has been further suggested that a reduction in oestrogen concentrations could result in imbalances of these neurotransmitters (57).

In humans, cutaneous circulation of the head, limbs and trunk is controlled by two types of sympathetic neurones: adrenergic neurones control cutaneous vasoconstriction and cholinergic neurones control vasodilation (172). Skin blood flow changes in during the menstrual cycle in women suggest that female sex steroids modulate the sensitivity and responses of these circuits (234).

Whilst these circuits might control the response, the effectors of vasodilation include the endothelium, and there is considerable evidence that oestrogen influences the endothelium. Oestrogen receptors ER α and ER β have been demonstrated to be present on endothelial cell plasma membrane (235, 236) and studies consistently demonstrate that oestradiol binding to ER α can induce endothelium-dependent vasodilation in vivo. While the classical model of

steroid action involves a ligand that is bound to steroid receptors then binding to specific response elements on target genes to regulate gene transcription, more recently focus has been on the pathways involved in non-genomic oestrogen-induced activation of endothelial nitric oxide synthase (74), however the role of ERB is less clear.

Cutaneous vasodilatation is one of the hallmarks of flushing and in the rat, where cutaneous vasodilatation of the tail is a primary mechanism of thermoregulation; ovariectomy has been shown to increase tail skin temperature, and treatment with oestrogens can abolish this effect (197).

What, then, is the effect of treatment with oestrogen (HRT) on increased cutaneous vasodilatation in postmenopausal flushing women?

Hypothesis

The altered endothelial function of women who flush is reversed by successful treatment of the flushes by HRT. This is reflected in altered skin blood flow and vascular response to vasoactive agents, acetylcholine and sodium nitroprusside.

METHODS

Participants

A total of 15 women who were commencing hormone replacement therapy were recruited.

Recruitment of volunteers followed Scottish media coverage, as well as from gynaecology and menopause clinics. A number of women were also recruited from the West of Scotland Breast Screening Centre in Glasgow.

Participating women were all aged 45-65 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function. Menopausal status was determined by either an FSH greater than 20 Units/Litre or amenorrhoea for 1 year or longer.

Study Design

Study participants were all seen at baseline when skin blood flow was assessed using LASER Doppler imaging (LDI) with iontophoresis (ION) of vasoactive compounds. Participants returned for repeat assessment following 8 weeks of treatment with hormone replacement therapy. Qualitative measures were obtained at the same time points.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 09/MRE00/40). All patients gave written informed consent.

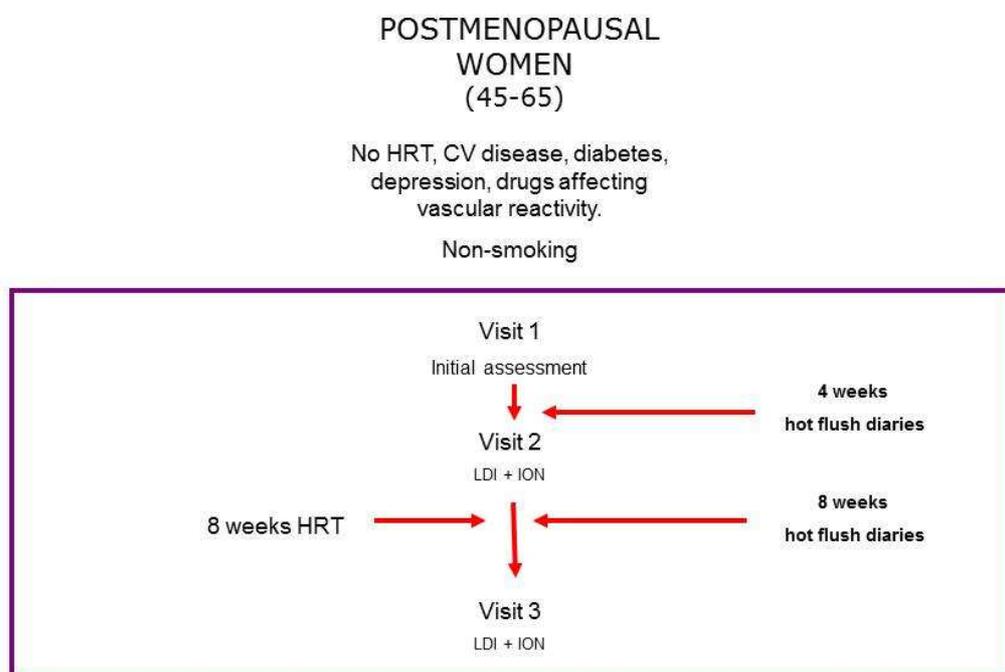


Figure 55: Study design

Body composition

Measurements of body mass, height, and blood pressure were made as described in Chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made at baseline and after 8 weeks of treatment with hormone replacement therapy. This technique is described in detail in Chapter 2.

Hot flush diary

Each participant was asked to keep a 'Hot Flush' diary for 4 weeks prior to initial assessment and throughout the study, as described in detail in Chapter 2.

Qualitative Measurements

Greene Climacteric Scale

Each participant completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Becks Depression Inventory

Each participant completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Hospital Anxiety and Depression Scale

Each participant who participated in the study after 16 August 2010 completed this questionnaire at visit 2 and visit 3 as described in Chapter 2.

Plasma analysis

A fasting blood sample was taken from each participant using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetra-acetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

A small volume was removed from the 6ml lithium heparin sample before centrifuge and storage as described in Chapter 2.

ICAM, VCAM, TNF - α , IL-6, Insulin and Adiponectin were measured by ELISA technique as described in Chapter 2.

CRP, Cholesterol, Glucose, HDL, LDL, and Trig were measured by standardised methods at the SAS laboratory for cardiovascular biomarkers at The Institute of Cardiovascular and Medical Sciences at Glasgow University, as described in Chapter 2.

Statistical analysis

Measurement of vascular responses was performed using raw values.

Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis

Diary data, HFRDIS and qualitative data were compared using Wilcoxin Signed Rank test of paired non-parametric data. Diary data included analysis of mean number of flushes per day, hot flush score and night time awakening.

Results

15 women with severe hot flushing were recruited. Following recruitment, 2 women declined continued participation in the study; 13 women attended for baseline assessment, however a further 2 women did not attend for treatment LDI + Ion (see Figure 56). Complete demographic and perfusion data are available for 11 participants (see Table 17).

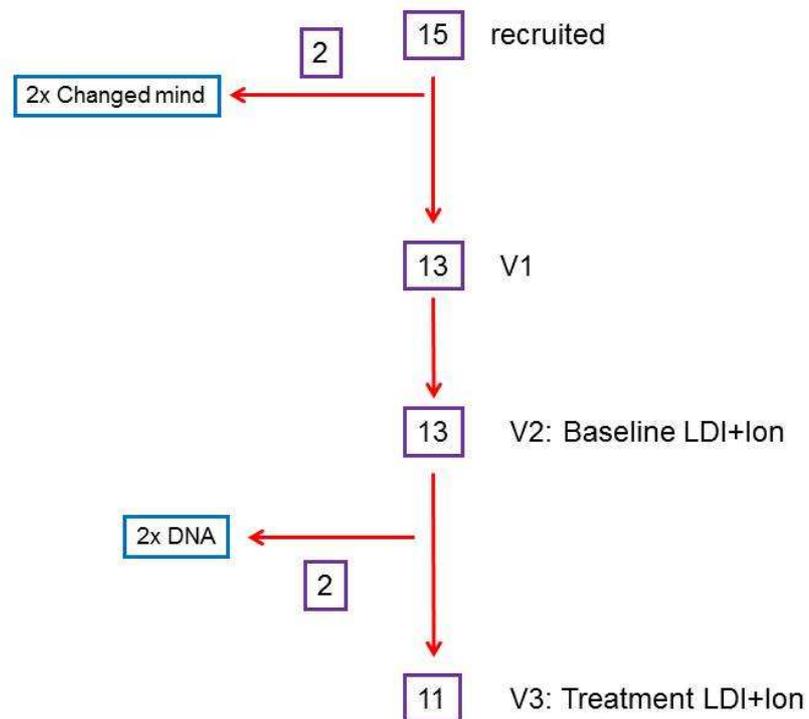


Figure 56: Recruitment and Participant numbers

Demographic characteristics

n	11
Age	52 (51-54)[48-58]
Years since LMP	3.5 (0.5-16.8)[0.1-20]
BMI	30.1 (24.5-35.9)[19.4-69.5]
Parity	1 (0-2)[0-3]
*SBP	138.7 ± 14.5
*DBP	84.5 ± 13.2

Table 15: Demographic characteristics

Data are median (interquartile range)[range]. All women are non-smokers. *Data are mean ± SD

Responses to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Following 8 weeks of hormone replacement therapy, enhanced vascular responses were seen following administration of ACh ($p < 0.001$) and SNP ($p = 0.01$).

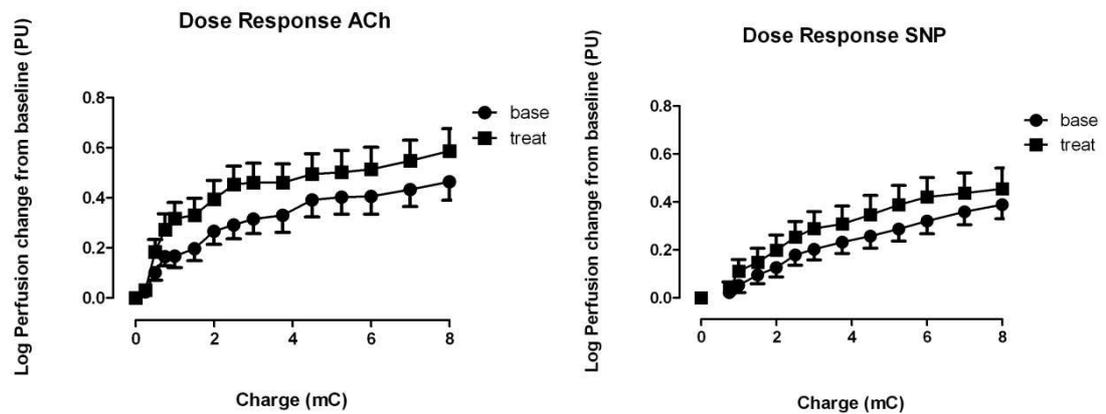


Figure 57: Dose response curve

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in control women compared with flushing women. $P < 0.001$ and $P = 0.01$ respectively. Data are mean \pm SEM.

Hot flush diaries

Only 5 participants completed baseline diary data, the overwhelming reason for this was an urgency to commence treatment. All 11 participants who returned for LDI + Ion assessment following 8 weeks of treatment had completed a full complement of treatment diaries.

Diary data was calculated as follows; flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed.

Data is presented as median (interquartile range)[range] as the data were non-parametric. Statistical comparisons were therefore made by Wilcoxin Signed Rank Analysis of matched pairs, but also by Mann Whitney-U analysis* as a result of limited complete pairs of data.

Treatment with HRT did not appear to significantly reduce the number of, or severity of, flushes, the frequency of night time awakenings, or the perception of daily hot flush interference (see Table 16 and Figure 58).

	Baseline n=5	Treatment n=11	P
Flush frequency (mean flush/day)	8.1 (1.7-11.0) [1.0-12.9]	2.5 (0.7-4.5) [0.4-12.0]	0.31 *0.17
Night time awakening (NTA)	2.3 (0.9-5.3) [0.6-7.6]	0.95 (0.3-2.3) [0.1-4.1]	0.13 *0.15
Hot flush related daily interference score (HFRDIS)	60.0 (35.0-77.8) [20.0-83.7]	29.4 (16.7-48.5) [3.0-68.6]	0.13 *0.10
Hot flush score	20.0 (2.3-29.7) [1.8-38.6]	4.8 (0.9-11.4) [0.4-32.9]	0.31 *0.21

Table 16: Hot flush diary data

Flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed. Placebo and Clonidine weeks, diaries were completed for 4 weeks each, and baseline diaries were completed for 3 weeks.

Data are P values determined by Wilcoxin Signed Rank analysis of paired data. *p values by Mann Whitney test.

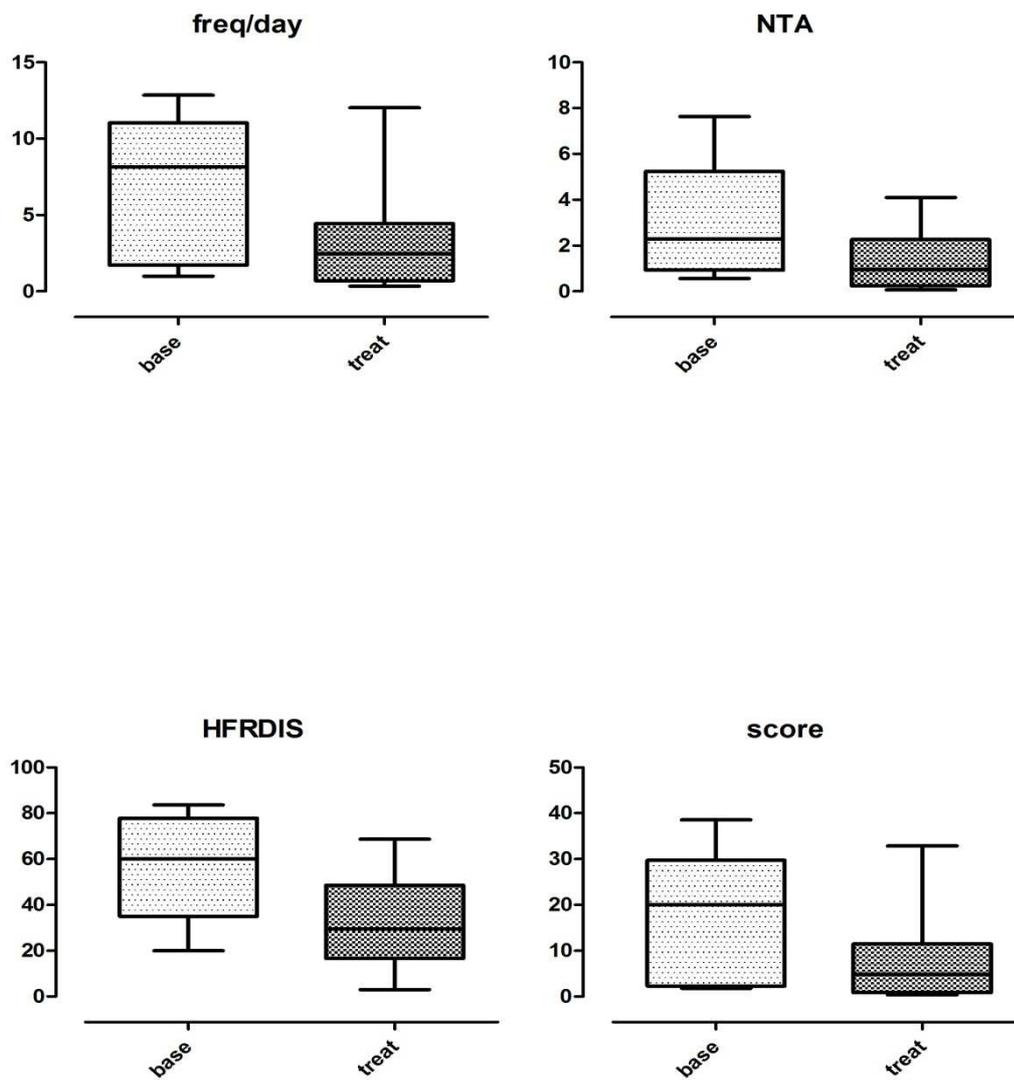


Figure 58: Diary data
Data are shown as box and whisker plots with median, IQR and range.

Qualitative Data

Unlike diary data, qualitative data were completed at assessment visits and for this reason is available for all participants.

Greene Climacteric Scale

Assessment of climacteric symptoms, as described by J.G. Greene (see Chapter 2), were made at baseline and following 8 weeks of treatment and total scores were reduced ($p=0.003$).

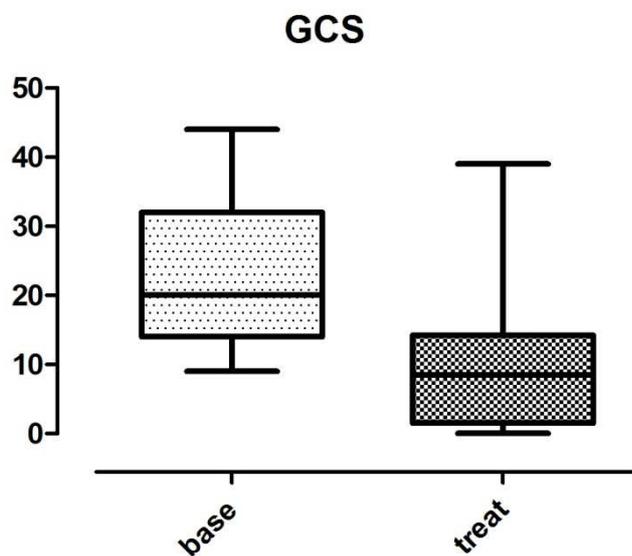


Figure 59: GCS
Data are shown as box and whisker plots with median, IQR and range.

Becks Depression Inventory

Becks Depression Inventory assessments were also made at baseline and following treatment, total scores were reduced ($p=0.02$) (See Figure 60).

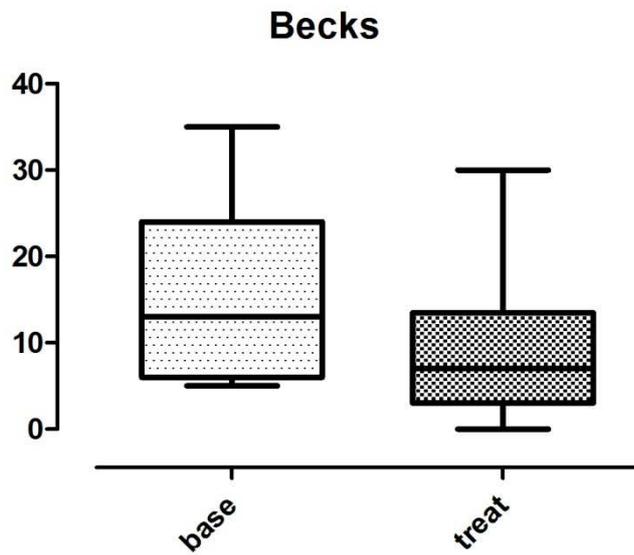


Figure 60: Becks Depression Inventory II
Data are shown as box and whisker plots with median, IQR and range.

Hospital Anxiety and Depression Scale

Becks Depression Inventory II is not designed to assess anxiety as a separate entity from depression. Therefore an amendment to ethical approval was sought and granted on 16 August 2010 to include Hospital Anxiety and Depression Scale (HADS). Each participant who participated in the study after this date completed this questionnaire at visit 2 and visit 3.

Total scores were reduced following 8 weeks of treatment with HRT ($p=0.01$) (see Figure 61).

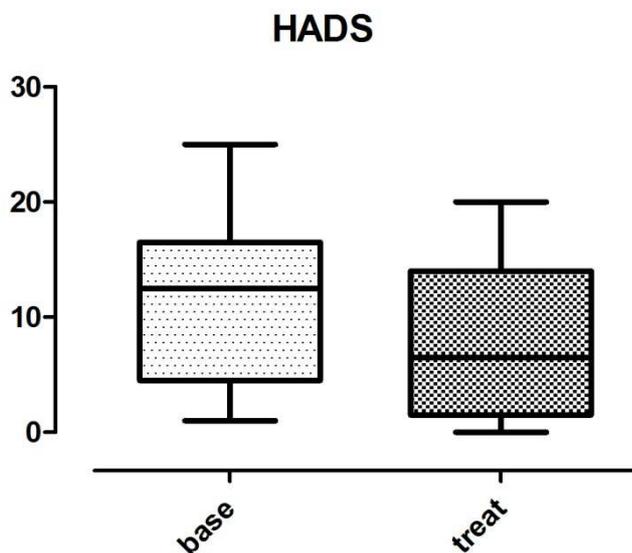


Figure 61: Hospital Anxiety and Depression Scale
Data are shown as box and whisker plots with median, IQR and range.

Blood analysis

Serum haematocrit was analysed as described in Chapter 2, there was no difference observed between baseline and treatment.

Plasma was analysed for HDL, LDL, Triglycerides, Glucose, total Cholesterol, Apolipoprotein A1 and B, ICAM and VCAM, TNF α , IL6, Adiponectin and CRP. Samples were taken at visit 2 before treatment was commenced (base) and following 8 weeks of treatment with HRT (treat). The methods are described in detail in Chapter 2.

ApoA1 and HDL were found to be significantly lower when measured after 8 weeks of treatment ($p=0.004$ for both), when compared to samples taken at baseline. No other significant differences were observed. See Figure 62.

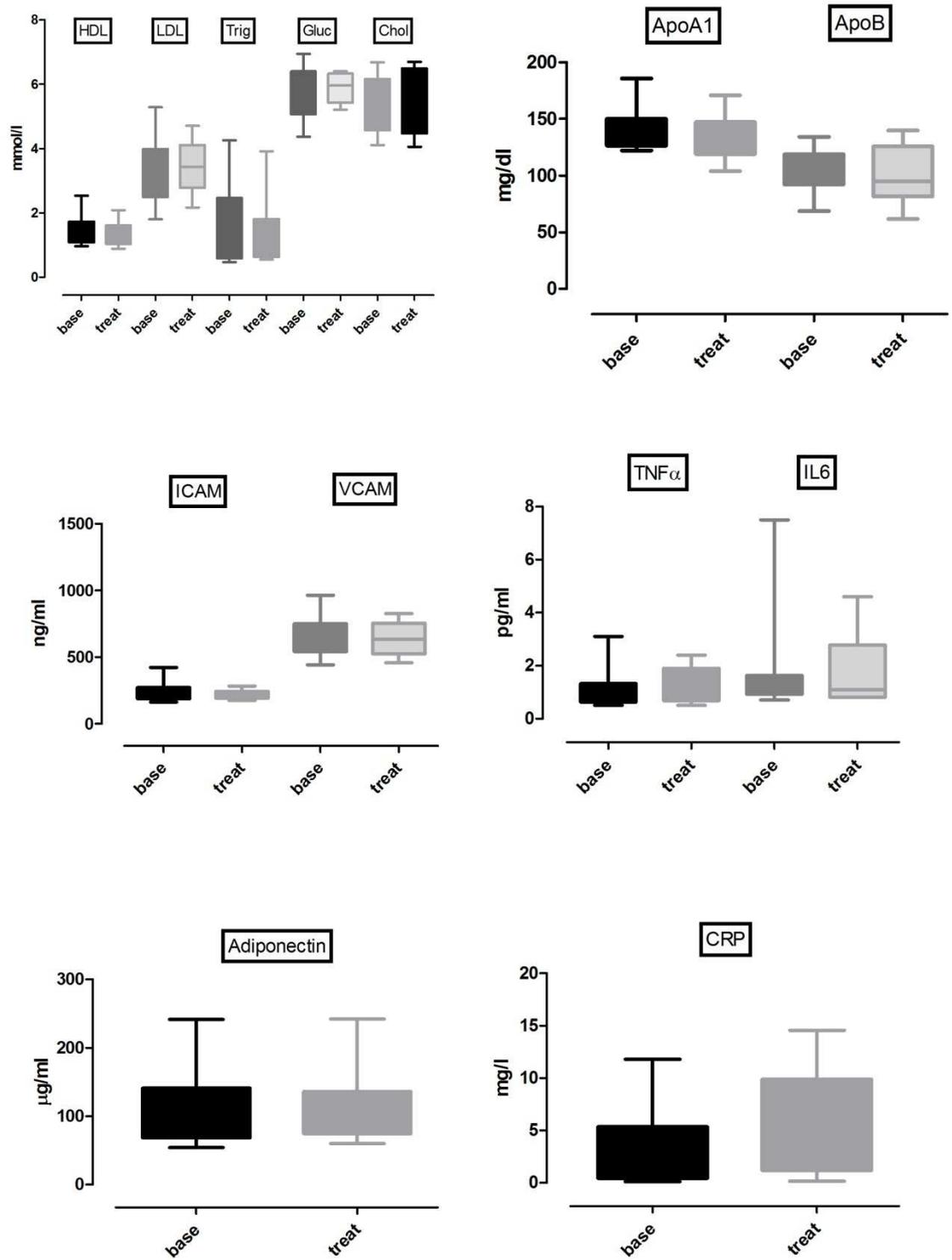


Figure 62: Plasma analysis

Data are shown as box and whisker plots with median, IQR and range.

Summary of results

Perfusion response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

HRT increased the cutaneous microvascular response to vasoactive agents, acetylcholine and sodium nitroprusside.

Qualitative Data

Green Climacteric Scores, Becks Depression Inventory Scores, and Hospital Anxiety and Depression Scores were all reduced following 8 weeks of treatment with HRT.

Blood analysis

Haematocrit

No differences within the group between baseline and treatment assessments.

Plasma analysis

ApoA1 and HDL were found to be significantly lower when measured after 8 weeks of treatment with HRT. No other significant differences were determined.

Discussion

In this study cutaneous microvascular perfusion responses to vasoactive agents are increased following 8 weeks of treatment with hormone therapy. If postmenopausal women with severe flushing have an increased risk of cardiovascular disease (see Chapter 3), improved cutaneous microvascular perfusion with treatment with HT may indicate 'better' endothelial function and a lower cardiovascular risk.

Endothelial dysfunction, characterised by a failure to vasodilate, or indeed to vasoconstrict, in the presence of endothelial nitric oxide, is a significant predictor of cardiovascular (cv) events. Oestrogen was thought to be cardioprotective (237), perhaps because of the disparity in cv-related deaths between men and women before midlife. The Heart and Estrogen/Progestin Replacement Study (HERS) (238) and Women's Health Initiative (WHI) publications failed to demonstrate a protective effect, and in fact, WHI showed a nonsignificant increase in risk associated with conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA) (239), but this was in women starting treatment some 12 years after the menopause. Many of these women would have had significant asymptomatic atherosclerosis, with adverse effects of oestrogen on thrombosis and inflammation (240).

More recent clinical trial data have found no adverse effect or any beneficial effect on atherosclerosis progression with HT versus placebo when it is employed in recently menopausal women (241, 242). However, it is plausible based on studies in nonhuman primates that initiating hormone therapy (HT) at the time of menopause may be cardioprotective (243). Indeed, it has been suggested that there may be a critical window, most likely the late perimenopausal transition during which HRT may be of benefit in the modification of cardiovascular disease risk. The Danish Osteoporosis Trial (DOPS trial) supports this timing hypothesis (window of opportunity); data demonstrated a significant reduction in coronary heart disease (CHD) and mortality in women who were randomised to HRT before age 60 or within 10 years of the menopause (244).

HT has also been shown to have beneficial effects on vascular relaxation in post-menopausal women with type 2 diabetes (85). In general, it is accepted that oestrogens promote cutaneous vasodilation (83). Oestrogen receptors ER α and ER β have been demonstrated to be present on the surface of endothelial cells and the classical model of steroid action involves binding to specific response elements on target genes to regulate gene transcription, with effects observed in hours to days (74). However, over the last 2 decades it has been shown that sex steroids can regulate cell function through the rapid activation of multiple signalling cascades and second messenger systems. More recently, focus has been on non-genomic sex-steroid signalling and one of the best-characterised examples is the activation of endothelial nitric oxide synthase (eNOS) in endothelial cells by oestradiol (84).

In addition to this direct modulation of vascular function through regulation of endothelium-derived factors (nitric oxide), sex steroid hormones can also regulate intracellular calcium and calcium sensitivity of the vascular smooth muscle. This vascular smooth muscle is innervated by efferent autonomic neurones, primarily sympathetic. Consequently alterations in sympathetic vasoconstrictor activity and the transduction of this activity into vasomotor tone have significant effects on the regulation of blood flow; this may be the explanation for the vasodilation in the skin blood vessels (130).

Hart et al suggest 3 sites at which sex hormones could influence the transduction of nerve activity into changes in vascular smooth muscle tone; 1) decreased synthesis and release of the main neurotransmitter, noradrenaline by inhibition of tyrosinase and tyrosine hydroxylase activity and decreased tyrosine by 17 β -oestradiol, 2) decreased non-neuronal degradation of noradrenaline by competitive binding of catecholestrogens to catechol-O-methyltransferase (COMT) in vascular smooth muscle thereby increasing noradrenaline in the synaptic cleft and the amount available to be removed by capillaries and 3) post-junctional activation of adrenergic receptors on the smooth muscle (130).

The fall in oestrogen levels at menopause, therefore, in addition to the impact of NO release, may have its thermoregulatory effect directly by receptor binding (245) or indirectly by non-receptor mediated effects.

The lack of effect on flush frequency and severity is likely to be as result of limited diary data; it is well accepted that HT is effective at reducing flush frequency and severity when compared to placebo (199), and GCS data in this study, support this.

Chapter 7

Obesity

Introduction

In the past it was thought that overweight women flushed less because androgen is converted to oestrogen in adipose tissue (246). Dubbed the 'thin hypothesis', thinner women with less adipose tissue should therefore have more hot flushes and overweight women should be afforded some protection secondary to higher levels of oestrogen. However, findings from multiple large epidemiological studies have challenged this hypothesis. Evidence now suggests that overweight women have 1.5 to 2.0 times increased odds of reporting hot flushes and this risk increases with severity of obesity (247).

Women tend to progressively gain weight over much of their adult lives. According to the Study of Women's Health across the Nation (SWAN), the Healthy Women Study, women on average can expect to gain 1.5 pounds (680g) a year in their 40s and 50s. In 2006, 24 per cent of adults (aged 16 or over) in England were classified as obese. This represents an overall increase from 15 per cent in 1994.

Women are more likely to be morbidly obese than men (3 per cent compared to 1 per cent) and to have a raised waist circumference (41 per cent and 32 per cent respectively). Using both BMI and waist circumference to assess risk of health problems, particularly cardiovascular disease and diabetes, 14, 16, and 23 per cent of the women are at increased risk, at high risk and at very high risk respectively (248).

Despite the magnitude of this health problem, it is still unclear why obesity is linked to an increased risk of hot flushes.

Body fat is, amongst other things, an insulator. In response to heat stress, individuals with higher body fat show greater elevations in core body temperature. In response to cold challenge, they show slower reductions in core body temperature than do their leaner counterparts. Subcutaneous adiposity (the fat between skin and the abdominal muscle wall) rather than visceral abdominal adiposity (the fat behind the muscle wall and in the peritoneal space around the organs) is probably most related to hot flushes according to one study (54). Thurston suggests that this subcutaneous adiposity, which is

especially insulating, may prevent the heat dissipation action of flushes and heavier women may require to have more dissipation events to dissipate a given amount of heat (247).

In early menopausal transition, obesity has been linked to lower levels of E_2 (249) and inhibin B (250), chronic menstrual dysfunction (251), and anovulatory cycles (252). It has also been suggested, however, that women who developed hot flushes entered the menopausal transition with high oestrogen levels which dropped over time, but that women with low to average oestrogen levels did not develop hot flushes (253).

In addition to low levels of E_2 , postmenopausal women with high body mass index (BMI) have also been shown to have significantly higher levels of testosterone and lower total oestrone, progesterone and sex hormone binding globulin (SHBG) (124). This is surprising because adipose tissue produces and stores oestradiol. Circulating oestrogen in blood is lower even if adipose tissue levels are elevated and it is this that enters the brain and the thermoregulatory centres, which govern hot flushes (254). When we consider that the leading model of hot flushes is thermoregulatory dysfunction governed by the hypothalamus, circulating oestrogen that is capable of breaching the blood-brain barrier is certainly likely to be of more interest than adipose tissue production and storage.

Obesity also significantly increases the risk of cardiovascular disease (CVD), which is linked to impaired endothelial function. Adipose tissue excess is associated with a continuous production of mediators that impair insulin action (insulin resistance), which in turn is associated with type 2 diabetes, hypertension, dyslipidaemia, and altered coagulation/fibrinolysis, all of which create a state of constant and progressive damage to the vascular wall leading to endothelial dysfunction (129).

The endothelium plays a vital role in vascular homeostasis, including vascular tone regulation. Obesity associated with endothelial dysfunction is characterised by decreased availability of NO. Tesouro et al found that patients with metabolic syndrome secondary to obesity have impaired forearm vasodilator response to acetylcholine (255). Flow mediated dilation (FMD) of the

brachial artery has also been used to demonstrate that weight loss in extremely obese patients leads to an improvement of endothelial function.

It has already been shown that there is an increase in perfusion responses in those postmenopausal women who flush when compared to their matched contemporaries, but these were all lean women (see Chapter 3). A group of women with BMI>30 were examined to determine whether the increased incidence of flushing in obese postmenopausal women might be due to altered endothelial function.

Hypothesis

Vasomotor symptoms in obese postmenopausal women are due to altered endothelial function as assessed by peripheral microvascular reactivity.

Methods

This chapter describes a sub-analysis comparison of women with a BMI greater than or equal to 30 to women with a BMI of less than 30. These women were all recruited to either group 1 (see Chapter five; treated with venlafaxine) or group 2 (see Chapter six; treated with HRT) of the study.

Participants

15 women with a BMI greater than or equal to 30 were included in this sub-analysis. 9 of these women were in Group 1 and 6 were in Group 2. The results presented in this chapter will compare those women with a BMI \geq 30 to those women with a BMI $<$ 30. See Figure 63.

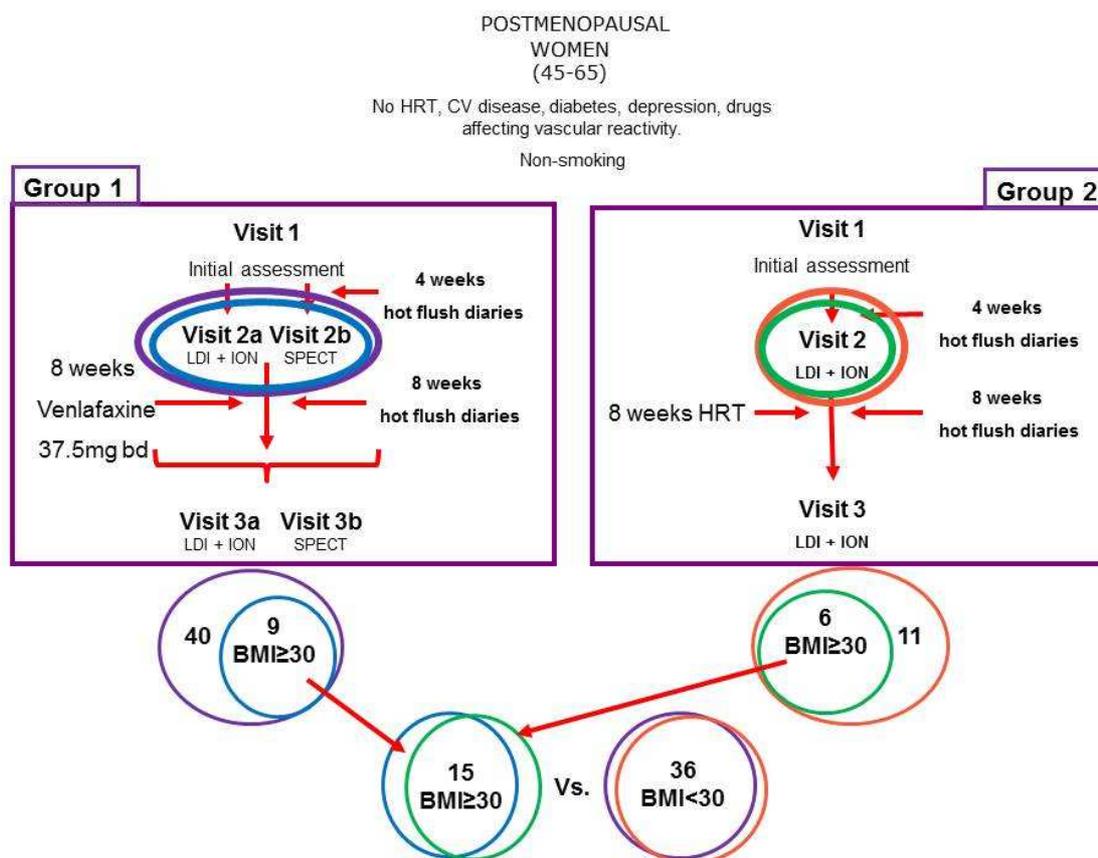


Figure 63: Participants

Purple circle represents total number of women seen at visit 2 in group 1 (venlafaxine), blue circle within represents those women with a BMI \geq 30 in the same group. Orange circle represents total number of women seen at visit 2 in group 2 (HRT), green circle within represents those women with a BMI \geq 30 in the same group. Total participants with BMI \geq 30 is 15. 36 women at visit 2 have a BMI $<$ 30.

Study Design

Study participants were all seen at baseline when skin blood flow was assessed using LASER Doppler imaging (LDI) with iontophoresis (ION) of vasoactive compounds. Qualitative measures were obtained at the same time points.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 09/MRE00/40). All patients gave written informed consent.

Body composition

Measurements of body mass, height, and blood pressure were made as described in Chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made using LASER Doppler imaging with iontophoresis. This technique is described in detail in Chapter 2.

Hot flush diary

Each participant was asked to keep a 'Hot Flush' diary for 4 weeks prior to assessment, as described in detail in Chapter 2.

Qualitative Measurements

Greene Climacteric Scale

Each participant completed this questionnaire as described in Chapter 2.

Becks Depression Inventory

Each participant completed this questionnaire as described in Chapter 2.

Hospital Anxiety and Depression Scale

Each participant who participated in the study after 16 August 2010 completed this questionnaire as described in Chapter 2.

Plasma analysis

A fasting blood sample was taken from each participant using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetraacetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

A small volume was removed from the 6ml lithium heparin sample before centrifuge and storage as described in Chapter 2.

ICAM, VCAM, TNF - α , IL-6, Insulin and Adiponectin were measured by ELISA technique as described in Chapter 2.

CRP, Cholesterol, Glucose, HDL, LDL, and Trig were measured by standardised methods at The Institute of Cardiovascular and Medical Sciences at Glasgow University, as described in Chapter 2.

Statistical analysis

Measurement of vascular responses was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis

Demographic data were compared using Student's t-test to compare blood pressure and Mann Whitney U test for non-parametric data.

Diary data, HFRDIS and qualitative data were compared using Wilcoxon Signed Rank test of paired non-parametric data. Diary data included analysis of mean number of flushes per day, hot flush score and night time awakening.

Results

Demographic Characteristics

There were 36 women with a BMI<30 and 15 women with a BMI≥30.

	BMI<30	BMI>30	P
n	36	15	
Age (years)	56 (53-63) [46-66]	54 (51-58) [45-63]	0.14
Years since LMP	7 (1-11) [0.08-20]	4.5 (1-7.5) [0.42-20]	0.50
BMI	25.3 (23.5-26.7) [19.4-29.8]	33.8 (31.2-35.9) [30.1-38.7]	<0.001
WHR	0.79 (0.75-0.82) [0.70-0.91]	0.84 (0.81-0.89) [0.75-0.96]	0.001
Parity	2 (1-3) [0-4]	1 (0-2) [0-3]	0.23
*SBP	129.6 ± 16.86	134.9 ± 16.27	0.32
*DBP	79.81 ± 9.35	88.86 ± 11.49	0.03

Table 17: Demographic characteristics Group 1 + Group 2

Data are median (interquartile range)[range]. Statistical analysis was performed using the Mann-Whitney U test. All women are non-smokers. *Data are mean ± SD, analysis by unpaired T-test.

	BMI<30	BMI>30	P
n	31	9	
Age (years)	58 (54-63) [46-66]	57 (53-61) [51-63]	0.39
Years since LMP	8 (0.33-20)	6 (1-15)	0.48
BMI	25.2 (23.4-26.9) [20.7-29.8]	33.8 (31.3-35.1) [30.3-37.1]	<0.001
Parity	2 (0-4)	2 (0-3)	0.44
SBP	128.4 ± 17.28	128.89 ± 11.97	0.94
DBP	79.29 ± 9.45	82.67 ± 6.89	0.34

Table 18: Demographic characteristics of Group 1 (venlafaxine treatment)
Data are median (interquartile range)[range]. Statistical analysis was performed using the Mann-Whitney U test. All women are non-smokers. *Data are mean ± SD, analysis by unpaired T-test.

	BMI<30	BMI>30	P
n	5	6	
Age (years)	52 (50-53) [49-54]	52 (47-55) [45-58]	1.0
Years since LMP	6 (0.08-19)	2 (0.42-20)	0.73
BMI	25.8 (22.0-27.8) [19.4-29.8]	33.7 (30.5-37.1) [30.1-38.7]	0.008
Parity	2 (1-2) [0-3]	1 (1-2) [0-3]	0.45
SBP	137 ± 12.92	145.8 ± 18.55	0.41
DBP	83 ± 8.94	94.40 ± 14.94	0.18

Table 19: Demographic characteristics of Group 2 (HRT)
Data are median (interquartile range)[range]. Statistical analysis was performed using the Mann-Whitney U test. All women are non-smokers. *Data are mean ± SD, analysis by unpaired T-test.

Responses to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

There was no difference in the endothelium dependent (ACh) baseline response, however there was a significantly lower endothelium independent (SNP) response when comparing participants with BMI<30 (n=36) to participants with BMI>30 (n=15) (ACh, $p=0.962$ and SNP, $p=0.025$). See Figure 64.

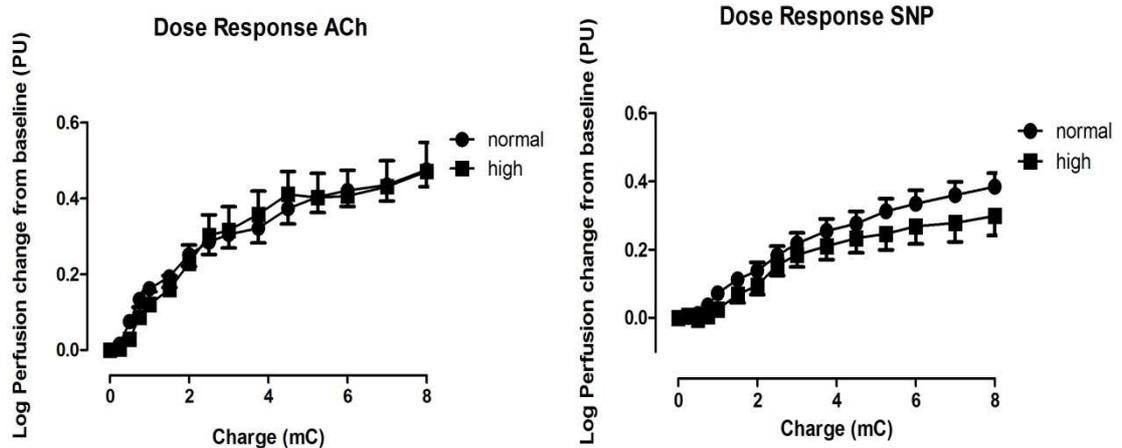


Figure 64: Dose Response Curve Group 1 + 2
 Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in women with BMI<30 (normal) compared with women with BMI≥30 (high). Data are mean ± SEM. General Linear Model.

It was already shown in chapter five that treatment with venlafaxine decreases the increased cutaneous perfusion responses seen in flushing women. This group was subdivided into those women with BMI<30 and those with a BMI>30 to determine whether their treatment responses varied.

Participants in group 1 as a whole experienced a reduction in peripheral perfusion responses following eight weeks of treatment with venlafaxine (see Chapter five). This response is maintained in those participants with a BMI<30 (n=31), and is present for both endothelium dependent and independent measurements. (P=0.001 and 0.04 respectively). See Figure 65.

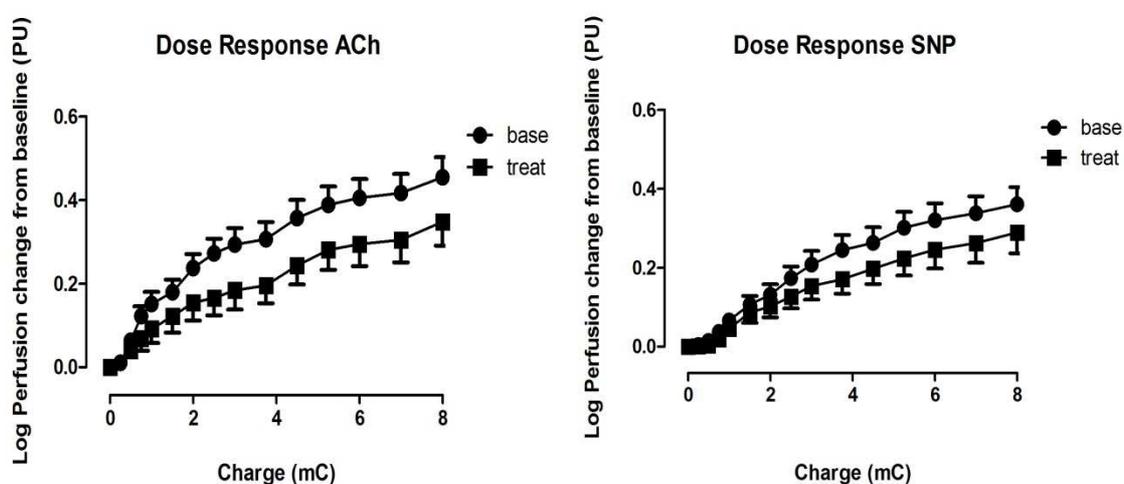


Figure 65: Dose Response Curve Group 1 BMI <30

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in women with BMI<30, prior to treatment (base) and following 8 weeks venlafaxine treatment (treat). Data are mean \pm SEM. General Linear Model.

However, in those participants with a BMI>30 in Group 1 (n=9), this reduction in perfusion response is lost in endothelium dependent measurements and reversed in endothelium independent measurements, that is, following eight weeks of treatment with venlafaxine, endothelium independent cutaneous microvascular perfusion appears to be increased. (ACh, $P=0.468$ and SNP, $P=0.001$). See Figure 66.

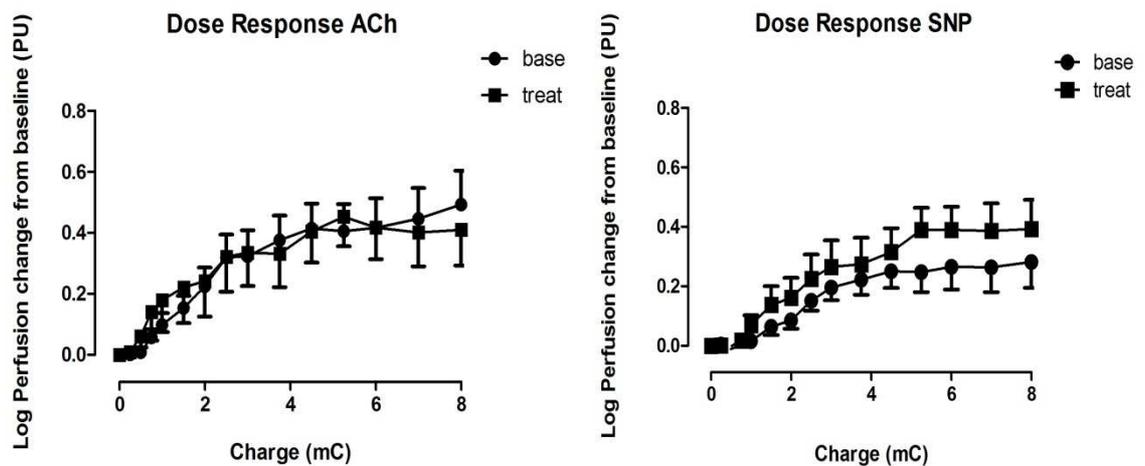


Figure 66: Dose Response Curve Group 1 BMI>30

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in women with BMI>30, no treatment (base) compared with 8 weeks venlafaxine (treat). Data are mean \pm SEM. General Linear Model.

It was already shown in Chapter six that treatment with HRT increases cutaneous perfusion responses seen in flushing women. This group was subdivided into those women with BMI<30 and those with a BMI>30 to determine whether their treatment responses varied.

Again, in keeping with the group as a whole (see Chapter six), participants with a BMI<30 (n=5) who were treated with HRT for eight weeks demonstrated an increase in perfusion responses to iontophoresis of both ACh and SNP (P<0.001 and P<0.001 respectively). See Figure 67.

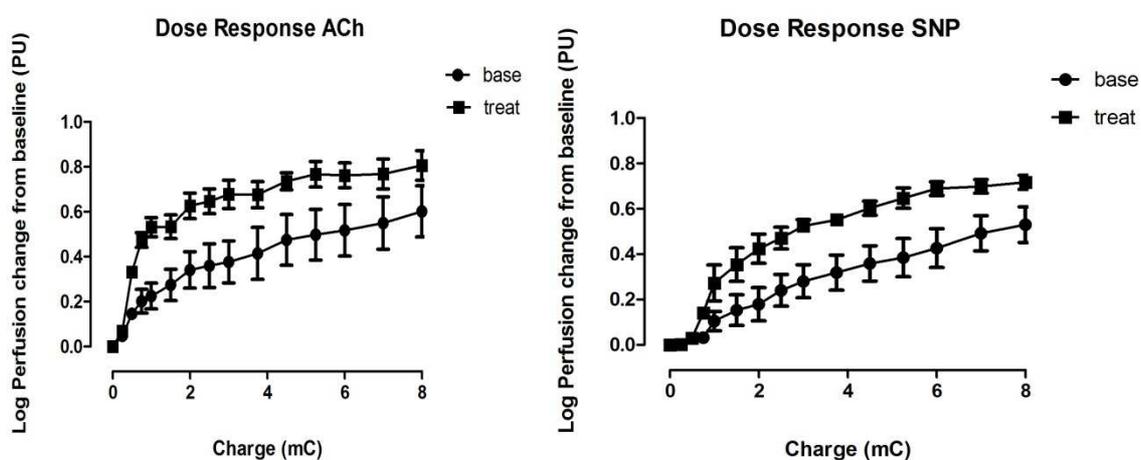


Figure 67: Dose Response Curve Group 2 BMI<30

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in women with BMI<30, no treatment (base) compared with 8 weeks HRT (treat). Data are mean \pm SEM. General Linear Model.

And again, in Group 2 (HRT) when participants with BMI \geq 30 (n=6) were examined, there was no significant change to endothelium dependent (ACh) responses (P=0.63) following eight weeks of treatment with HRT, but a reversal in the response seen to SNP. That is, there was a decrease in endothelium independent responses (SNP, P=0.04). See Figure 68.

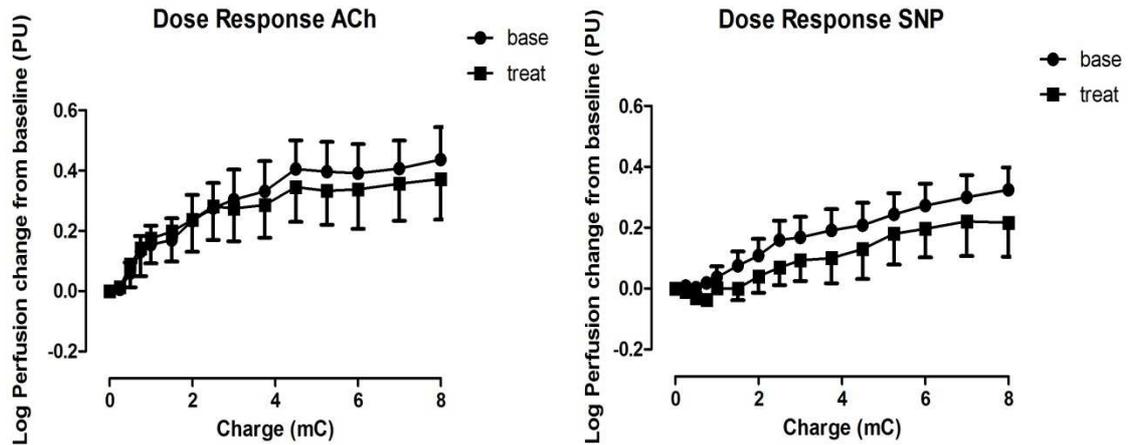


Figure 68: Dose Response Curve Group 2 BMI>30

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in women with BMI>30, no treatment (base) compared with 8 weeks HRT (treat). Data are mean \pm SEM. General Linear Model.

Hot flush diaries

Unfortunately, despite recruiting a total of 15 (9 from group 1 and 6 from group 2) women with a body mass index (BMI) of 30 or more, only 10 women recorded pre-treatment (baseline) hot flush diaries. 8 from group 1 and 2 from group 2. The overwhelming reason for two thirds of group 2 participants not recording pre-treatment diaries was an urgency to begin treatment. Often these ladies had been waiting a considerable length of time already to begin treatment and did not wish to delay any longer. This has had an impact on our results. The participant from group 1 who did not complete diaries refused as it was claimed to be impossible to count the number of flushes experienced as they were simply too frequent, and long-lasting, that they almost seemed to merge into one flush.

There were 36 participants with a BMI less than 30. 31 from group 1 and 5 from group 2. Baseline diaries were completed in 35, 31 from group 1 and 3 from group 2. Again the reason for poor compliance with pre-treatment diaries was an urgency to begin treatment.

In table 20; there were no significant differences in the number or severity of flushes experienced by participants when comparing two groups; BMI less than 30 and BMI greater than 30. This was also true of night time awakening and hot flush related daily interference score.

	Baseline BMI<30 (n=35)	Baseline BMI>30 (n=10)	P
Flush frequency (mean flush/day)	7.8 (5.8-9.5) [1.0-19.6]	6.7 (4.0-10.1) [2.1-21.2]	0.97
Night time awakening (NTA)	2.3 (1.6-3.4) [0.5-4.6]	2.8 (1.0-3.3) [0.3-7.6]	0.57
Hot flush related daily interference score (HFRDIS)	40.8 (22.0-59.7) [5.3-84.7]	45.4 (25.1-70.3) [5.5-83.7]	0.45
Hot flush score	17.0 (10.4-20.9) [1.8-43.1]	13.8 (7.8-24.9) [1.9-42.4]	0.95

Table 20: BMI<30 vs. BMI>30 baseline diary data.

Group 1 + Group 2. Data are median (interquartile range)[range]. Statistical analysis was performed using Mann Whitney test.

Flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed. NTA (night time awakening) = total number of NTA/week divided by the number of diary days completed, and a mean taken of the weeks completed. HFRDIS (hot flush related daily interference scale) was completed once per diary week, and a mean was taken of the weeks completed. Placebo and Clonidine weeks, diaries were completed for 4 weeks each, and baseline diaries were completed for 3 weeks.

Correlation of diary data with demographics

There was no correlation between BMI or waist hip ratio (WHR) and number or severity of flushes (see Figure 69).

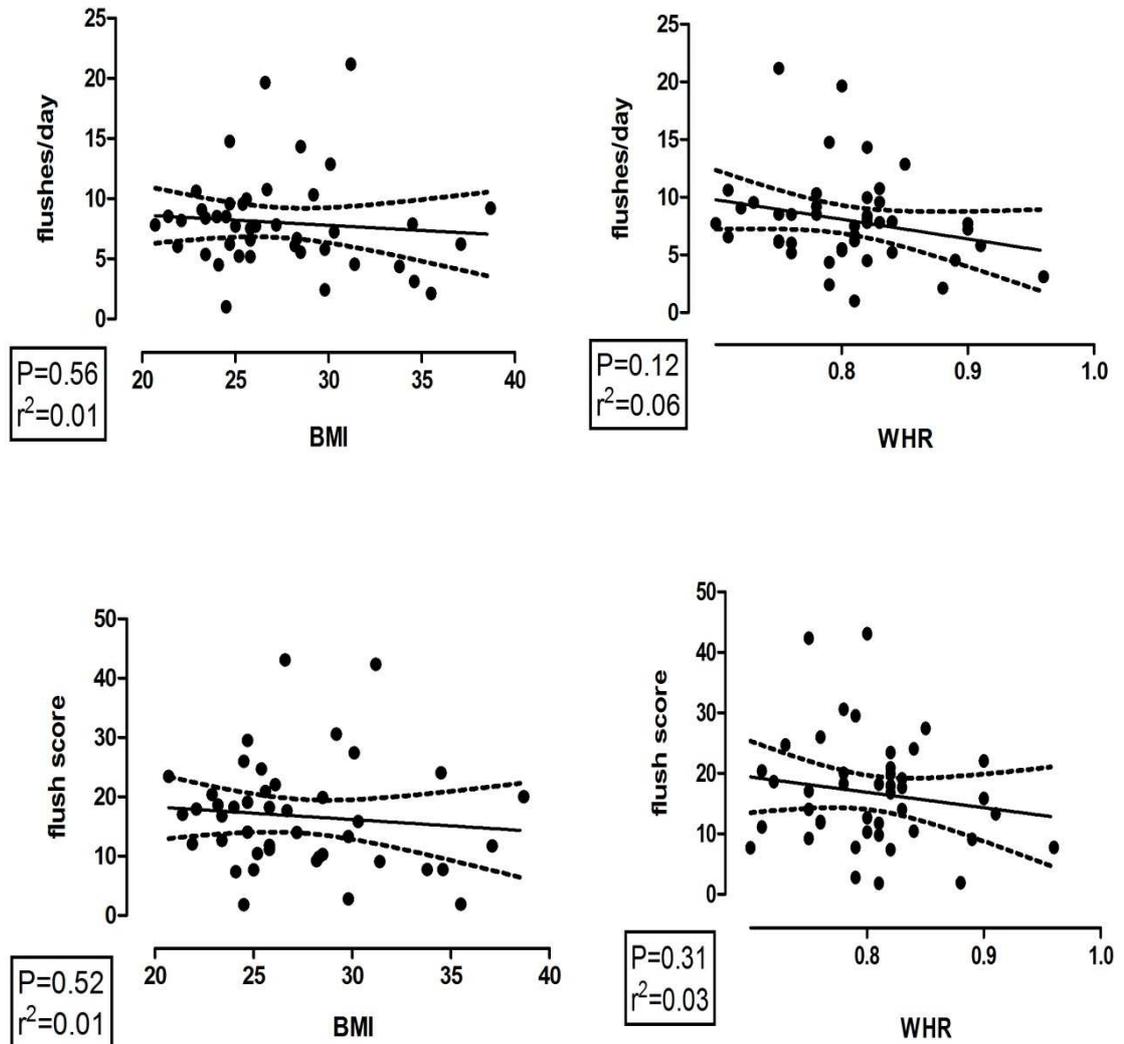


Figure 69: Correlation of flush frequency and score with BMI and WHR
Correlation plots and regression lines with 95% confidence intervals.

Qualitative Data

Greene Climacteric Scale

There was no difference in baseline GCS scores between those participants with a BMI<30 (lean) when compared to those with a BMI>30 (obese).

There was a significant reduction in GCS scores following treatment with venlafaxine, in group 1 participants, in both lean and obese subgroups, however there was no improvement for either lean or obese participants in group 2 following treatment with HRT. Unfortunately, despite improvements in compliance with these questionnaires, numbers were still small and this may be the reason for lack of significance in this group, as there does appear to be a trend towards reduction in scores (see Figure 70 and Table 21).

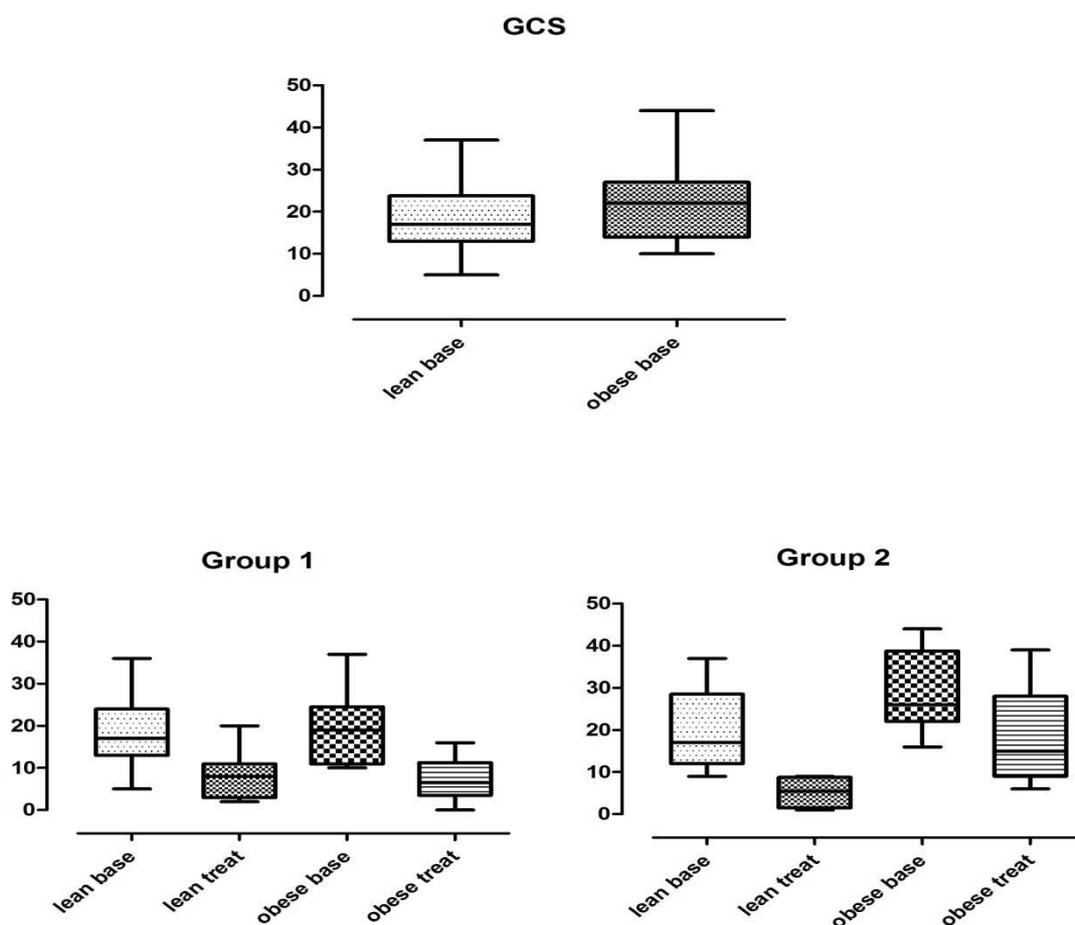


Figure 70: Greene Climacteric Scale
Data are shown as box and whisker plots with median, IQR and range.

Lean n=36						Obese n=15						p
17.0 (13.0-23.8) [5.0-37.0]						22.0 (14.0-27.0) [10.0-44.0]						*0.14
Group 1						Group 2						
Lean N=31			Obese N=9			Lean N=5			Obese N=6			
base	treat	p	base	treat	p	base	treat	p	base	treat	p	
17.0 (13.0-24.0) [5.0-36.0]	8.0 (3.0-11.0) [2.0-20.0]	<0.001	19.0 (11.0-24.5) [10.0-37.0]	6.5 (3.5-11.25) [0.0-16.0]	0.03	17.0 (12.0-28.5) [9.0-37.0]	5.5 (1.5-8.8) [1.0-9.0]	0.09	26.0 (22.0-38.8) [16.0-44.0]	15.0 (9.0-28.0) [6.0-39.0]	0.13	

Table 21: Greene Climacteric Scale

Data are median (interquartile range)[range]. P values are determined by Wilcoxon Signed Rank analysis of paired data. *P value is determined by Mann Whitney test.

Becks Depression Inventory

Results here are similar to results with GCS scores; there was no significant difference in baseline results for lean versus obese participants. Again, there was a significant reduction in BDI scores following treatment with venlafaxine in group 1 participants, in both lean and obese subgroups, however no improvement for either lean or obese participants in group 2 following treatment with HRT (see Figure 71 and Table 22).

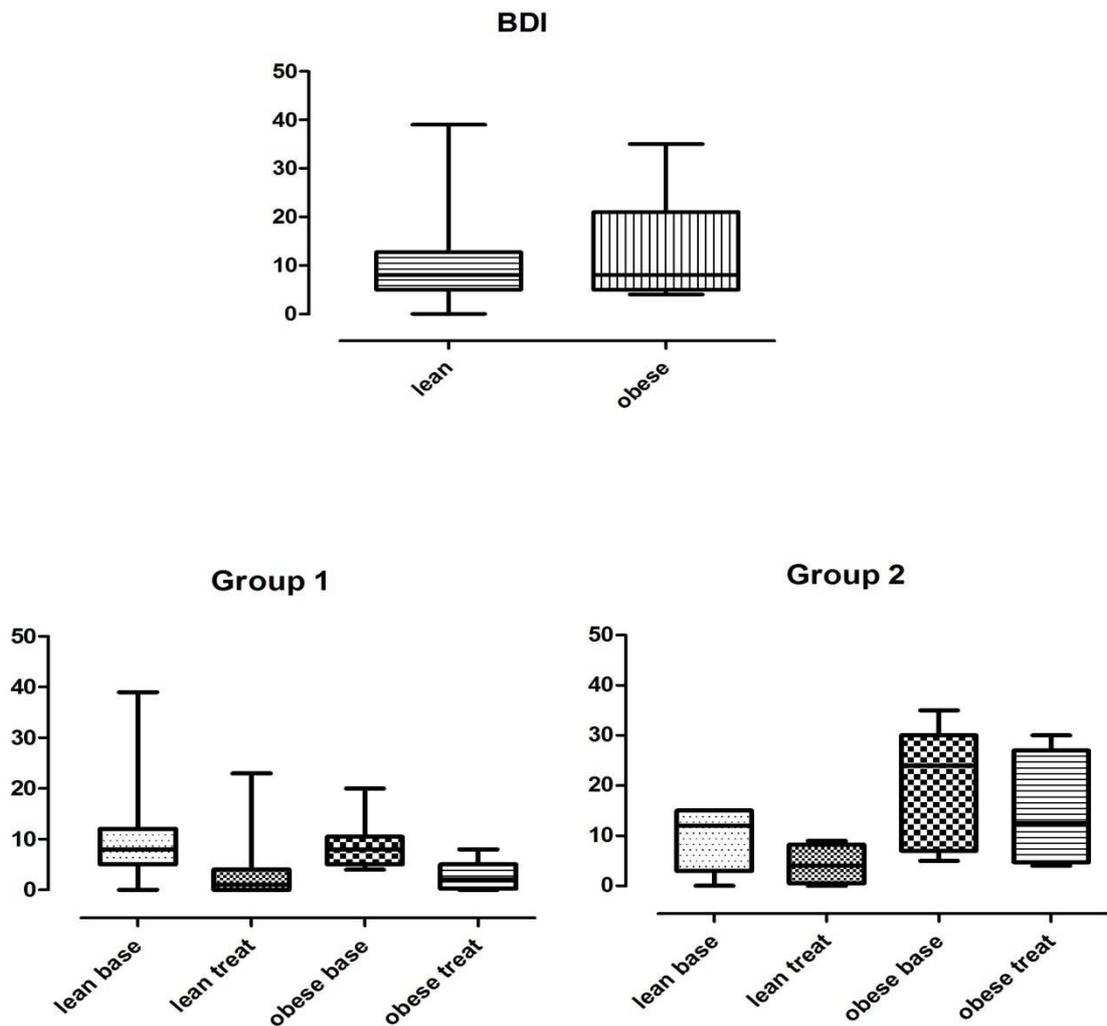


Figure 71: Becks Depression Inventory
Data are shown as box and whisker plots with median, IQR and range.

Lean n=36						Obese n=15						p
8.0 (5.0-12.8) [0.0-39.0]						8.0 (5.0-21.0) [4.0-35.0]						*0.49
Group 1						Group 2						
Lean N=31			Obese N=9			Lean N=5			Obese N=6			
base	treat	p	base	treat	p	base	treat	p	base	treat	p	
8.0 (5.0-12.0) [0.0-39.0]	1.0 (0.0-4.0) [0.0-23.0]	<0.001	8.0 (5.0-10.5) [4.0-20.0]	2.0 (0.3-5.0) [0.0-8.0]	0.04	12.0 (3.0-15.0) [0.0-15.0]	4.0 (0.5-8.3) [0.0-9.0]	0.20	24.0 (7.0-30.0) [5.0-35.0]	12.5 (4.8-27.0) [4.0-30.0]	0.10	

Table 22: Becks Depression Inventory

Data are median (interquartile range)[range]. P values are determined by Wilcoxon Signed Rank analysis of paired data. *P value is determined by Mann Whitney test.

Hospital Anxiety and Depression Scale

As already stated in Chapter 2, this was a questionnaire that was introduced late in the study, therefore numbers of participants who completed this were small in comparison to the other questionnaires. The only significant improvement seen with this score was in group 1 lean participants following treatment with venlafaxine (see Figure 72 and Table 23).

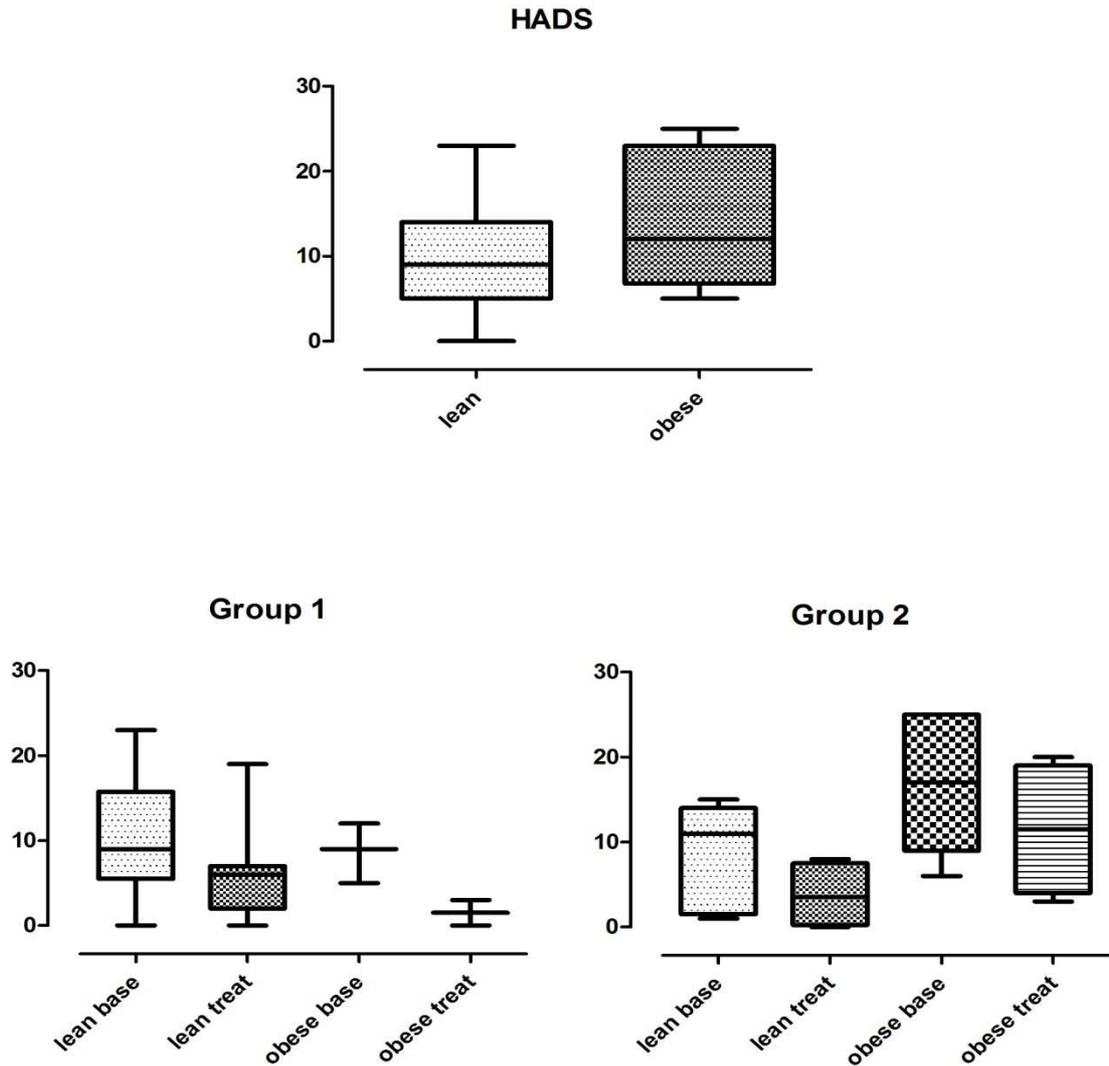


Figure 72: Hospital Anxiety and Depression Score
Data are shown as box and whisker plots with median, IQR and range.

Lean n=17						Obese n=8						p
9.0 (5.0-14.0) [0.0-23.0]						12.0 (6.8-23.0) [5.0-25.0]						*0.27
Group 1						Group 2						
Lean N=12			Obese N=3			Lean N=5			Obese N=5			
base	treat	p	base	treat	p	base	treat	p	base	treat	p	
9.0 (5.5-15.8) [0.0-23.0]	6.0 (2.0-7.0) [0.0-19.0]	0.03	9.0 (5.0-12.0) [5.0-12.0]	1.5 (0.0-3.0) [0.0-3.0]	0.5	11.0 (1.5-14.0) [1.0-15.0]	3.5 (0.3-7.5) [0.0-8.0]	0.10	17.0 (9.0-25.0) [6.0-25.0]	11.5 (4.0-19.0) [3.0-20.0]	0.10	

Table 23: Hospital Anxiety and Depression Score

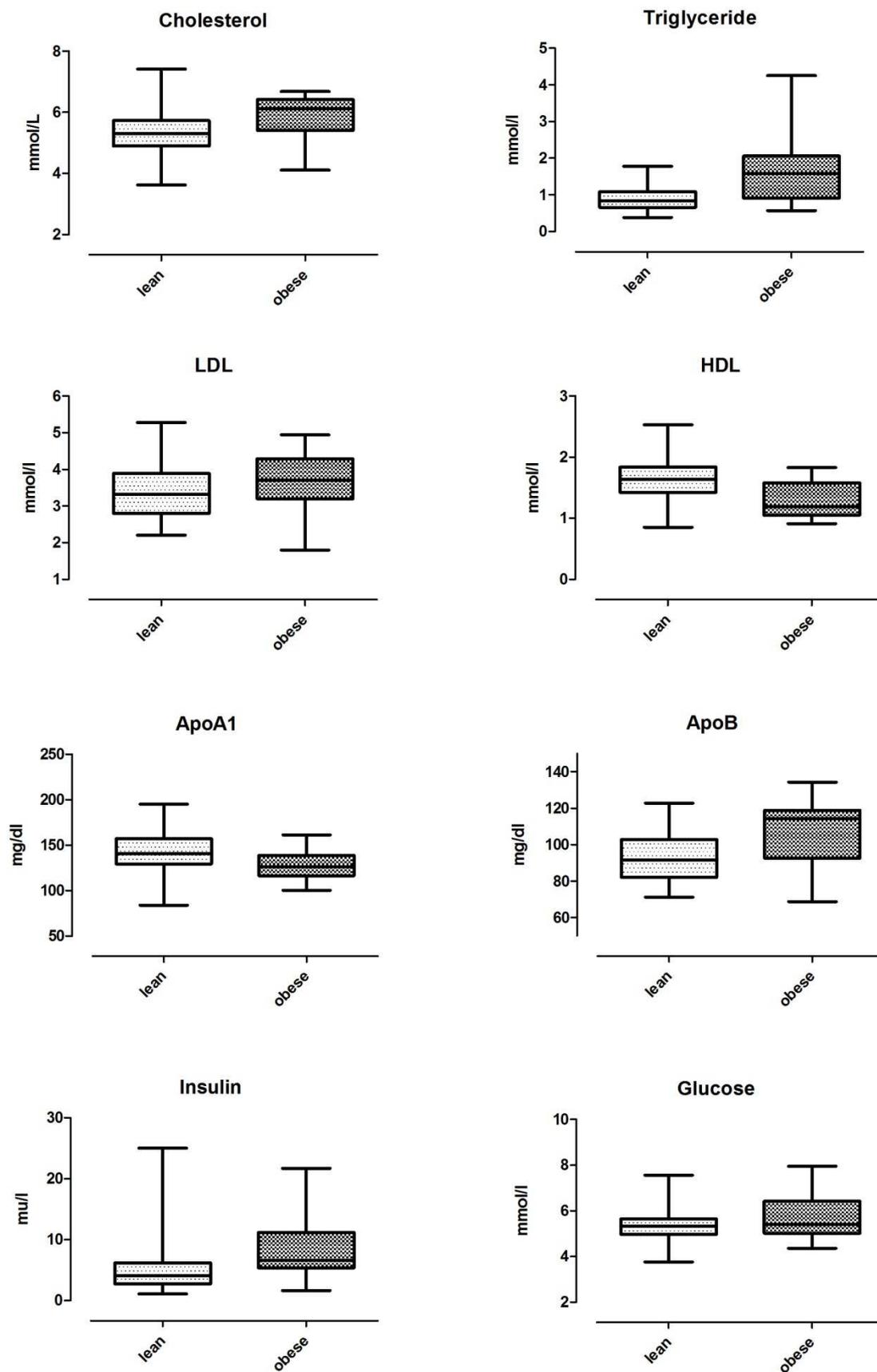
Data are median (interquartile range)[range]. P values are determined by Wilcoxon Signed Rank analysis of paired data. *P value is determined by Mann Whitney test.

Plasma analysis

As one might expect obese women had significantly higher total cholesterol ($p=0.04$), triglycerides ($p<0.001$), apolipoprotein B ($p=0.005$), and CRP ($p=0.01$) levels. They also had significantly lower HDL-cholesterol ($p=0.002$) and apolipoprotein A1 ($P=0.01$).

These results were not affected by adjustment for age, years since last menstrual period (YSLMP) and parity: total cholesterol ($p=0.03$), triglycerides ($p<0.001$), apolipoprotein B ($p<0.001$), CRP ($p=0.01$), HDL-cholesterol ($p=0.001$), and apolipoprotein A1 ($P=0.01$).

Insulin was significantly higher in the obese group ($p=0.003$), however this was not significant when adjusted as above ($p=0.17$), see Figure 73 and Table 24.



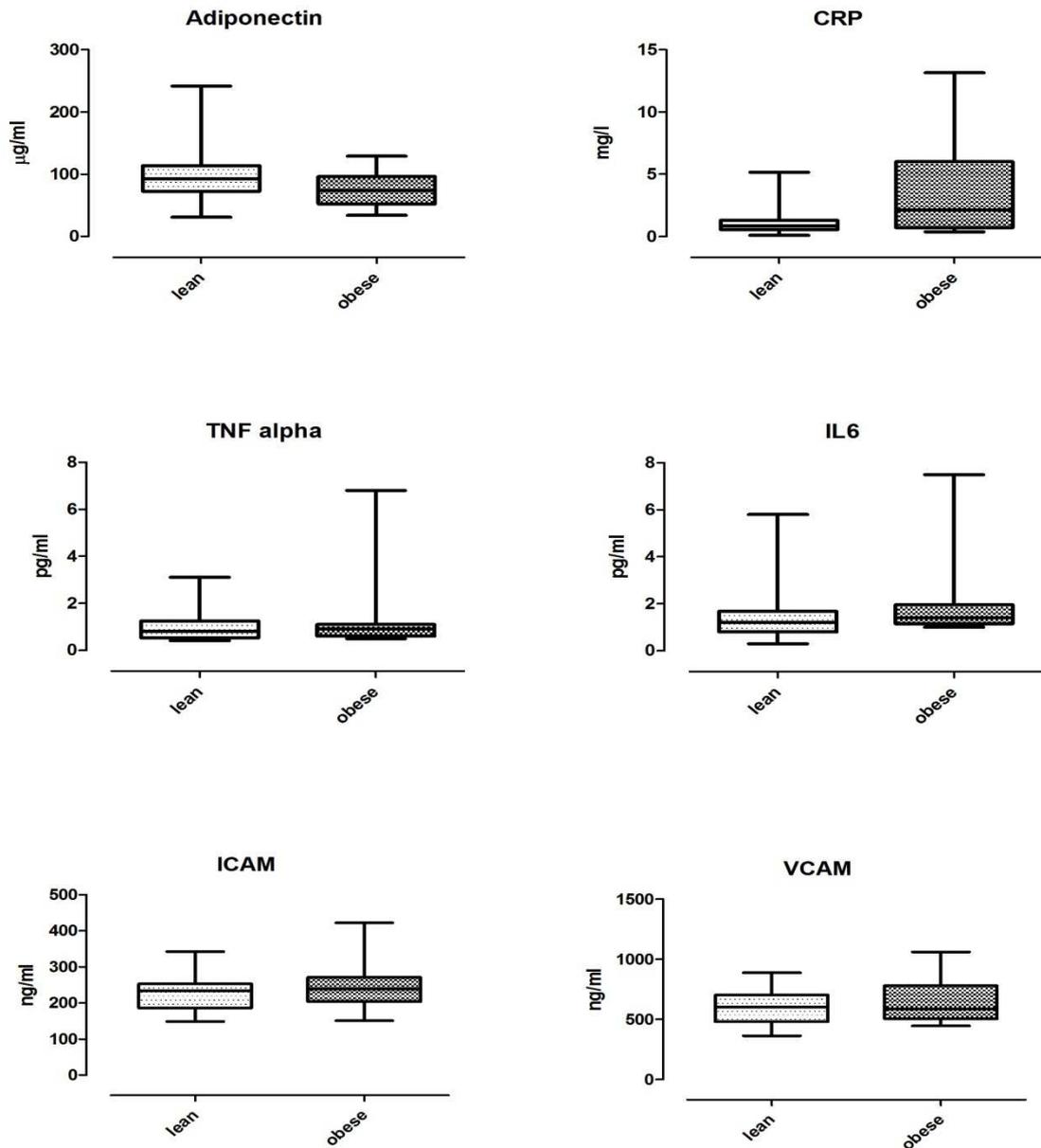


Figure 73: Plasma analysis

Results of plasma analysis, a comparison of obese and lean participants. Adjusted results; Cholesterol, $p=0.03$; Trig, $p<0.001$; ApoB, $p<0.001$; CRP, $p=0.01$, HDL, $p=0.001$; ApoA1, $p=0.01$. Data are shown as box and whisker plots with median, IQR and range.

	BMI<30 n=36	BMI>30 n=15	P	Adj. P	R-Sq (%)
Cholesterol	5.3 (4.9-5.7)[3.6-7.4]	6.1 (5.4-6.4)[4.1-6.7]	0.04	0.03	13.7
Triglyceride	0.9 (0.7-1.1)[0.4-1.8]	1.6 (0.9-2.1)[0.6-4.3]	<0.001	<0.001	45.6
LDL-cholesterol (mmol/l)	3.3 (2.89-3.9)[2.2-5.3]	3.7 (3.2-4.3)[1.8-4.9]	0.11	0.05	12.6
HDL-cholesterol (mmol/l)	1.6 (1.4-1.8)[0.9-2.5]	1.2 (1.1-1.6)[0.9-1.8]	0.002	0.001	25.9
Total Cholesterol to HDL-cholesterol ratio	3.2 (2.9-3.9)[2.1-5.1]	1.1 (0.9-1.3)[0.6-1.5]	<0.001	<0.001	75.3
Apolipoprotein A1 (mg/dl)	141.1 (129.6-157.1) [83.9-195.3]	126.3 (116.3-138.9) [100.4-161.3]	0.01	0.01	17.1
Apolipoprotein B (mg/dl)	91.8 (82.1-103.0)[71.3-122.9]	114.4 (92.6-118.8)[68.8-134.4]	0.005	<0.001	32.49
Insulin (mu/l)	4.1 (2.7-6.2)[1.1-25.0]	6.7 (5.4-11.2)[1.7-21.7]	0.003	0.17	12.4
Glucose (mmol/l)	5.3 (5.0-5.7)[3.8-7.6]	5.4 (5.0-6.4)[4.4-8.0]	0.30	0.07	15.9
Adiponectin (µg/ml)	92.7 (72.3-113.8)[30.9-241.4]	74.0 (52.3-96.5)[34.1-129.0]	0.05	0.03	11.6

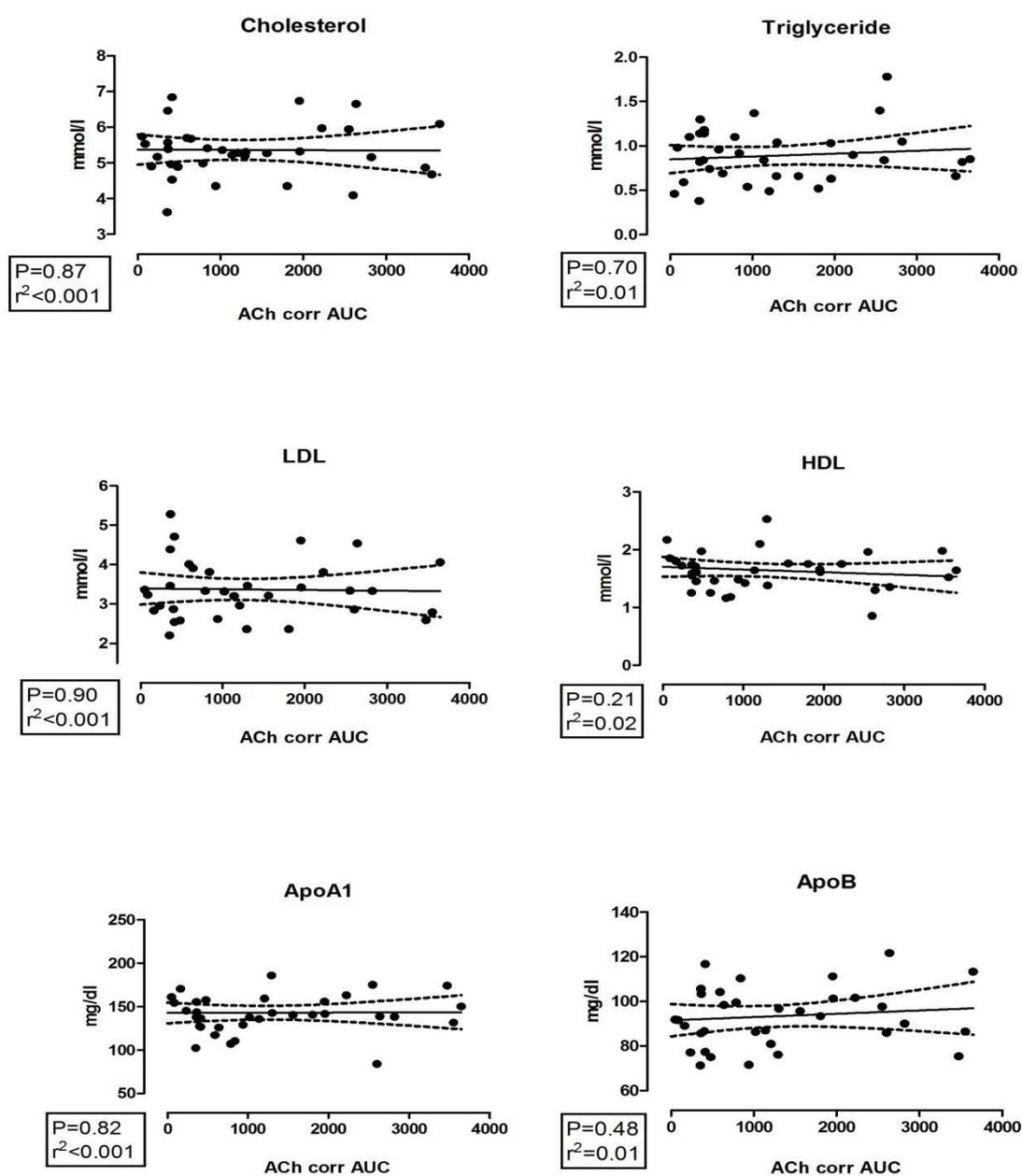
C-Reactive Protein (CRP) (mg/l)	0.9 (0.5-1.3)[0.1-5.2]	2.1 (0.7-6.0)[0.4-13.2]	0.01	0.01	21.3
TNF alpha (pg/ml)	0.8 (0.5-1.3)[0.4-3.1]	0.9 (0.6-1.1)[0.5-6.8]	0.61	0.16	13.2
IL-6 (pg/ml)	1.2 (0.8-1.7)[0.3-5.8]	1.4 (1.2-2.0)[1.0-7.5]	0.16	0.39	7.2
ICAM	233.7 (186.9-253.1) [149.1-343.0]	239.7 (204.1-271.0) [150.5-422.4]	0.31	0.33	11.2
VCAM	604.7 (483.0-703.8) [362.9-889.5]	587.4 (507.1-779.4) [448.1-1060.0]	0.80	0.70	5.6

Table 24: Plasma analysis

Results of plasma analysis, a comparison of obese and lean participants. Data are median (interquartile range)[range]. Statistical analysis was performed using Mann-Whitney test. Adjusted P calculated using general linear model to adjust for Age, Years since last menstrual period (LMP) and Parity.

Perfusion response and plasma analysis correlations

A negative correlation between CRP and endothelium dependent perfusion responses was demonstrated in the lean group ($p=0.05$) (see Figure 73). A negative correlation between endothelium dependent perfusion and TNF alpha in the obese group ($p=0.03$) (see Figure 75) and a negative correlation between VCAM and endothelium independent response in the obese group ($p=0.05$) (see Figure 76). No other associations have been demonstrated. See Figures 74-77.



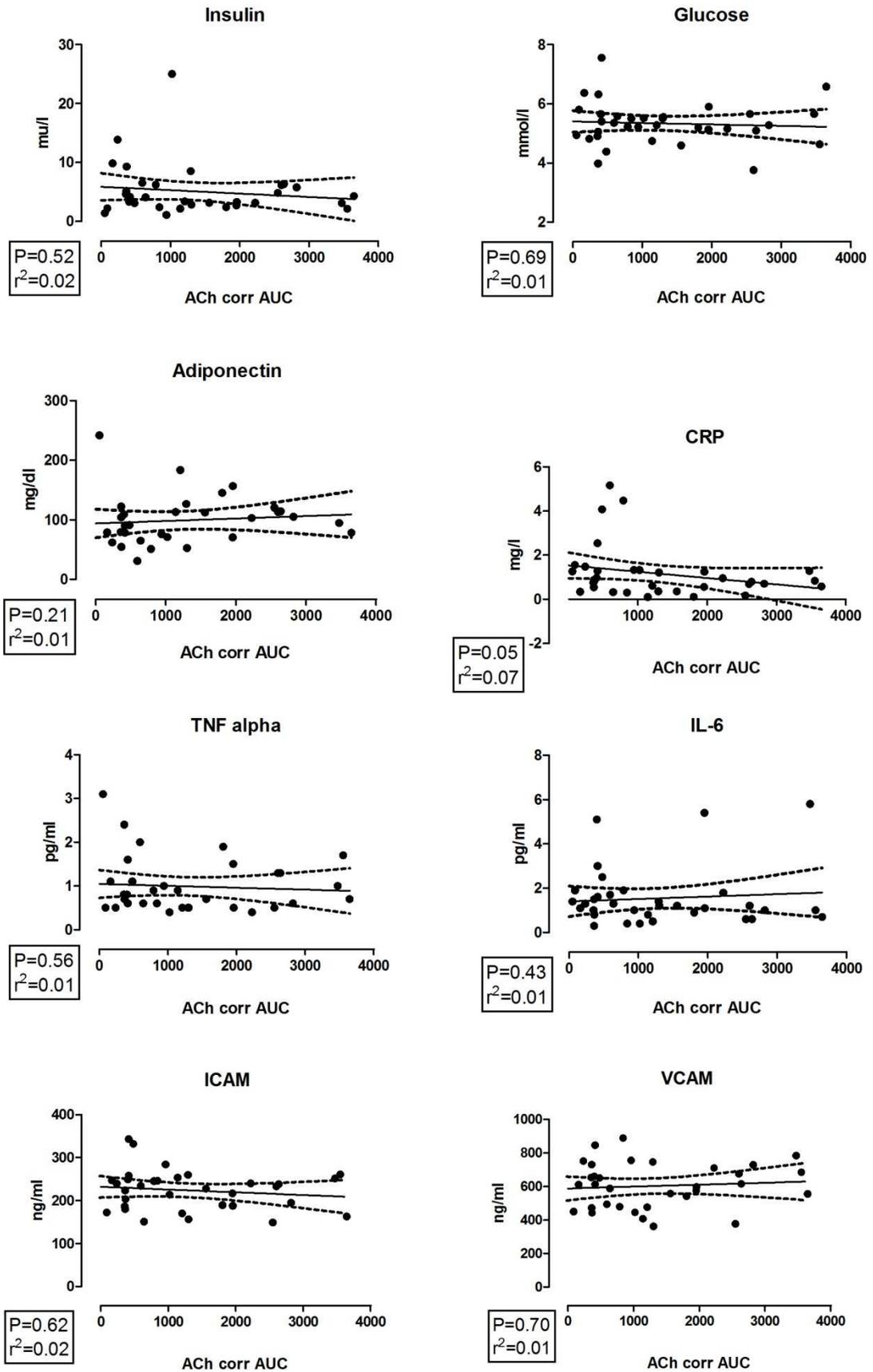
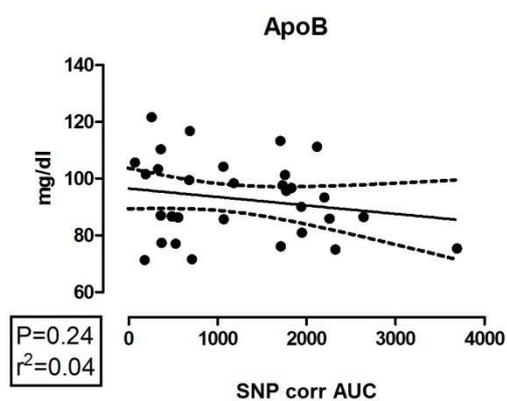
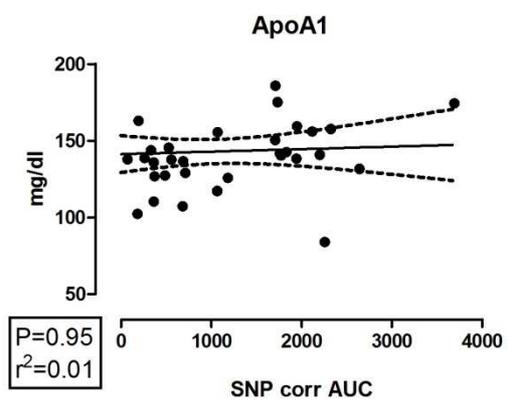
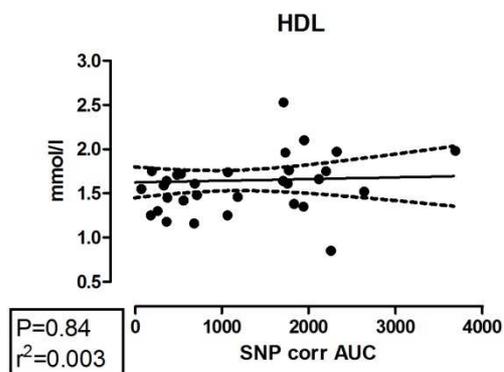
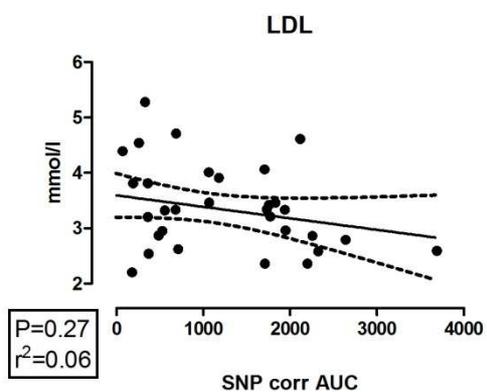
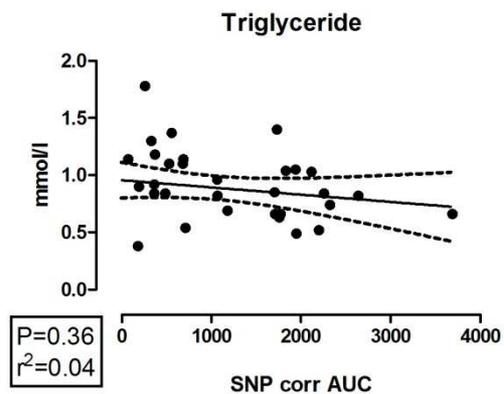
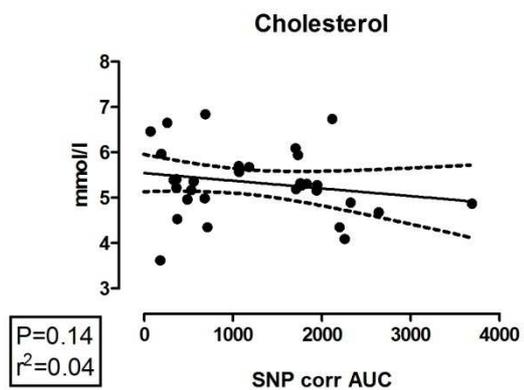


Figure 74: Lean ACh perfusion response and plasma correlations
Correlation plots and regression lines with 95% confidence intervals for endothelium dependent (ACh) perfusion response and its association with results of plasma analysis.



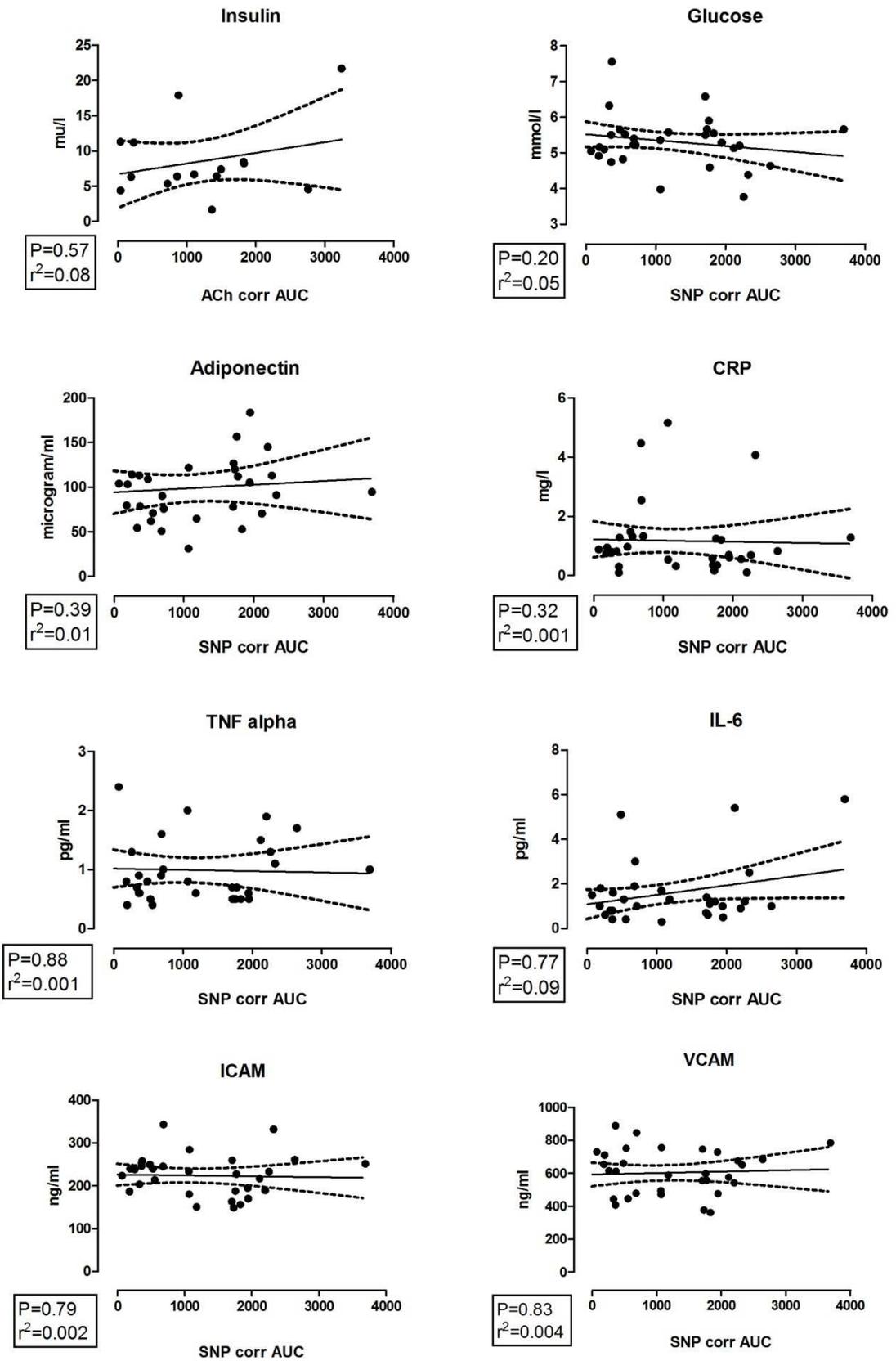
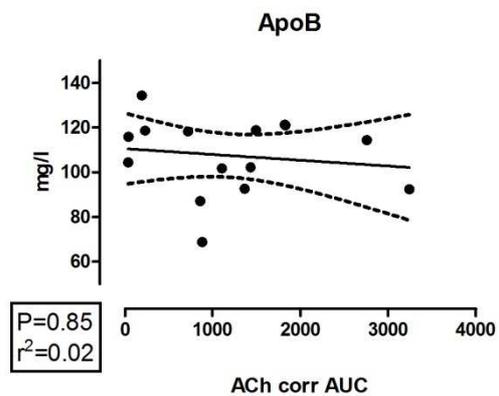
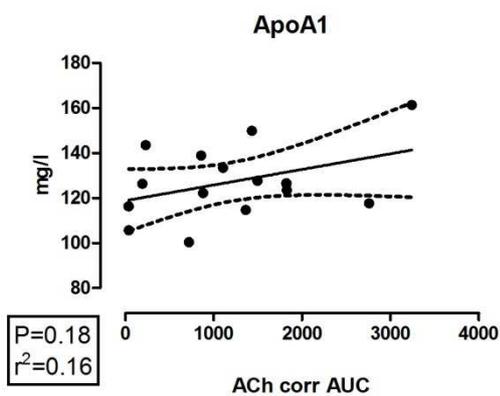
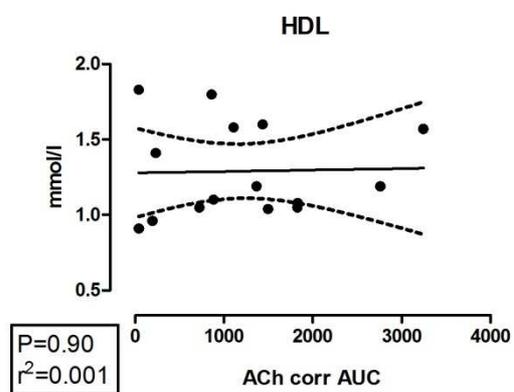
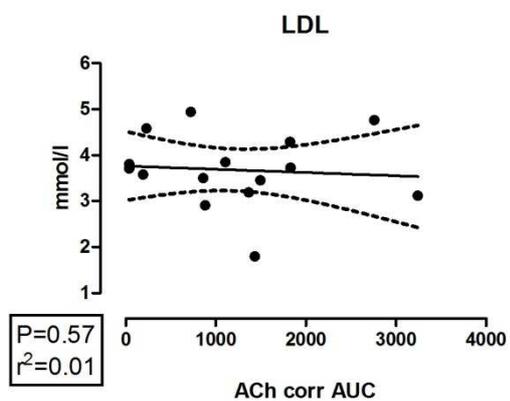
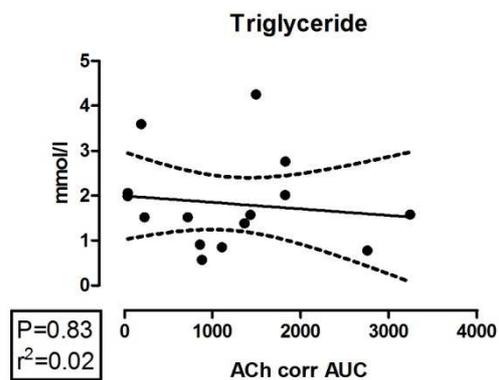
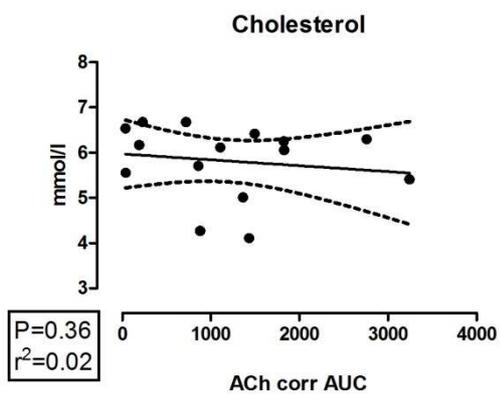


Figure 75: Lean SNP perfusion response and plasma correlations
 Correlation plots and regression lines with 95% confidence intervals for endothelium independent (SNP) perfusion response and its association with results of plasma analysis.



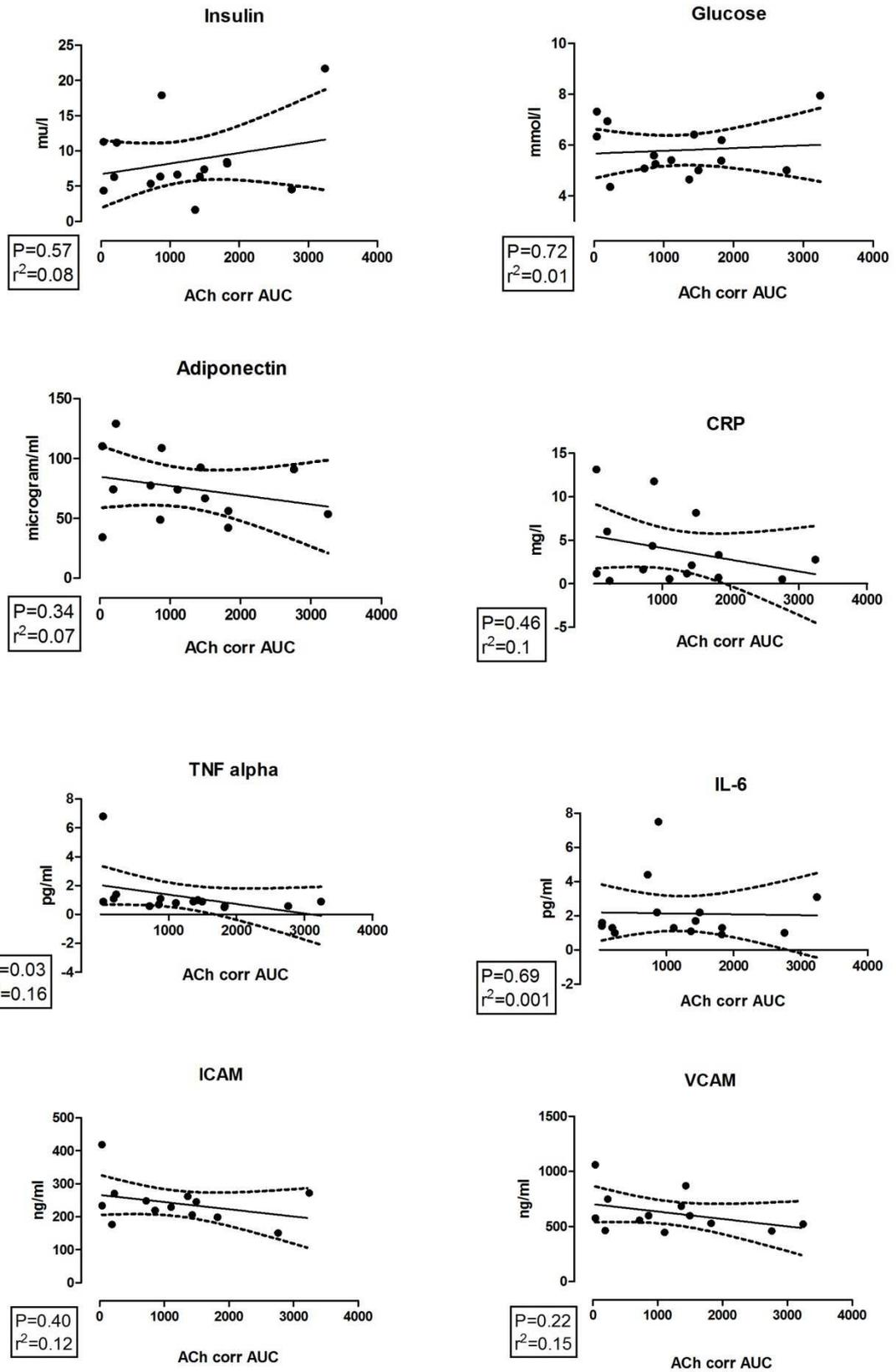
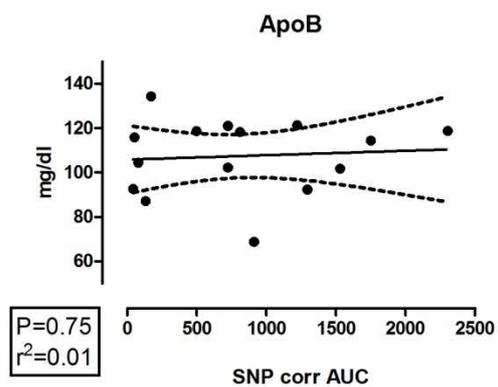
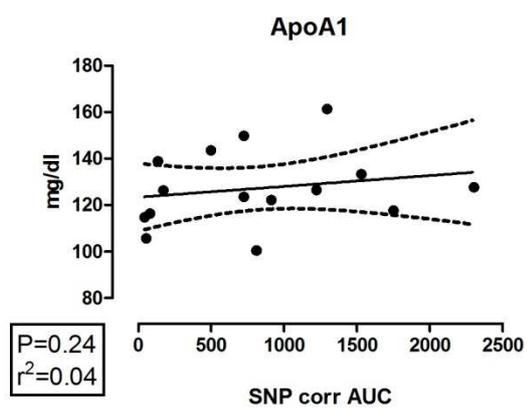
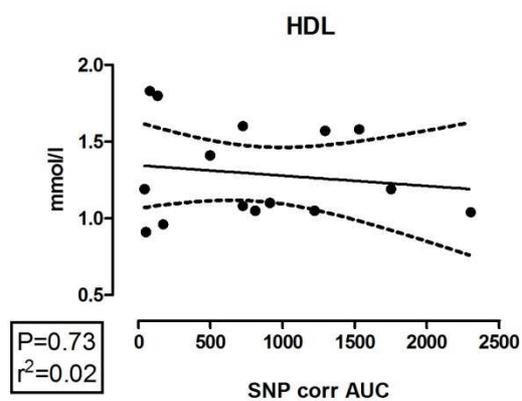
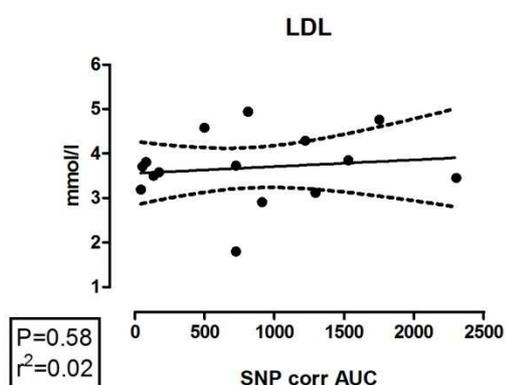
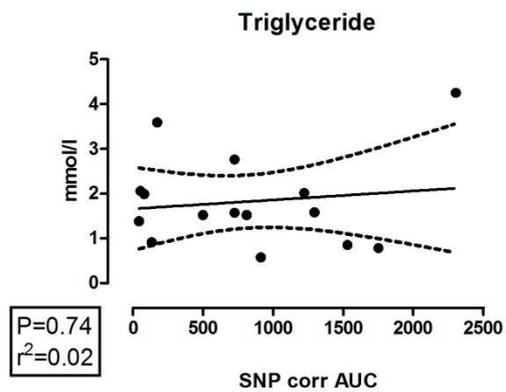
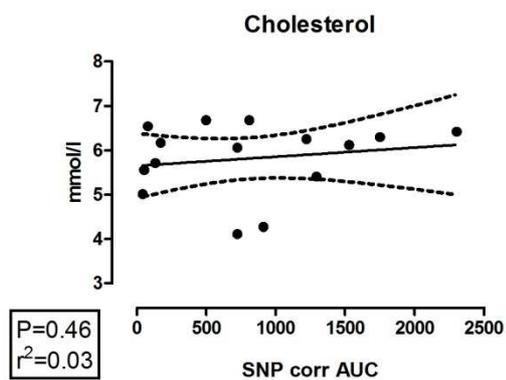


Figure 76: Obese ACh perfusion response and plasma correlations
 Correlation plots and regression lines with 95% confidence intervals for endothelium dependent (ACh) perfusion response and its association with results of plasma analysis.



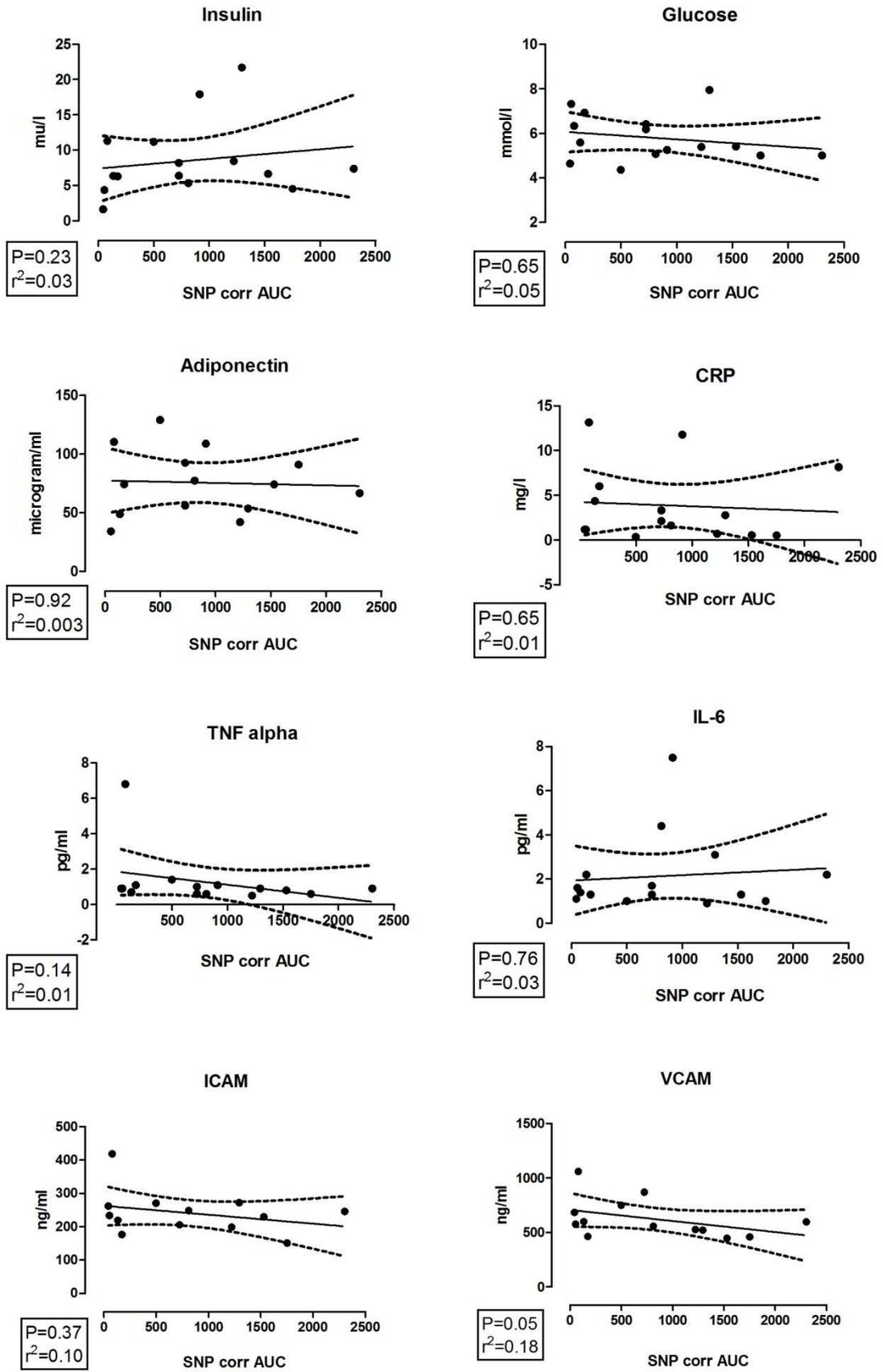


Figure 77: Obese SNP perfusion responses and plasma correlations
 Correlation plots and regression lines with 95% confidence intervals for endothelium dependent (ACh) perfusion response and its association with results of plasma analysis.

Summary of results

Perfusion response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Baseline

No differences were detected between obese and lean participants, in response to vasoactive agents for endothelium dependent perfusion; however there was reduced endothelium independent perfusion in the obese group.

Group 1 perfusion responses following treatment (with venlafaxine)

Participants with a BMI<30 had results in keeping with whole group responses, that is, perfusion responses were reduced following 8 weeks of treatment with venlafaxine. Those participants with a BMI>30 appeared to have an increased endothelium independent response following 8 weeks of treatment with venlafaxine.

Group 2 perfusion responses following treatment (with HRT)

Participants with BMI<30 had results in keeping with whole group responses, in that perfusion responses (endothelium dependent and independent) were increased following 8 weeks of treatment with HRT. Those participants with BMI>30 appeared to have decreased responses to both acetylcholine and sodium nitroprusside following 8 weeks of treatment with HRT.

Diary data

Baseline

No differences were detected in flush frequency, severity, night time awakening or HFRDIS between obese and lean participants.

Correlation of demographic data with diary data

There was no correlation between BMI or WHR with flush frequency or score.

Qualitative Data

Greene Climacteric Scores

No differences were detected between obese and lean participants at baseline.

Group 1 responses to treatment (with venlafaxine)

There were significant improvements in both obese and lean participants.

Group 2 responses to treatment (with HRT)

There was no significant improvement demonstrated in either lean or obese participants following treatment.

Becks Depression Inventory II

No differences were detected between obese and lean participants at baseline.

Group 1 responses to treatment (with venlafaxine)

There were significant improvements in both obese and lean participants.

Group 2 responses to treatment (with HRT)

There was no significant improvement demonstrated in either lean or obese participants following treatment.

Hospital Anxiety and Depression Scores

No differences were detected between obese and lean participants at baseline.

Group 1 responses to treatment (with venlafaxine)

There were significant improvements demonstrated in lean participants, however not in obese participants.

Group 2 responses to treatment (with HRT)

There was no significant improvement demonstrated in either lean or obese participants following treatment.

Serum analysis

Total cholesterol, triglycerides, apolipoprotein B, insulin and CRP were significantly higher in the obese group. They also had significantly lower HDL-cholesterol and apolipoprotein A1.

Serum analysis and their correlation with perfusion responses

A negative correlation between CRP and endothelium dependent perfusion responses was demonstrated in the lean group. In the obese group, there was a negative correlation between endothelium dependent perfusion and TNF alpha and a negative correlation between VCAM and the endothelium independent response.

Discussion

Obesity is the result of adipocyte hypertrophy during a positive caloric balance. It has been suggested that excessive hypertrophy causes a pathological change in the adipose tissue, creating adverse paracrine, endocrine and immune responses which may lead to cardiovascular diseases.

During this hypertrophic process, adipocytes grow out of proportion and angiogenesis cannot keep pace, resulting in a relative lack of blood flow leading to cellular and adipose tissue hypoxia. This contributes to pro-inflammatory responses, which in turn also lead to regional changes in blood flow. Local hypoxia also increases levels of inflammatory adipokines such as IL6 and TNF α , which induce dysfunction of adipose tissue arterioles, leading to further microcirculation dysfunction, compounding the hypoxic effects.

Insulin resistance is also induced by a number of adipokines, as is recruitment and infiltration of monocytes, lymphocytes and neutrophils into the blood vessel wall, which instigates local inflammation. Nitric oxide production in endothelial cells is also impaired, which further compromises endothelial-dependent vasodilatation.

Unexpectedly then, there was no difference detected, between obese (BMI \geq 30) and lean participants (BMI<30), in endothelium dependent cutaneous microvascular perfusion responses. There was however reduced endothelium independent perfusion in the obese participants when compared to the lean participants.

This absence of any difference in the endothelium dependent response is particularly surprising when coupled with elevated cholesterol, triglycerides, apolipoprotein B and CRP, and significantly lower HDL-cholesterol and apolipoprotein A1 in the obese group, all measures which are associated with vascular disease in both men and women.

Insulin was also significantly higher in the obese group, however did not withstand adjustment for age, years since last menstrual period or parity.

Serum adiponectin levels, however, were lower in obese participants after adjusting for age, years since menstrual period and parity.

Adiponectin is an adipocyte-derived plasma protein of 244 amino acids, and differs from the other adipocyte hormones in that its concentration declines with increasing obesity. Besides inhibiting inflammatory pathways, recombinant adiponectin increases insulin sensitivity and enhances lipid clearance. It also stimulates NO production in endothelial cells through an AMP-activated protein kinase (AMPK) mediated phosphorylation and activation of eNOS.

Vascular smooth muscle responsiveness to NO has been shown in healthy adults to be negatively correlated with total adiposity, but more specifically with abdominal adiposity (256).

Body fat distribution (subcutaneous versus visceral, pericardial, perivascular etc.) may be the underlying explanation for distinct metabolic and cardiovascular disease risk in individuals with a similar BMI. Different locations of adipose tissue may express different profiles of adipokines (257) and not all fat depots may be pathological (“adipopathy”).

Men often store fat in visceral tissue by the more pathologic hypertrophy of adipocytes, whereas women store excess fat in the subcutaneous peripheries by hyperplasia (258). This may explain why, for the same age and weight, men have higher rate of cardiovascular disease when compared with women.

Cardiovascular disease risk in women, however, rises markedly after the menopause, and although the aetiology is unclear it may be due to the distribution of the fat, which not only becomes more central (259) but increases in proportion to total fat mass (260). There may also be a steeper increase in visceral adipose tissue and a steeper decrease in subcutaneous fat (261).

Perivascular adipose tissue (PVAT) represents an important role in the pathophysiology of vascular disease, both smooth muscle and endothelial cell dysfunction. Almost all vessels are surrounded by this fat depot, and it has been shown to secrete an as yet unidentified anti-contractile substance, perivascular adipose tissue derived relaxing factor (PVADRF), in parallel to the amount of

healthy adipose tissue. Not only does it appear to be abolished in obesity (262), but it has been reported to exert pro-contractile effects.

Perhaps these effects, as a result of increased deposits in PVAT, explain the differences seen in endothelium independent responses and not in endothelium dependent responses. However, the anti-contractile effect of PVAT appears to be both endothelium dependent via NO, and endothelium independent via generation of hydrogen peroxide.

Changes seen in adipose tissue as a consequence of obesity include infiltration and activation of macrophages leading to inflammation characterised by upregulation of tumour necrosis factor alpha (TNF α). It is this that Greenstein has suggested has resulted in the impaired ability of PVAT to regulate vascular tone (262). Indeed, there was a negative correlation between TNF α and endothelium dependent perfusion in obese participants.

Thurston suggested obesity resulted in a greater number of hot flushes to dissipate a given amount of heat as a result of the insulating effect of subcutaneous adiposity (247). It is possible that the metabolic effects associated with increases in visceral fat seen in obesity, and leading to impaired endothelial function, result in a diminished ability to peripherally vasodilate, thereby compounding the need for more flushes to dissipate the given amount of heat.

There was no correlation between the perfusion response and the flush frequency or severity in this study, therefore one cannot speculate that those women who are obese may have had greater increased perfusion responses compared to lean women, but for the endothelial dysfunction, which has inhibited this response.

There was no difference in flush frequency, severity, night time awakening or HFRDIS between obese and lean participants. Published data suggest that women who are overweight are more likely to experience vasomotor symptoms, however as the presence of severe flushing was necessary for inclusion in the study; it is not possible to discuss the presence or absence of vasomotor symptoms in relation to BMI. What can be stated is that for postmenopausal

women who experience severe vasomotor symptoms, there appears to be no difference in the frequency or severity when comparing lean with obese. There is also no correlation between number or severity of hot flushes and waist hip ratio.

There was, however a negative correlation identified between CRP and endothelium dependent (ACh) perfusion in the lean group and TNF α and ACh perfusion in the obese group, as well as VCAM and endothelium independent perfusion in the obese group. These suggest that whilst we have been unable to demonstrate an expected difference between lean and obese in peripheral perfusion responses, the relationship between the perfusion response and biomarkers of chronic inflammation associated with endothelial dysfunction are as expected. Perhaps, thermoregulatory heat loss requirements, in obese women with flushing, are able to overcome early endothelial dysfunction.

Perfusion responses following treatment with venlafaxine in those participants with a BMI<30, were in keeping with overall group response. That is, endothelium dependent and independent responses were reduced following 8 weeks of treatment. However, in the obese group, there was no change in endothelium dependent perfusion responses, but a significant increase in endothelium independent (vascular smooth muscle) response. Yet, diary data confirmed an improvement in symptoms.

Equally, lean participants treated with HRT had similar effects to the HRT group as a whole; obese participants again appeared to exhibit an opposite response to vascular smooth muscle perfusion, but no apparent differences in endothelium dependent perfusion. There are insufficient diary data to comment on any change following HRT administration.

There certainly appears to be significant differences in the behaviour of the cutaneous vessels when treatment responses in obese versus lean are compared, despite a beneficial effect of treatment. It may be easier to explain the HRT treatment effect on perfusion in obese women; where the vasodilator effect of oestrogen may have been lost in vessels whose PVAT has a diminished ability to regulate tone, and in fact, may now be pro-contractile. Should caution be exercised even during the window of opportunity for cardiovascular disease

prevention, in women who are obese? It is more difficult to propose a possible mechanism for the vasodilator effect of venlafaxine on obese cutaneous vessels, given its strong association with vasoconstriction.

No differences were detected in measures of GCS, BDI or HADS between lean and obese participants. Again, treatment with venlafaxine resulted in improvements in all measures irrespective of BMI. The exception to this was HADS in Group 1 obese participants and treatment with HRT where data was most likely insufficient. Further, much larger, studies would be required to identify differences in lean and obese responses to treatment and the effect on peripheral vasculature, ideally also comparing methods of perfusion assessment.

Chapter 8

Hypogonadal Men

Introduction

Prostate cancer is the most common cancer in men. Over 40,000 men are diagnosed every year, and it is estimated that by 2030, it will be the most common cancer (263).

Before the 1940s, there was no effective treatment for advanced prostate cancer, but in 1939 Charles Huggins, aware of the sensitivity of the prostate gland, proposed orchidectomy for the control of prostate cancer. He also demonstrated that it reduced pain and produced stabilisation or regression of metastatic osseous lesions (134). In 1941, he discovered that the same results could be achieved by the administration of female sex hormones, stilbestrol and hexestrol (264).

Now, 90% of androgen deprivation therapy is achieved chemically, primarily luteinising-hormone-releasing hormone (LHRH) agonists, and 5-year survival is 81% (265) for all prostate cancer. However, there are side effects associated with treatment.

In 1896, Cabot was the first to report flushes as an effect of castration for enlarged prostate (266), even before the association between androgens and the prostate, and Huggins and Hodges also reported flushes in 9 out of 21 castrated patients 2 to 3 weeks following surgery (267).

Hot flushes occur in some 44-80% (268) of men, and are usually reported a few weeks after initiation of therapy, thought to coincide with achievement of castrate levels of testosterone (135). A proportion of patients (15-27%) describe hot flushes as the most significant adverse quality of life (QOL) effect from androgen deprivation therapy (ADT) (269, 270), with those experiencing more frequent or severe hot flushes reporting greater distress (271, 272). Hot flushes tend to become more frequent 3 months after starting treatment, half will still experience them 5 years after starting therapy (147) and in some they may persist long term (273), and even after discontinuation of therapy.

In addition, hot flushes can occur as night sweats, negatively impacting upon sleep (274), and are associated with nausea, anxiety and embarrassment.

In common with postmenopausal women, hot flushes appear to occur at a time of hormone withdrawal and, as with women, alterations in skin blood flow have been demonstrated during a flush 'attack' (148).

In addition, improvements seen in symptoms with hormone replacement are common to both men (149) and women and suggest a dependence on sex steroid levels.

Despite this and although hormone withdrawal unquestionably plays a major role in the development of hot flushes, the physiological mechanism remains obscure. Alterations in the hypothalamic thermoneutral zone have been suggested as the mechanism driving the heat loss responses seen in flushing postmenopausal women. We have demonstrated increases in peripheral perfusion responses to a vasoactive agent in postmenopausal women, suggesting a peripheral involvement. Is there a similar response seen in men?

Hypothesis

The contribution of altered peripheral vascular function to hot flushes in postmenopausal women is also found in hypogonadal men.

Methods

Participants

A total of 12 men with a diagnosis of prostate cancer who would receive androgen ablation therapy were recruited. Recruitment followed identification of suitable participants at the urology oncology multi-disciplinary meeting (MDT). Potential participants were approached with information about the study at their clinic appointment, and written information was given to take home. A follow up telephone call was made more than 24 hours later for the decision to participate.

Participating men were all aged 55-75 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function.

Study Design

Study participants were all assessed at visit 1, prior to baseline skin blood flow assessment using LASER Doppler imaging (LDI) with iontophoresis (ION) of vasoactive compounds (visit 2). Participants returned for repeat assessment 8 weeks after initiation of luteinising hormone releasing hormone (LHRH) agonist therapy (visit 3) and again at 24 weeks (visit 4) (see Figure 78). Qualitative measures were obtained at the same time points.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 09/MRE00/40). All patients gave written informed consent.

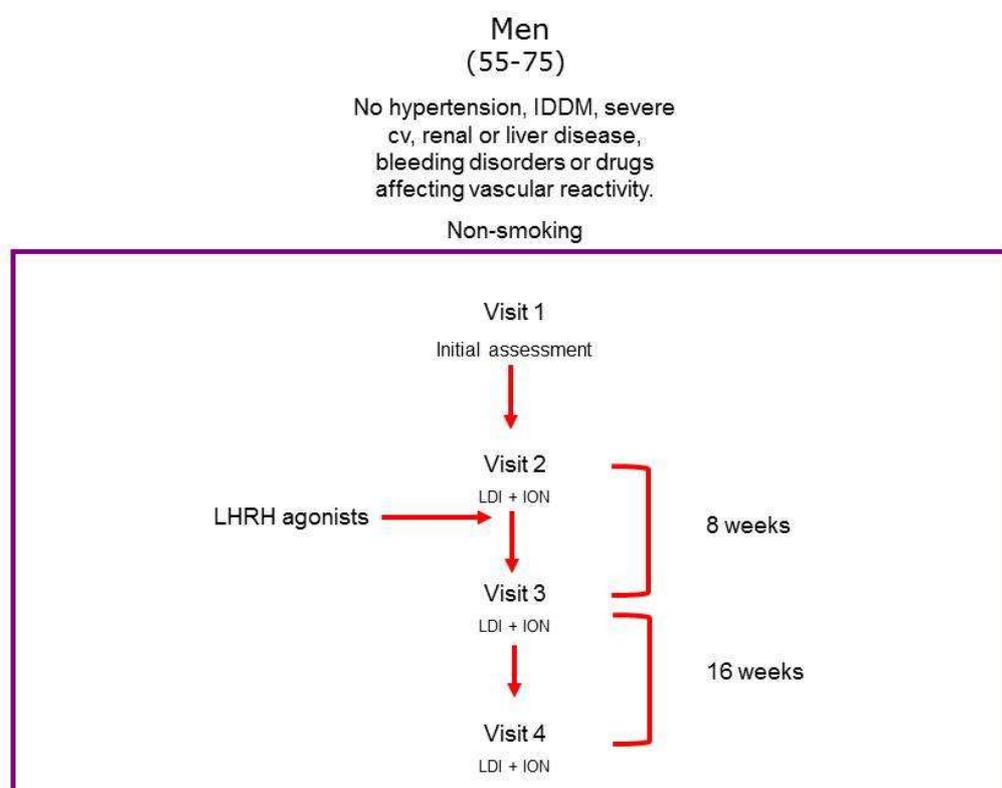


Figure 78: Study design

Body composition

Measurements of body mass, height, and blood pressure were made as described in Chapter 2.

LASER Doppler Imaging with Iontophoresis

Assessment of cutaneous microvascular perfusion was made at baseline and 8 weeks after initiation of GnRH agonist therapy. This technique is described in detail in Chapter 2.

Hot flush diary

Each participant was asked to keep a 'Hot Flush' diary as soon as/if they developed flushing during the course of their treatment and for the remainder of the study, as described in detail in Chapter 2.

Plasma analysis

A fasting blood sample was taken from each participant using 21G Vacuette® SAFETY blood collection set + luer adapter from an antecubital vein. Three samples were collected in Vacuette® gel tubes, one 9ml ethylenediamine tetraacetic acid (EDTA - lilac top), one 6ml lithium heparin (LiHep - green top) and one 4ml citrate (blue top).

A small volume was removed from the 6ml lithium heparin sample before centrifuge and storage as described in Chapter 2.

ICAM, VCAM, TNF - α , IL-6, Insulin and Adiponectin were measured by ELISA technique as described in Chapter 2.

CRP, Cholesterol, Glucose, HDL, LDL, and Trig were measured by standardised methods at The Institute of Cardiovascular and Medical Sciences at Glasgow University, as described in Chapter 2.

Statistical analysis

Measurement of vascular responses was performed using raw values.

Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, \log_{10} transformation of the data was performed to equalise the variances and thereby permit parametric data analysis

Comparison of demographic data was by the Mann-Whitney U test, except for blood pressure comparisons, which were analysed using unpaired T-test.

Diary data included analysis of mean number of flushes per day and hot flush score.

Results

Demographic Characteristics

A total of 12 men were recruited. Recruitment of gentlemen with prostate cancer who were to receive androgen ablation therapy, but who had little in the way of other co-morbidities, particularly cardiovascular, was challenging.

n	12
Age (years)	70 (66-74) [54-75]
Body Mass Index (BMI)	26.8 (24.4-29.6) [23.3-40.2]
Systolic Blood Pressure (SBP)*	147.0 ± 20.1
Diastolic Blood Pressure (DBP)*	85.4 ± 7.9

Table 25: Demographic characteristics.
Data are median (interquartile range)[range]. *Data are mean ± SD

Unfortunately there were 2 gentlemen who did not return following commencement of androgen ablation therapy, and therefore data are available for 10 participants.

Participants who developed flushing following commencement of androgen-ablation therapy had higher diastolic blood pressure than those without flushing.

	Non-flush	Flush	P
n	6	4	
Age (years)	69 (63-72) [54-72]	74 (70-75) [68-75]	0.07
Body Mass Index (BMI)	24.9 (23.5-27.1) [23.3-29.1]	30.1 (26.4-38.2) [25.9-40.2]	0.07
Systolic Blood Pressure (SBP)	137 ± 22.3	155.0 ± 18.8	0.24
Diastolic Blood Pressure (DBP)	79.4 ± 2.2	90.5 ± 9.6	0.04

Table 26: Demographic characteristics of Group 3 flush vs. non-flush
Data are median (interquartile range)[range]. Statistical analysis was performed using the Mann-Whitney U test. All men are non-smokers. *Data are mean ± SD, analysis by unpaired T-test.

Responses to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Vascular reactivity for 10 men was measured using LDI + Ion at baseline and following 8 weeks of androgen ablation therapy. 4 men developed flushes, and 6 did not. Data were analysed as described above.

There was no difference detected between men who developed flushes and those who did not when examining the perfusion responses at baseline, i.e. before any treatment had been commenced. This was true for both the endothelium-dependent (ACh) and independent vasodilators (SNP), (ACh, $p = 0.55$, SNP, $p = 0.62$). See Figure 79.

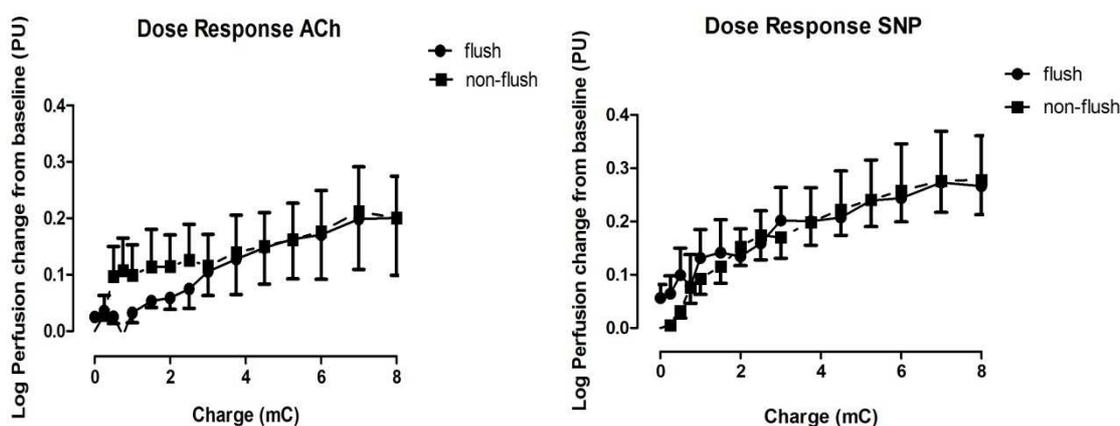


Figure 79: Dose Response Curves

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in flushing men compared with non-flushing men before commencing treatment. Data are mean \pm SEM.

However, after 8 weeks of treatment, the response of subcutaneous vessels was greater in the men who developed flushing than in those who did not. The enhanced vascular response occurred following administration of both the endothelium-dependent (ACh) and independent vasodilators (SNP), (ACh, $p < 0.001$, SNP, $p = 0.005$). See Figure 80.

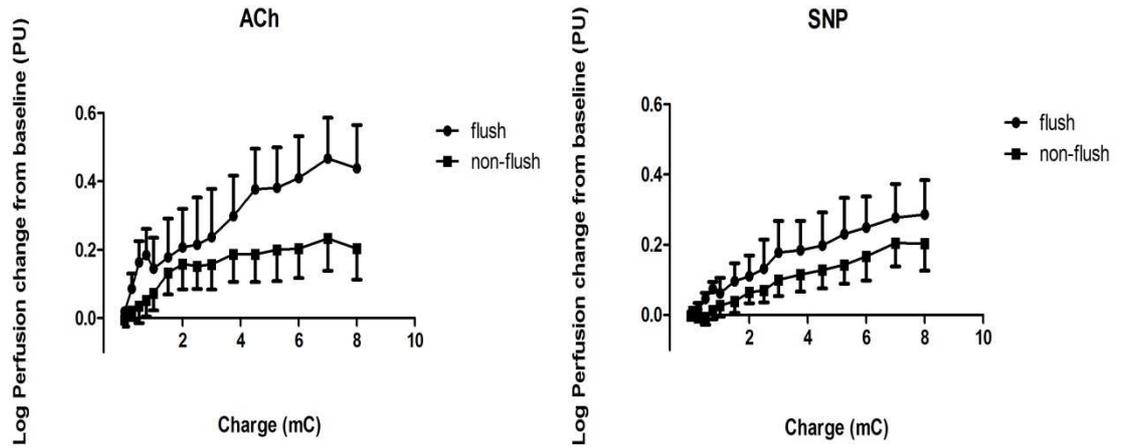


Figure 80: Dose Response Curves

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in flushing men compared with non-flushing men on androgen ablation therapy. Data are mean \pm SEM.

Hot flush diaries

Of the four participants who developed flushing, only two completed hot flush diaries. One gentleman reported that “every day was the same, so there is no point in filling out the diaries” and the other gentleman stated that he had “2 every day and 2 every night”, and therefore did not feel the need to complete the diaries.

Data are therefore too limited to analyse, however are presented below. As previously described, flushes are most likely to coincide with achievement of castrate levels of testosterone a few weeks after commencement of therapy and increase in frequency at approximately three months. For this reason, participants were asked to return at 8 weeks following commencement of therapy, and again at 24 weeks.

Table 27 demonstrates the flush frequency and severity in the two participants who completed diaries. Figure 81 demonstrates frequency of flushes with time. Both participants appeared to follow the expected pattern, with peak of flushing at approximately 8 weeks. Participant A had complete arrest of flushes at around 22 weeks, whilst participant B continued to have a few flushes per day following the initial peak.

	A.		B.	
	8 weeks	24 weeks	8 weeks	24 weeks
Flush frequency (flush/day)	3.5 ± 3.8 2.7 (0-6.9) [0-10]	7.7 ± 4.6 9.3 (3.6-11.0) [0-14.1]	1.3 ± 0.8 1.1 (0.9-1.8) [0.3-2.9]	0.3 ± 0.3 0.1 (0.1-0.4) [0-1.1]
Hot flush score	6.8 ± 7.8 5.0 (0-13.9) [0-20]	15.1 ± 9.6 18.0 (4.7-22.7) [0-28.3]	1.6 ± 1.3 1.3 (0.8-2.3) [0.2-4.3]	0.3 ± 0.3 0.1 (0.1-0.4) [0-1.1]

Table 27: Hot flush diary data.

Flush/day = total number of flushes/week divided by the number of diary days completed, and a mean taken of the weeks completed. Hot flush score = mean daily flush frequency multiplied by mean score. A mean was taken of the weeks completed.

Data are mean ± SD, and median (interquartile range)[range].

A= first participant. B=second participant. 8 weeks and 24 weeks = time from commencement of chemical androgen ablation therapy.

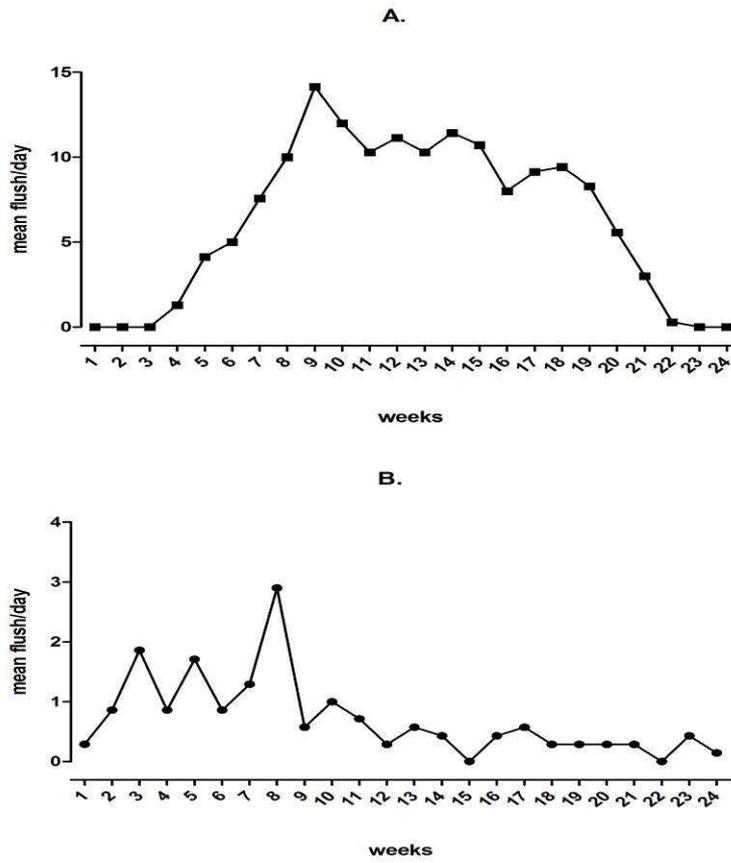


Figure 81: mean flush/day
 Graph demonstrates mean number of flushes per day for each week of participation in the study. A. = participant A. B. = participant B. Flush/day = total number of flushes/week divided by the number of diary days completed.

Four men with flushing returned for assessment at 24 weeks, and whilst there appeared to be a reduction in the number of flushes towards the end of the 24 week study period (see Figure 82), there was no difference in perfusion responses at this time point when compared with perfusion responses at 8 weeks. Endothelium dependent (ACh) $p=0.76$. and endothelium independent (SNP) $p=0.49$.

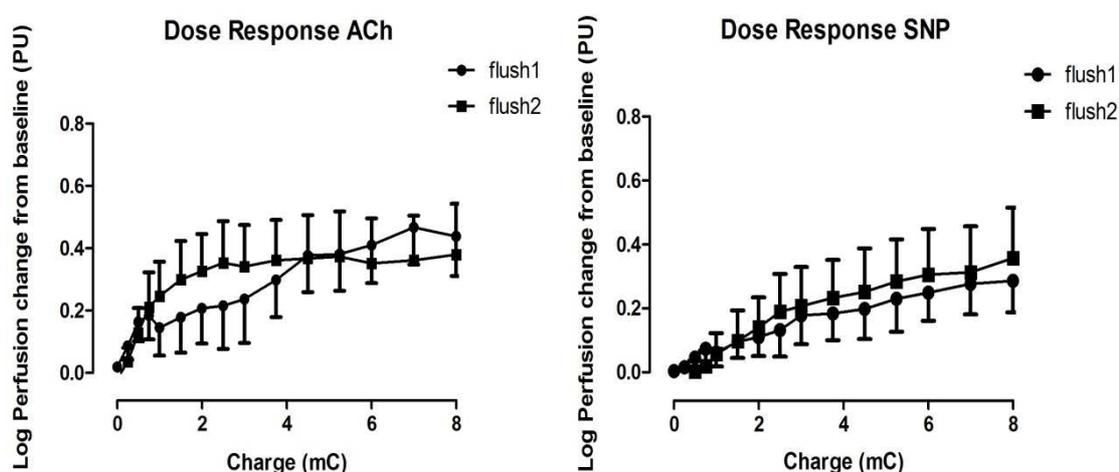


Figure 82: Dose response curves

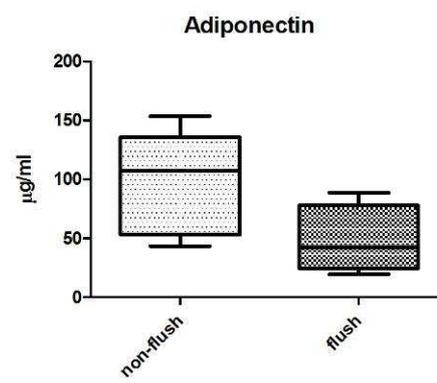
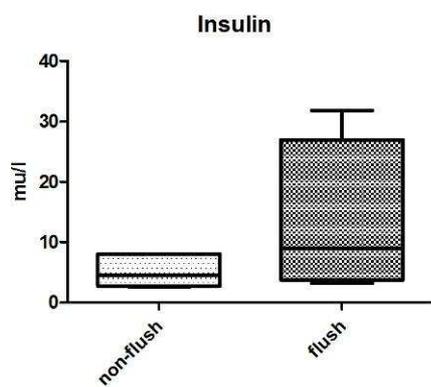
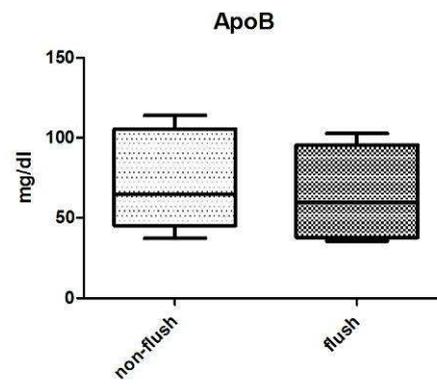
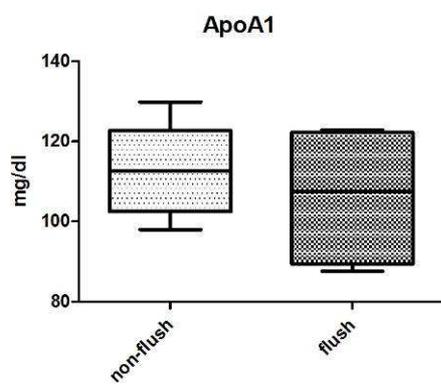
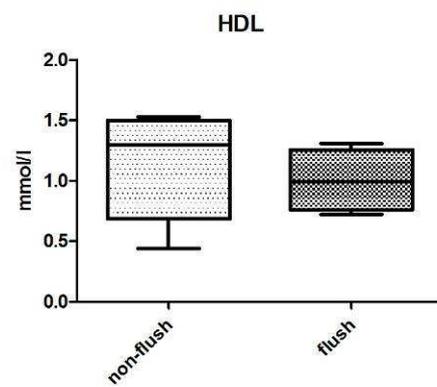
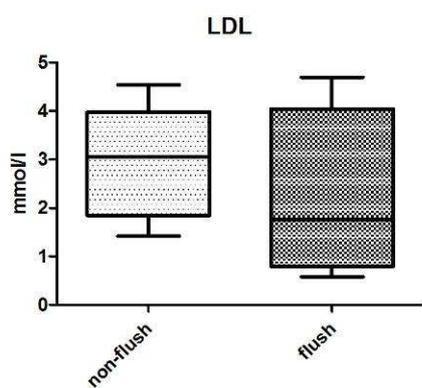
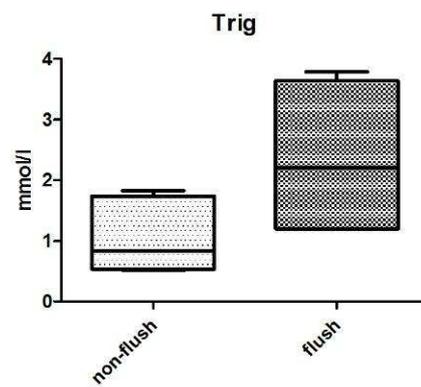
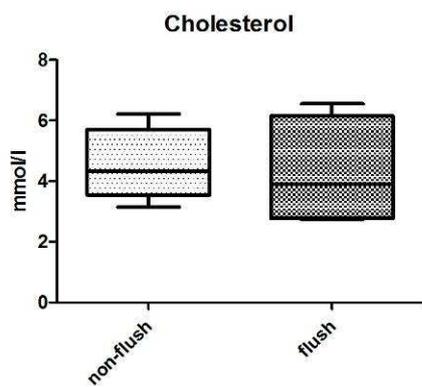
Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in flushing men following commencement of androgen deprivation therapy at week 8 (flush 1) and week 24 (flush 2). Data are mean \pm SEM.

Plasma analysis

In Chapter 3, it was demonstrated that women with flushing had significantly lower HDL-cholesterol levels, lower apolipoprotein A1 and higher I-CAM levels than their non-flushing controls.

As the perfusion responses for flushing hypogonadal men when compared to their non-flushing counterparts were similar to that seen with flushing versus non-flushing postmenopausal women, the same measures were analysed in this group of men (see Figure 83 and Table 28).

Tumour necrosis factor (TNF) alpha was higher in men who developed flushing (P=0.03) when compared to those men who did not develop flushing.



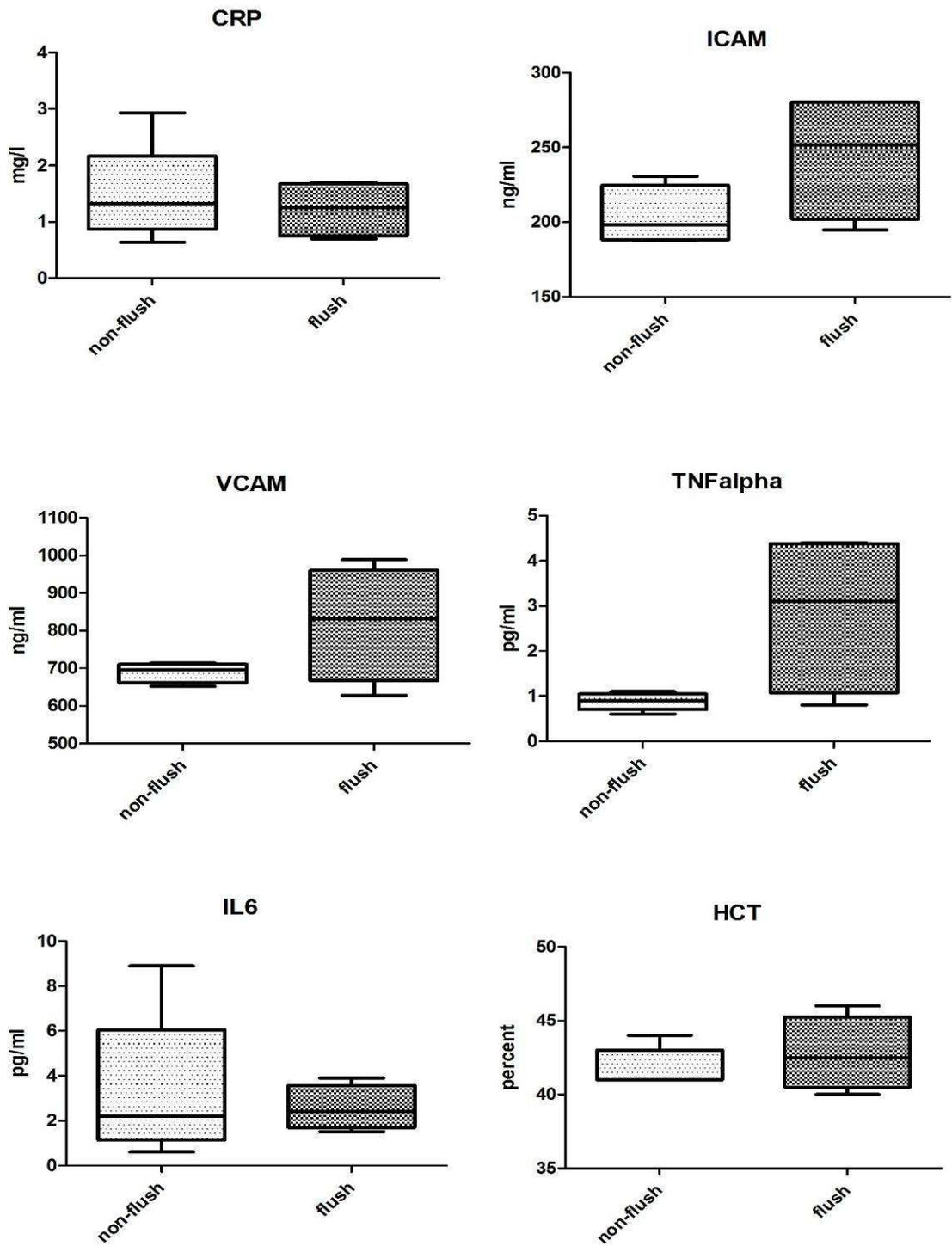


Figure 83: Plasma analysis

Data are shown as box and whisker plots with median, IQR and range.

	Flush n=4	Non-flush n=6	P
Cholesterol (mmol/l)	4.3 (3.6-5.7) [3.1-6.2]	3.9 (2.8-6.1) [2.8-6.6]	0.65
Triglyceride (mmol/l)	0.8 (0.5-1.7) [0.5-1.8]	2.2 (1.2-3.6) [1.2-3.8]	0.90
Low Density Lipoprotein (LDL)-cholesterol (mmol/l)	3.1 (1.9-4.0) [1.4-4.5]	1.8 (0.8-4.0) [0.6-4.7]	0.31
High Density Lipoprotein (HDL)-cholesterol (mmol/l)	1.3 (0.7-1.5) [0.4-1.5]	1.0 (0.8-1.3) [0.7-1.3]	0.85
Apolipoprotein A1 (mg/dl)	112.6 (102.5-122.7) [97.9-129.8]	107.5 (89.5-122.2) [87.6-122.9]	0.37
Apolipoprotein B (mg/dl)	65.0 (45.3-105.6) [37.3-113.9]	59.8 (38.0-95.6) [35.5-102.7]	0.68
Insulin (mu/l)	4.6 (2.8-8.1) [2.6-8.1]	9.0 (3.8-27.0) [3.2-31.8]	0.24
Adiponectin (µg/ml)	107.6 (53.3-135.7) [43.6-153.6]	42.3 (24.5-77.9) [19.6-88.8]	0.09
C-Reactive Protein (CRP) (mg/l)	1.3 (0.9-2.2) [0.6-2.9]	1.2 (0.8-1.7) [0.7-1.7]	0.71
Inter-Cellular Adhesion Molecule 1 (ICAM-1) (ng/ml)	198.3 (188.2-224.7) [187.6-230.7]	251.7 (201.9-280.5) [194.8-280.6]	0.14

Vascular Cell Adhesion Molecule 1 (VCAM-1) (ng/ml)	696.1 (661.5-651.7) [651.7-713.7]	831.6 (667.6-960.1) [627.1-988.7]	0.15
Tumour Necrosis Factor alpha (TNF α) (pg/ml)	0.9 (0.7-1.1) [0.6-1.1]	3.1 (1.1-4.4) [0.8-4.4]	*0.03
Interleukin 6 (IL6) (pg/ml)	2.2 (1.2-6.1) [0.6-8.9]	2.4 (1.7-3.6) [1.5-3.9]	0.93
Haematocrit (percent)	41 (41-43)[41-44]	43 (41-45)[40-46]	0.25

Table 28: Plasma analysis

Data are median (interquartile range)[range]. Statistical analysis was performed using students t-test on log transformed data. *statistically significant.

Medical student's intercalated BSc project

Acknowledgement

The work presented in this section, formed the basis of a project carried out by Jennifer Cruikshank as part of her intercalated BSc project under my supervision. Jennifer recruited a number of healthy volunteers to act as controls for the prostate cancer group. Participant assessments were supervised, and full analyses of the data have been completed as part of the work that I present in this thesis.

Introduction and aim of project

Endothelial dysfunction is associated with both acute and chronic inflammation (275-277), and it is now thought that cancer, irrespective of its trigger, is accompanied by a chronic inflammation, which supports the progression of most, if not all, tumours.

Autoimmune disease (e.g. inflammatory bowel disease) and inflammatory conditions of unknown origin (e.g. prostatitis is associated with prostate cancer) are recognised as triggers of chronic inflammation associated with cancer development (278).

This project investigated endothelial function, as assessed by cutaneous microvascular perfusion, at baseline (i.e. before androgen ablation therapy) in men with prostate cancer and compared it to healthy matched controls.

Hypothesis

Cutaneous microvascular perfusion responses to vasoactive agents are reduced in men with prostate cancer compared to healthy controls.

Methods

Participants

6 healthy controls without prostate cancer were recruited from within the medical faculty. Participating men are all aged 55-75 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function.

LASER Doppler Iontophoresis, Plasma analysis, and Statistical analysis

As described above and in Chapter 2.

Results

Demographics

A total of 12 men with prostate cancer and 6 healthy controls were recruited. All had baseline cutaneous perfusion measurements carried out using LASER Doppler imaging and iontophoresis.

	Prostate cancer	Healthy controls	P
n	12	6	
Age (years)	70 (66-74) [54-75]	62 (62-71) [61-72]	0.11
Body Mass Index (BMI)	26.8 (24.4-29.6) [23.3-40.2]	27.8 (23.4-28.7) [21.9-29.1]	0.75
Systolic Blood Pressure (SBP)*	147.0 ± 20.1	136.0 ± 9.8	0.27
Diastolic Blood Pressure (DBP)*	85.4 ± 7.9	85.6 ± 2.9	0.95

Table 29: Demographic characteristics of men with prostate cancer and healthy controls
Data are median (interquartile range)[range]. Statistical analysis was performed using the Mann-Whitney U test. All men are non-smokers. *Data are mean ± SD, analysis by unpaired T-test.

Responses to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

Vascular reactivity for 12 men with prostate cancer at baseline (i.e. before androgen ablation) and 6 healthy controls was measured using LDI + Ion. Data was analysed as described above.

The response of the subcutaneous vessels was greater in the healthy control group, following administration of both the endothelium-dependent (ACh, $p < 0.001$) and independent vasodilators (SNP, $p < 0.001$) (see Figure 84).

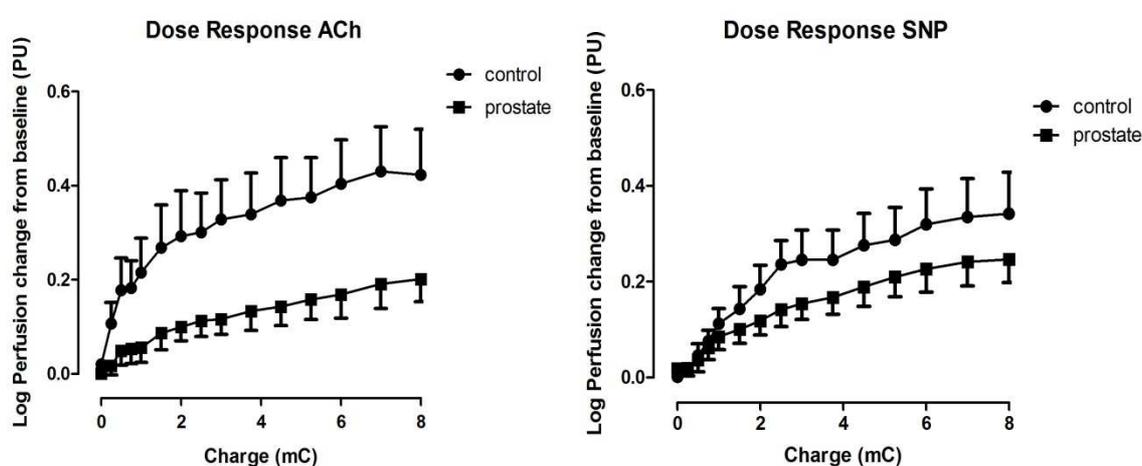


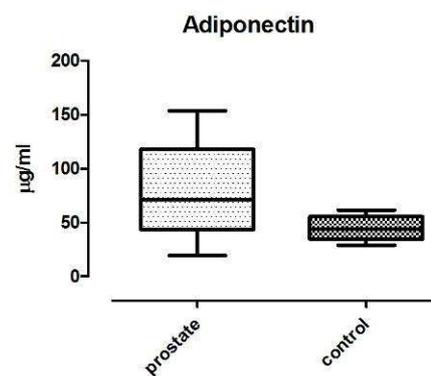
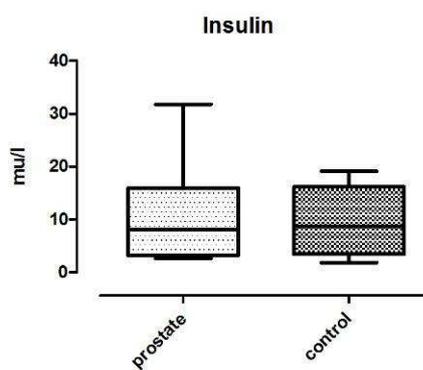
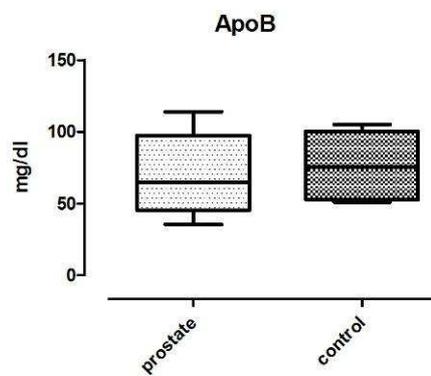
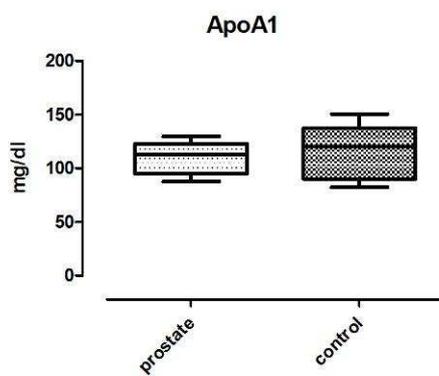
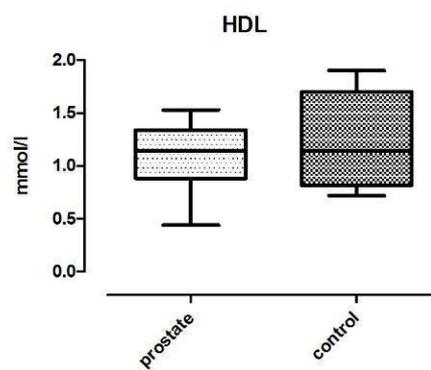
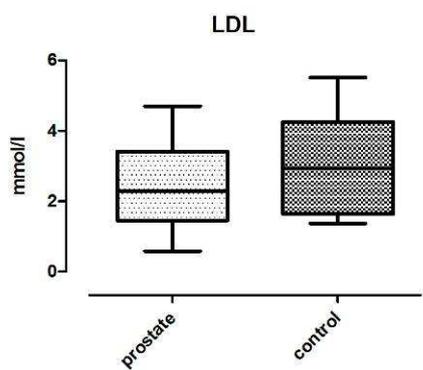
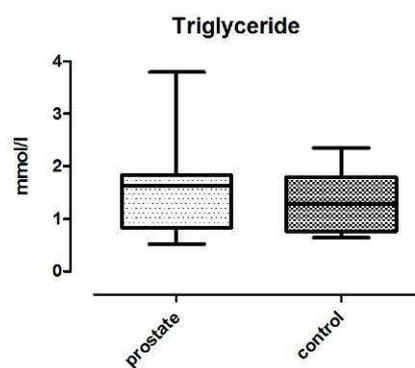
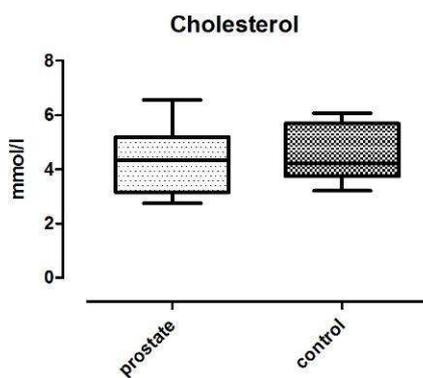
Figure 84: Dose Response Curves

Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in men with prostate cancer before androgen ablation therapy (prostate) and in healthy matched controls (control). Data are mean \pm SEM.

Plasma analysis

Inflammation with associated release of inflammatory markers is closely linked to endothelial dysfunction, and is also thought to play a role in the development and progression of tumours.

In these groups, VCAM-1 ($p=0.02$) and IL-6 ($p=0.048$) were both significantly higher in the prostate group than in healthy controls, see Figure 85 and Table 30.



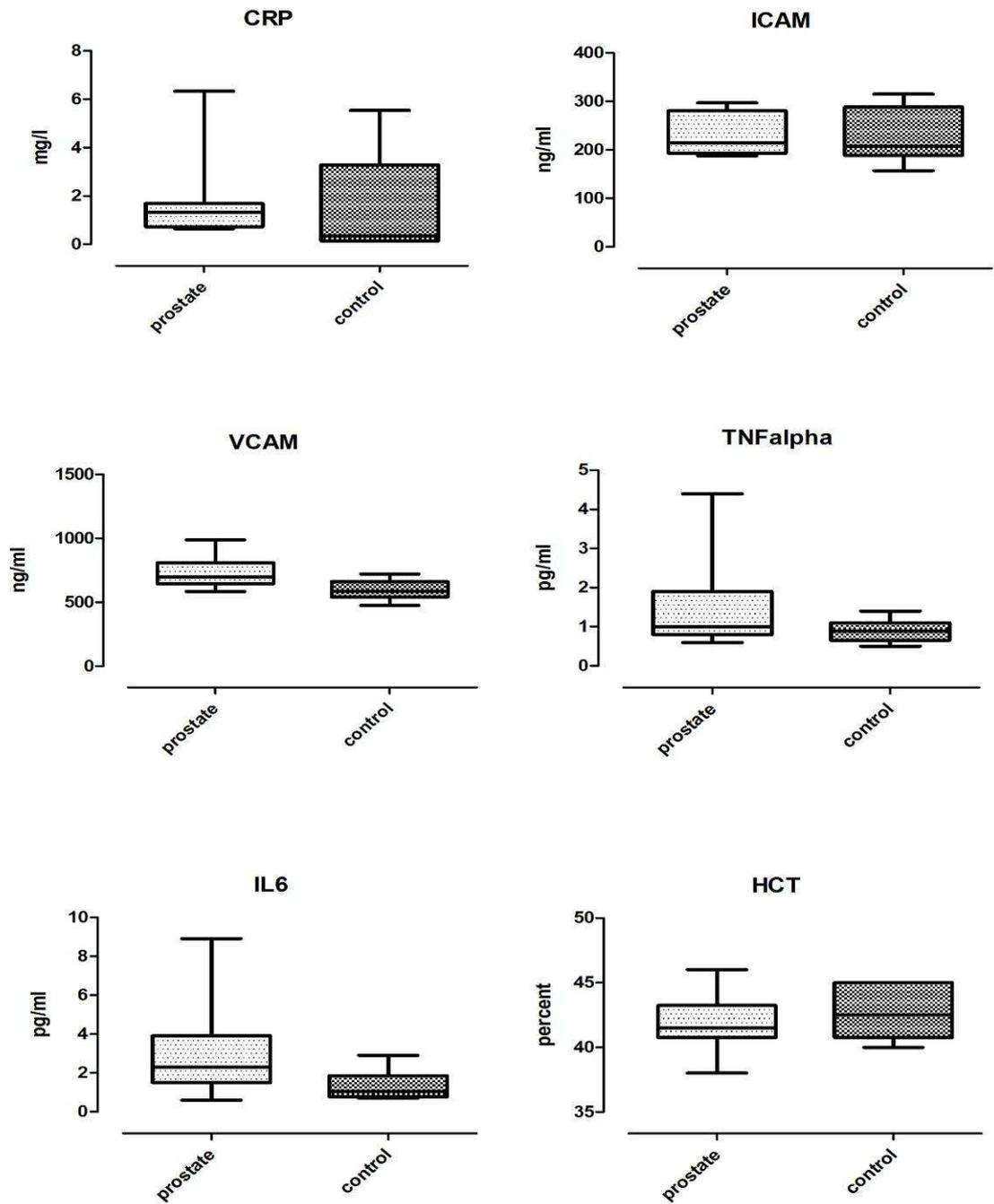


Figure 85: Plasma analysis, men with prostate cancer compared with healthy controls
Data are shown as box and whisker plots with median, IQR and range.

	Prostate n=12	Control n=6	P
Cholesterol (mmol/l)	4.3 (3.1-5.2) [2.7-6.6]	4.2 (3.7-5.7) [3.2-6.1]	0.76
Triglyceride (mmol/l)	1.6 (0.8-1.8) [0.5-3.8]	1.3 (0.8-1.8) [0.6-2.4]	0.67
Low Density Lipoprotein (LDL)-cholesterol (mmol/l)	2.3 (1.5-3.4) [0.6-4.7]	2.9 (1.6-4.3) [1.4-5.5]	0.47
High Density Lipoprotein (HDL)-cholesterol (mmol/l)	1.1 (0.9-1.3) [0.4-1.5]	1.1 (0.8-1.7) [0.7-1.9]	0.59
Apolipoprotein A1 (mg/dl)	112.6 (95.0-122.9) [87.6-129.8]	120.3 (90.1-137.3) [82.4-150.4]	0.58
Apolipoprotein B (mg/dl)	65.0 (45.4-97.3) [35.5-113.9]	75.3 (52.8-100.5) [51.1-105.0]	0.52
Insulin (mu/l)	8.1 (3.2-15.9) [2.6-31.8]	8.6 (3.4-16.2) [1.8-19.1]	0.86
Adiponectin (µg/ml)	71.6 (43.6-117.9) [19.6-153.6]	43.8 (34.5-55.7) [28.9-61.7]	0.11
C-Reactive Protein (CRP) (mg/l)	1.3 (0.7-1.7) [0.6-6.3]	0.3 (0.1-3.3) [0.1-5.5]	0.11
Inter-Cellular Adhesion Molecule 1 (ICAM-1) (ng/ml)	214.9 (193.2-280.2) [187.6-296.3]	208.1 (188.8-288.5) [157.1-314.8]	0.89

Vascular Cell Adhesion Molecule 1 (VCAM-1) (ng/ml)	696.1 (645.6-810.4) [584.2-988.7]	584.8 (540.4-660.7) [474.1-720.8]	*0.02
Tumour Necrosis Factor alpha (TNF α) (pg/ml)	1.0 (0.8-1.9) [0.6-4.4]	0.9 (0.7-1.1) [0.5-1.4]	0.21
Interleukin 6 (IL6) (pg/ml)	2.3 (1.5-3.9) [0.6-8.9]	1.1 (0.8-1.9) [0.7-2.9]	*0.048
Haematocrit (percent)	42 (41-43)[38-46]	43 (41-45)[40-45]	0.46

Table 30: Plasma analysis

Data are median (interquartile range)[range]. Statistical analysis was performed using students t-test on log transformed data. *statistically significant.

Summary of Results

Perfusion response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP)

- The response of the subcutaneous vessels was greater in men who flushed after commencement of androgen ablation therapy than in those who did not.
- There was no difference detected between men who developed flushes and those who did not when examining the perfusion responses at baseline, i.e. before any treatment had been commenced.
- In those participants who flushed, there was no difference in perfusion responses at week 24 when compared with perfusion responses at week 8.
- Perfusion responses were greater in the healthy control group than in the prostate cancer group at baseline.

Diary data

Data are too limited to analyse.

Plasma analysis

Tumour necrosis factor (TNF) alpha was higher in flushing men compared to those men who did not develop flushing.

VCAM-1 and IL-6 were both significantly higher in the prostate group than in healthy controls.

Discussion

It would appear that testosterone depleted gentlemen who develop hot flushes, do indeed have similar cutaneous microvascular responses to that seen in postmenopausal women with severe flushing when compared to their non-flushing contemporaries. These results would appear to be consistent with flushing as a result of hormone withdrawal, as onset of flushes appear to coincide with when one would expect levels of testosterone to reach castrate levels.

Frödin (148) demonstrated that the hot flush is associated with increases in skin blood flow and water evaporation at the time of the 'attack'. We have demonstrated increases in cutaneous microvascular perfusion in response to vasoactive substances outwith a flushing episode. This suggests that following withdrawal of testosterone, in this case as a result of androgen ablation therapy, cutaneous vessels become more readily vasodilated in some than in others. What is the mechanism underlying this change?

The absence of a demonstrable difference at baseline between those that developed hot flushes and those who did suggest that whatever this change is, it occurs following the commencement of androgen ablation therapy.

Steroid hormones may alter vascular reactivity; and several studies have shown that short-term administration of testosterone in men with coronary artery disease improves endothelial vasomotor function (279, 280). So too in women; higher total testosterone and SHBG have been found to be inversely related to carotid atherosclerosis in postmenopausal women (281), and parenteral testosterone in women taking oestrogen increased FMD (282).

Oestrogens and dehydroepiandrosterone (DHEA), an important prohormone of oestrogens and androgens, both stimulate nitric oxide (NO) synthesis via induction of eNOS (283, 284). Testosterone has been shown to act as a direct vasodilator (285-287), but the precise mechanisms by which it is acting are still unknown. Some suggest the role of nitric oxide (NO) (288, 289), but others have denied this (290, 291). It has also been suggested that the key mechanism

underlying testosterone-induced vasodilatation may be modulation of vascular smooth muscle ion channel function (292).

Because 17beta-estradiol causes acute and long-term vasodilatation and testosterone and estrogens share the same biosynthetic pathway, it has been suggested that testosterone-induced vasodilatation might be an indirect effect of the local conversion of testosterone to 17beta-estradiol by vascular P450-aromatase. However, this has been excluded, because, inhibition of P450 does not inhibit testosterone vasodilatation, estrogen receptor antagonism does not inhibit, and non-aromatisable metabolites of testosterone also cause vasodilatation.

Why then, in our group of men, who are all testosterone deplete as a result of ADT, do those with flushes have increased perfusion responses compared to those without flushes?

It has been suggested that the pathophysiological mechanism underlying a hot flush following hormone withdrawal is well defined (293); an LHRH agonist-induced noradrenaline flood of the hypothalamus, resulting in poorly regulated vasodilation. This still does not explain the absence of flushing in some individuals, when all are hormone-deplete or the improvement in symptoms with the passage of time.

It has been well described in postmenopausal women by Freedman et al, that there is a narrowing of the thermoregulatory zone, so that heat loss responses are triggered by only small increases in core temperature. Given the similar peripheral responses seen in men with flushing as a result of hormone withdrawal to that seen in postmenopausal women, it is possible that this mechanism underpins the responses seen here too.

In chapter 3, we demonstrated that postmenopausal women with flushing had decreased levels of HDL-cholesterol and apolipoprotein A1, and increased levels of ICAM-1. Low levels of HDL-cholesterol are associated with an increased risk of cardiovascular disease, which is thought to be the end result of atherosclerosis. ICAM-1 is a biomarker, stimulated by inflammatory cytokines, associated with the chronic inflammation thought to play a role in

atherosclerosis. Paradoxically, it was the women with flushing who had a worse cardiovascular risk profile, with biomarkers for endothelial dysfunction that had 'better' endothelial function than their non-flushing counterparts.

Whilst we have not demonstrated differences in HDL, apolipoprotein A1 or ICAM-1, we have demonstrated that levels of TNF-alpha, which plays a role in endothelial dysfunction and mediating cardiovascular disease, were higher in those men who developed flushing compared to those who did not. Again in the presence of 'better' endothelial function.

While an association between total cholesterol, elevated LDL-C and low HDL-C and endothelial dysfunction has not been consistently demonstrated, TNF-alpha is a proinflammatory cytokine which induces vascular dysfunction by endothelial cell activation, barrier dysfunction and increases in endothelial cell permeability. It also has deleterious effects on endothelium-dependent and nitric oxide (NO)-mediated vasodilatation, and it diminishes the production of and enhances catabolism of NO. Like the paradoxical results seen in women (described in chapter 3), increased levels of TNF-alpha and 'better' endothelial function in men who flushed might further support the hypothesis that the presence of flushing may signpost underlying cardiovascular disease.

Certainly, in contrast to the well-established concept that testosterone had deleterious effects on the heart and vasculature, carotid intima-media thickness has been shown to be inversely correlated with testosterone levels (294), and low serum testosterone levels have been associated with an increased risk of death from cardiovascular disease (295). Despite this, a contribution of androgen deprivation therapy (ADT) to CVD and diabetes mellitus (DM) is uncertain. An association has been shown for ADT with CVD, fatal myocardial infarction and DM (296-298), but an analysis of randomised controlled trials has found no dose-response relationship of ADT to CVD (299).

Mixed information surrounding ADT exists, but even mildly reduced testosterone levels have been shown to be associated with type 2 diabetes, obesity and dyslipidaemia (300). Some animal studies (301) have suggested attenuation of atherosclerosis development with testosterone treatment, however there is no supporting human evidence. It has been suggested, however, that there may be

an “optimal window” of testosterone concentration (302), where adverse effects may be expected in concentrations outside of this window. This might be similar to the “window of opportunity” and cardiovascular disease in newly postmenopausal women.

Despite uncertainty surrounding the trigger and pathophysiology of flushing, as a result of hormone withdrawal in both men and women, treatments are certainly similar. Both hormonal and non-hormonal treatments are common to both. Transdermal oestrogen patches (303), diethylstilbestrol (DES) (304) and synthetic progestagens (305) have been used with good effect, and as have gabapentin, venlafaxine (306) and other selective serotonin reuptake inhibitors (307), with poor results from clonidine (308). Equivocal results are reported for alternative therapies such as acupuncture (309) and vitamin E (149).

Specific to this group, however, is the likely presence of chronic inflammation, widely accepted to play a role in the promotion of cancer cell survival. We have demonstrated that perfusion responses to acetylcholine and sodium nitroprusside are decreased in men with prostate cancer when compared to healthy controls. Underlying endothelial dysfunction, in the presence of chronic inflammation associated with cancer, resulting in reduced vascular responses to vasoactive agents may explain this result. However, whether this difference is specific to prostate cancer, or is as a consequence of the presence of cancer-associated inflammation, is unknown. Ethical approval has been granted to study peripheral vascular reactivity in a group of men with a non-hormone dependent cancer, but work is not yet completed.

Discussion and Future Research

Final Discussion

Menopause is a word used by many to describe, not the defined singular time point identified in retrospect, but a time period. The STRAW Working Groups have described reproductive ageing as a process, and identified its seven stages (11), but for women it is 'the menopause' and it is often met with dread for the symptoms that accompany it.

Whilst we now know that cessation of menstrual cycles is a result of ovarian follicle decline and associated reduction in ovarian follicular hormones, we are still somewhat uncertain as to the exact mechanism underlying the most commonly reported symptom of this process. Bloodletting, thankfully, has been discontinued as a therapy for the "hysterical fits" associated with the syndrome of the menopause, and we now know that oestrogen is effective for vasomotor symptoms.

However, beyond replacing that which we know to be reduced at the same time as the symptoms occur, our understanding is somewhat limited.

We know that flushing bears a resemblance to physiological heat loss mechanisms, that is, peripheral vasodilation and sweating, and there has been much focus on central thermoregulatory mechanisms. Freedman's group has described a narrowing of the thermoregulatory zone so that only small increases in core temperature are required to trigger heat loss mechanisms. They also found that oestrogen replacement increased the lowered sweating threshold.

However, this mechanism proposes that central control is primarily/solely responsible. There has been little work demonstrating the role of the effectors of this mechanism. We know that there is increased blood flow to the hand during a flushing episode, and we also know that there is diminished vasoconstrictor response to cold. It has further been suggested that the rate of temperature change may play a role. Could there be an independent role of cutaneous microvasculature beyond merely acting as an effector?

In this thesis I have focused on alterations in cutaneous microvascular perfusion in postmenopausal women with severe flushing, factors which may influence that vascular function, and the effect of successful treatment of those flushes.

A peripheral role

There appears to be increased cutaneous vasodilation in those women with severe flushing, suggesting 'better' endothelial function, despite a worse cardiovascular risk profile. Evidence exists to support peripheral vascular function acting as a prognostic tool for coronary vascular function; however a greater vasodilatory response is usually considered a sign of vascular health, suggesting that these results are contradictory. There are also suggestions that postmenopausal flushing may be a marker of underlying cardiovascular disease. It is possible that on a background of endothelial dysfunction, with associated reduced nitric oxide, and therefore increased sensitivity, that the introduction of nitric oxide via acetylcholine or sodium nitroprusside may result in an increased rebound vasodilatory response in women with flushing.

Whilst, there are reports of vasomotor symptoms and an association with cardiovascular risk, this is not universal.

A role for neurotransmitters

Additionally, there could be heat loss mechanisms activating vasodilation via alternate pathways despite endothelial function, or an overshadowing of peripheral response by the central control. Noradrenaline is thought to be one of the primary neurotransmitters responsible for alterations in this central 'thermostat'. Animal studies show increased levels of noradrenaline after castration, and in symptomatic postmenopausal women, hot flushes can be provoked by increasing levels of noradrenaline with alpha₂ antagonists. The sweating threshold can be increased, and the thermoneutral zone widened, with flushes ameliorated with the alpha₂ agonist, clonidine.

In our group, clonidine reduced the number and severity of flushes but this was not superior to placebo. Interestingly, whilst there was no difference in ACh- or SNP-stimulated cutaneous microvascular vasodilation following clonidine

treatment when compared to placebo treatment, there was an increase in perfusion response after clonidine and also after placebo when compared to no treatment.

The placebo effect is well recognised; in flushing related studies, there are reports of 25-75% reduction in hot flushes in those receiving placebo (310, 311), and as well as long-documented analgesic effects, studies have also shown placebo-related reduction in β -adrenergic activity in the heart, and immune modulation. This is clearly an area of particular interest in those women who cannot use hormones or other pharmaceutical therapies.

The effect of successful treatment

Serotonin, like noradrenaline, is associated with thermoregulation, and again a relationship with oestrogen withdrawal has been demonstrated. Furthermore, SSRIs, designed to increase serotonin availability, are known to be effective in hot flushing. We have demonstrated a reduction in flush frequency and severity with venlafaxine (SNRI, but SSRI activity at low doses), and a decrease in cutaneous microvascular vasodilation after treatment. No relationship was demonstrated between flush reduction and perfusion; however central serotonin reuptake transporter binding was correlated with depression score reduction following treatment.

Most studies have failed to show a direct relationship between antidepressant occupancy of SERT and treatment response, despite the efficacy of SSRIs being reasonably well established and a necessary minimum of 80% occupancy of SERT for SSRI treatment of depression stated by most pharmacokinetic studies (231). More recently, it has been suggested that SSRI treatment response in major depressive disorder can be predicted by pre-treatment binding in the median raphe nucleus (232) and the most recent work from Freedman's group has identified a rise in brainstem activity in fMRI preceding a hot flush (71). Additional activations were also noted in the forebrain, where projections from the raphe nuclei extend. If, as Freedman suggests, forebrain activity is associated with the phenomenological correlates following brainstem activation, could there be some dysfunction in the projections, i.e. the experience of the flush. If a hot flush is a dysfunction of the central thermostat as a result of

hormone withdrawal, this would be present in all, but if the response or experience of that central thermostat dysfunction is mediated by the projections to the forebrain and these are altered in some way, that may explain why some have flushes and others do not, when the same physiological event has occurred in all. If they can predict the anti-depressant effect, then perhaps we can predict, in some way, those who will suffer flushing.

The absence of correlation between peripheral perfusion and central serotonin reuptake transporters may reflect vasoconstriction as a result of direct 5HT receptor activation on endothelial cells with discrete central mood effects. The significant reduction in BDI scores following treatment, in the absence of severe depression is interesting, and its relationship with SERT binding, novel, suggesting the importance of venlafaxine in mood alteration beyond severe depression with or without vasomotor symptoms.

Oestrogen receptors are also present on endothelial cells and as the most commonly used, and most effective, treatment for vasomotor symptoms, cutaneous microvascular perfusion was assessed following 8 weeks of HRT, and demonstrated an increase in both endothelium dependent and independent vasodilation. Improvements in GCS scores were also demonstrated.

The effect of obesity

An obvious factor to consider when assessing endothelial function, and vasomotor symptoms, is the effect of obesity. The 'thin hypothesis' has all but been stamped out, and it is widely accepted that an increase in adiposity is associated with an increased risk of flushing. But, is it as a result of altered microvascular perfusion responses, or simply a consequence of excess insulation?

One might expect that endothelial function would be altered in obese participants; metabolic syndrome secondary to obesity has been shown to result in impaired vasodilator responses to acetylcholine, however there were no differences demonstrated between obese and lean participants at baseline. This may be in part due to the exclusion criteria, meaning that these were basically healthy women with no hypertension or diabetes.

If women with severe vasomotor symptoms are at increased cardiovascular risk, this apparent similarity in perfusion responses of obese and lean women may be explained by the underlying cardiovascular risk. Endothelial dysfunction seen in obesity may be similar to that seen in flushing women, thereby negating any differences in perfusion responses that we might expect to see at baseline.

We have, however, not assessed body fat distribution and this, in addition to consideration of perivascular fat and its ability to regulate vascular tone, may be important.

What about men?

Research suggests that hot flushes are common in androgen-deprived men; in prostate cancer therapy, flushes are the most significant adverse QoL symptom and in some may not resolve even after discontinuation of therapy. In contrast, most men in this group did not experience flushing, and for this reason, numbers available for comparison are small. There are other reasons, including difficulty in recruiting men without significant co-morbidities, however, despite this, hypogonadal men with flushing demonstrated similar cutaneous microvascular perfusion responses to ACh and SNP as postmenopausal flushing women. That is, perfusion responses in men were similar prior to treatment, in those without flushing compared to those who went on to develop flushing.

ACh- and SNP-stimulated vasodilation was, however, reduced when compared to healthy controls and this may reflect endothelial dysfunction associated with the chronic inflammation known to accompany or even drive malignancy.

Limitations

Sample size

The power calculation determined that a sample size of 20 would be required to determine a 25% change in ACh between groups. This would suggest that a number of groups within the study were too small to generate reliable findings, however, this calculation is based on a two sample t-test, and in fact smaller groups are required when a paired test is carried out.

A posthoc power calculation with adjustment for a paired t-test reveals that a sample size of 11 would be sufficient to determine a 25% change in ACh at $\alpha = 0.05$, with a power of 0.86. The sample size in the HRT group comparing perfusion response following treatment would therefore be sufficient, although a larger group would be preferable. Numbers in the subgroup analysis of obese versus lean are too small to generate reliable findings.

The numbers of men in this study are also small, and further recruitment is planned to confirm results.

Choice of drug

Venlafaxine

This drug was chosen as it has been widely studied in randomised controlled trials (RCTs) and is known to be effective in reducing the number and severity of flushes. Furthermore, this drug is used widely as an acceptable and safe treatment in women with breast cancer taking tamoxifen. Whilst it is thought to act as a pure serotonin reuptake inhibitor at low doses, we cannot completely exclude the role of noradrenaline entirely. Ideally the results seen here with this drug would be compared to a pure selective serotonin reuptake inhibitor (SSRI).

HRT

The type of HRT was not standardised for the purpose of this study, as the purpose was not to compare the results of varying preparations of HRT on vascular function but to assess changes in skin perfusion with improvements in flushing with treatment with HRT. However, I acknowledge that different preparations with different progestagens may have influenced the perfusion results and certainly may have had an impact on lipids in plasma analysis.

Diaries

The diary data for the HRT group are disappointing, and most likely the cause of a failure to demonstrate an improvement in flushing with treatment. HRT is widely accepted, and long-proven, to be the most effective treatment for vasomotor symptoms. Ideally those participants who had not completed pre-treatment diaries would have been excluded from the study.

Plasma analysis

Whilst the plasma analysis yielded some interesting results, the nature of this work was exploratory rather than strictly hypothesis driven. The aim was to study factors which may influence vessel function and therefore might impact upon the changes that we saw in perfusion responses as a result of treatment aimed at improving hot flushes. Therefore, before any definitive conclusions could be drawn, hypothesis directed studies would need to be designed.

Future Research

Though the work presented in this thesis has determined some novel concepts, confirmation through further exploration of these findings is required. Areas and suggestions for possible future research are outlined below.

In Chapter 5, the successful treatment of vasomotor symptoms with the serotonin and noradrenaline reuptake inhibitor demonstrated significant decreases in microvascular vasodilation, although a relationship between these was not shown. The duration of treatment is short relative to the time women may experience symptoms and it would be interesting to repeat LDI+ION at a other time points to determine whether there is a degree of initial vasoconstriction which may resolve with time and which may, as a consequence, result in a resurgence of symptoms, as receptor availability is altered.

There was no placebo group in this study, it may be considered unethical to perform SPECT scanning with placebo treatment, however the use of placebo in flushing, and its associated physiological responses, certainly warrant further exploration.

The absence of any stimulated vasodilation differences between obese and lean women defies what we might expect to see. Weight loss is known to have a beneficial effect on endothelial function and there is some data to suggest that it may decrease hot flushes. What effect would there be on perfusion responses and would there be a correlation with flush frequency?

The HRT group was, admittedly, poor, and should be repeated. There should also be completion of recruitment and further analysis of hypogonadal men with prostate cancer; the results have shown some interesting findings which would be better demonstrated with a larger cohort, particularly the resolution of flush symptoms over time and the relationship with perfusion responses. Recruitment to the group of men with a non-hormone dependent cancer should also be completed.

Calcitonin gene-related peptide (CGRP), and adrenomedullin, have also been of interest in flushing as both are potent vasodilators that can cause facial flushing,

and have been shown to be increased at times of flushing in both men and women, however a link with steroid hormones has yet to be established (312, 313). It has been suggested that CGRP and/or substance P may be involved in the perception of local skin warming by nociceptive afferent fibres, which have been shown to be more closely related to vasodilator response than actual temperature (314).

Breast cancer is the most common cancer in women in the U.K. and up to 85% of women treated for breast cancer are affected by hot flushes and night sweats. 10 years of tamoxifen significantly reduces the risk of recurrence and breast cancer mortality (315, 316), however over 50% of women do not adhere to the 5 years of endocrine treatment with an associated increase in mortality (317), perhaps, partly, as a result of ineffective management of vasomotor symptoms (318). SNRIs are commonly used as oestrogen is often contraindicated, cognitive behavioural therapy (CBT)(319) has also been shown to be of benefit. Further study of the physiological responses, both peripheral and central, seen after successful CBT treatment would be of interest. Research into other treatments for flushing in these women also requires urgent attention.

Conclusion

In this thesis I have explored the role of skin blood flow as a measure of a peripheral component in the underlying mechanism of menopausal hot flushes. I have provided evidence that alterations in vasodilation are present and there may be a suggestion that cardiovascular risk factors play a part.

Similarities in cutaneous microvascular perfusion responses are present in men, following hormone withdrawal, supporting the role of skin blood flow in the pathophysiology, but the effect of obesity does not appear to be modulated through alterations in vascular reactivity.

The role of neurotransmitters in thermoregulation is complex. Serotonin is certainly important and modification of this in the treatment of flushing appears to be the best alternative to hormones that we have at present. It appears to alter skin blood flow, and there are certainly positive mood effects, likely as a result of central modulation of SERT, however our understanding is still limited, and more work is required, both peripherally and centrally.

Appendices

Appendix I

Full publications containing work undertaken in this thesis

Appendix II

Press

Volunteers needed for hot flushes university research

HELEN McARDLE

RESEARCHERS in Glasgow want to recruit women for a study investigating the causes "hot flushing", a common symptom of the female menopause.

Dr Jenifer Sassarini, a clinical research fellow at the University of Glasgow, is looking for 40 volunteers aged 45 to 65 to take part in a clinical trial aiming to unravel the physiological mechanisms behind the ailment.

Around 70 to 80% of women in the menopausal transition will experience hot flushing and in 25% of these cases, symptoms will be so severe that they will affect daily life. While the problem generally resolves itself within a year or two, some 15 to 20% of women will continue to experience flushes for 20 years.

As life expectancy is increasing, Dr Sassarini fears this could become a significant problem.

Once admitted on to the study, candidates will undergo a 20-minute laser procedure allowing researchers to scan their arm and look at blood vessels beneath the skin.

They will then be asked to take an eight-week course of the drug, Venlafaxine, and keep a diary recording

their flushing during that period. At the end of the eight weeks, the scan would be repeated.

Data gathered during a study at the university three years ago appears to indicate key physiological differences between women who flush, and the lucky minority who do not.

To volunteer or for more information contact Dr Jenifer Sassarini on 0141 232 9533 or 0141 232 9537 or J.Sassarini@clinmed.gla.ac.uk)

HOT TOPIC FOR STUDY

A UNIVERSITY of Glasgow academic is recruiting women to for a study investigating what causes hot flushing in the female menopause. Dr Jenifer Sassarini, a clinical research fellow at the university, says up to 80 per cent of women will experience the problem. Sue Friel, below, took part in an earlier study and is now receiving effective treatment. The 49-year-old, from Linlithgow, West Lothian, said: "It sounds like a joke but hot flushing is so inconvenient and embarrassing." Dr Sassarini said: "We are looking for women aged 45 to 65, who experience flushing more than 20 times a week." To volunteer, or for more details, call Dr Sassarini on 0141 232 9533, 0141 232 9537 or email j.sassarini@clinmed.gla.ac.uk



HOT FLUSHES

Programme: MacAulay & Co
 Programme Start: 24/02/2010 10:05:00
 Presenter: Susan Calmin & Karen McKenzie
 Item Start: 10:44:51
 Duration: 8:20

STUDIO INTERVIEW: DR JENNIFER SASSERINI [PHONETIC] , GLASGOW UNIVERSITY - a hot flush is when someone feels incredibly warm. We are looking for people who are going through the Menopausal transition. STUDIO INTERVIEW: JOAN MCFADDEN, AGONY AUNT - I researched a piece for the Record on the menopause. Menopause Matters is a website to look at which was set up by Heather Curry.

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