The Effect of Estrogen on the Female Cardiovascular System

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by

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State Exam Med Saarland, FRG 1990
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MRCOG
to my husband
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IGNORANCE

Strange to know nothing, never to be sure
Of what is true or right or real,
But forced to qualify or so I feel
Or Well, it does seem so:
Someone must know.

Strange to be ignorant of the ways things work:
Their skill at finding what they need,
Their sense of shape, and punctual spread of seed,
And willingness to change;
Yes, it is strange,

Even to wear such knowledge - for our flesh
Surrounds us with its own decisions -
And yet spend all our life on imprecisions,
That when we start to die
Have no idea why.

Philip Larkin, 11 September 1955
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Abstract

The use of postmenopausal oestrogen replacement (HRT) has more than trebled over the last two decades. Apart from its effect on menopausal symptoms and consequently on quality of life, many women embark on HRT because of its presumed benefits on long term health, particularly on cardiovascular health. However, some of the effects of oestrogens on the cardiovascular system remain controversial, and the underlying mechanisms are yet to be fully understood.

The present investigation set out to examine the effect of unopposed oestrogen on certain aspects of the female cardiovascular system, both in vivo and in vitro. In the clinical arm of the study, transdermal oestrogen treatment in oophorectomised women was shown to be associated with an overall reduction in ambulatory blood pressure (ABP), whereas with oral therapy ABP remained unchanged. With both delivery systems, ABP increased significantly in a proportion of women. The reason for this is unclear, however, further investigation did not reveal a demonstrable association with molecular variants of the angiotensinogen genotype. Looking at carotid disease in postmenopausal women, oral oestrogen treatment was associated with significant plaque regression, as assessed by ultrasonography. However, the results of this pilot study await confirmation in a definitive trial.

In the in vitro arm of the study, a model for the induction of intimal hyperplasia in human ovarian veins was introduced. Intimal thickness increased significantly in culture, and was attenuated by the addition of oestrogen. The underlying mechanism may be linked to increased VEGF expression in vitro in response to oestrogen, which was also demonstrated in this experiment. When tested in vivo, however, serum VEGF were reduced following exposure to transdermal oestrogen, possibly by reciprocal regulation of VEGF and nitric oxide. Regrettably, the expression of adhesion molecules ICAM-1, VCAM-1 and E-Selectin, known to play a role in vascular response to damage, could not be adequately investigated as planned, because of methodological difficulties.

In conclusion, postmenopausal oestrogen replacement therapy is associated with demonstrable benefits on blood pressure and vascular disease, and is capable of vascular protection in vitro. Further work is needed to fully investigate underlying mechanisms.
1. GENERAL BACKGROUND

1.1 CARDIOVASCULAR RISK AND MENOPAUSE

Cardiovascular disease (CVD) remains the leading cause of mortality in women in the Western world, killing more women than all forms of cancer taken together. On average, women develop CVD about 10-12 years later in life than men. This lag may be even greater for more serious clinical manifestations of myocardial infarction (MI) and sudden death, with a difference of about 20 years. The gender difference is also evident in the pattern of presentation of CVD and its prognosis. In contrast to men, women seem to present more frequently with angina and less frequently with sudden death, but once ischaemic heart disease (IHD) has developed it may entail a worse prognosis for women than for men, with both medical and surgical therapies. Women have a higher rate of early death after MI than men, and in-hospital mortality also seems to be substantially increased. It is uncertain -and controversial- whether this reflects the on average older age of women at presentation, the presence of multiple risk factors, more frequent severe coexistent disease or suboptimal, possibly delayed care.

Arguably, the higher incidence of CVD in older women has been attributed to oestrogen depletion in the climacteric, with a rapid increase after the age of 55. In 1993, the Office of Population Census and Surveys reported the death rates per 1000 women from ischaemic heart disease (IHD) in England and Wales at 1.2 in the age group of 45-54, 4.6 at 55-64 years and 12.4 at 65-74 years, which would...
suggest a leap around ten years after menopause. Numerous studies have investigated the link between CVD and the menopause. Most, but not all, found that the age-adjusted rates rose after the menopause. The Framingham study, in which 1598 women were followed from menopause to death, seems to support the view that the menopause in itself is a significant cardiovascular risk factor. In each age group studied, the incidence of cardiovascular events was significantly higher in postmenopausal than premenopausal women. This seemed to be particularly true about women who have undergone surgical menopause by bilateral salpingo-oophorectomy. A study looking at the incidence of aortic calcification as a marker of atherosclerosis in 600 women described 3.4 fold increase in adjusted atherosclerotic risk following cessation of ovarian function, without a particular difference in the type of menopause.

However, the Nurses' Health Study reported that natural menopause did not seem to be associated with an increase in cardiovascular risk. Further, a study looking at all cause mortality and its relation to age at menopause in a population of around 20000 White Adventist women suggested that, although early age at menopause was associated with excess mortality, particularly from cardiovascular events, there was no evidence that this was related to oestrogen depletion, and oestrogen use at all ages was not associated with reduction in mortality figures. Age at surgical menopause in particular was not related to mortality. They concluded that earlier deaths in women with early menopause may be a reflection of accelerated ageing, of which premature ovarian failure may have been a consequence rather than a cause. Reviews of national mortality data in 1987 presented strong evidence against the role of menopause as an independent cardiovascular risk factor, demonstrating a mortality rate from CVD which was constant from young to old.
age, without a change in the slope of the curve at the time of menopause. Further, if we look at WHO data on mortality figures from circulatory diseases in the UK, the gender gap does not lessen at the time of menopause, or in higher age groups (Figure 1).

![Figure 1: WHO figures on mortality from circulatory disease in the UK 1992](image)

Although epidemiological evidence remains inconclusive, the body of opinion currently seems to favour the view that there may be an association between menopause and cardiovascular risk, as abundant data from animal and human
studies demonstrated adverse effects of oestrogen deficiency on cardiovascular risk factors, particularly the lipid profile. Postmenopausal women have on average a more atherogenic lipid profile than their premenopausal counterparts, with higher plasma levels of total cholesterol, triglycerides, low density (LDL)-cholesterol, very low density (VLDL)-cholesterol, apolipoprotein B and Lipoprotein (a), and with a decline in levels of high density (HDL)-cholesterol and apolipoprotein A.\textsuperscript{12}

Menopausal status may also adversely affect other cardiovascular risk factors, such as impaired glucose tolerance and insulin resistance. Insulin resistance and circulating insulin levels may be higher in older women, but are greater in postmenopausal women independent of age.\textsuperscript{13} Less consistent reports have described associations between menopause, body weight and blood pressure changes. How far weight gain and obesity contribute to a greater cardiovascular risk after the menopause remains unclear, as most studies investigating the subject did not actually confirm a significant weight increase in the menopause.

There is a progressive rise in blood pressure with age. Some authors suggest that menopause -independent of age- is accompanied by a rise in systolic and diastolic blood pressure, with a higher prevalence of hypertension in postmenopausal women.\textsuperscript{14} Postmenopausal women have been shown to display exaggerated stress-induced cardiovascular responses, and to have elevated daytime ambulatory pressures.\textsuperscript{15}
1.2 Cardiovascular Risk and Oestrrogen Replacement

Oral contraceptives containing ethinylestradiol and progestagens increase the risk of vascular events, to which both oestrogens and progestagens may contribute. The effect of unopposed natural oestrogens on the risk of vascular events is dramatically different. A considerable number of epidemiological studies looking at the effect of postmenopausal oestrogen replacement on cardiovascular risk, using recognisable endpoints such as myocardial infarction or ischaemic heart disease, has been published to date, including cohort, cross sectional and case-control studies. Although the studies are from different countries, they are largely consistent. In several prospective studies, postmenopausal oestrogen replacement has been shown to significantly decrease cardiovascular risk. In one study, 2270 females aged 40-69 were evaluated over 8.5 years, and a 66% reduction in fatal coronary events was noted in oestrogen users versus non-users. In the ten-year follow-up from the Nurses’ Health Study the adjusted relative risk of IHD in current oestrogen users was 0.56, and in ever-users 0.79, when compared to never-users.

Stampfer and Colditz reviewed evidence from 31 studies, and found that 25 of them had demonstrated a protective effect which was unlikely to be explained by confounding factors. The cumulative relative risk of CVD in all studies on ever-use of oestrogen was 0.56 (95% Confidence Interval 0.50, 0.61). A further meta-analysis by Grady et al, based on 32 studies, estimated a relative risk of IHD on oestrogen replacement at 0.65 (95% Confidence Interval 0.59-0.71).
Women with non-cardiac vascular disease also seem to benefit from ERT, although the evidence is less consistent. A large Scandinavian study has demonstrated a significantly reduced risk of stroke in women who have had exposure to sex steroids when compared to never-users. Further, several studies have reported on the benefits of oestrogen replacement in women with existing CVD, advocating its use in secondary prevention, to achieve a reduction in recurrent cardiovascular events and cardiovascular mortality. In a retrospective study, the all cause mortality was examined in 1900 women who had angiographically confirmed coronary stenosis of varying degree, and in 446 women with normal coronary arteries. Survival at ten years was significantly higher in oestrogen users than in never-users.

A figure widely quoted is a 50% reduction in the incidence if IHD with oestrogen replacement. This estimate stems from observational rather than randomised studies, thus comparing the outcome of women on HRT in every day clinical practice to that of untreated patients. The number of women involved in these studies would strongly suggest that these findings are true rather than due to chance. Unfortunately, we lack evidence from randomised prospective clinical trials, and it has been argued that the existing epidemiological studies are flawed, particularly with selection bias. Women who come forward requesting postmenopausal oestrogen replacement belong to a higher socio-economic class, may have generally healthier life styles, exercise more, have a lower body weight and access health professionals more frequently than women who do not consider hormone replacement. A recent cohort study looking at lifestyle and health characteristics of 840 women has confirmed that current oestrogen users do indeed
have healthier profiles than non-users. A further prospective study on 541 premenopausal women has shown that women who go on to use HRT have higher levels of HDL cholesterol and alcohol intake, and lower LDL cholesterol, systolic and diastolic blood pressure, body weight and fasting insulin, when compared with women who will not take HRT once postmenopausal.

Nevertheless, taking into account the effects of oestrogen on various cardiovascular risk factors and on blood vessels, which have been consistently reproduced in randomised trials, it would appear biologically conclusive that the observed reduction in cardiovascular risk in oestrogen treated women is genuine.
1.3 Effect of Estrogen on Cardiovascular Risk Factors

1.3.1 Lipid Factors

Cholesterol and triglycerides are essential components of a multitude of biochemical processes, such as hormone synthesis, maintenance of cell membrane integrity, energy metabolism, and inter- and intracellular signalling. Lipoproteins are particles serving as lipid transport vehicles in plasma. Four major classes are distinguished according to their density following ultracentrifugation: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Exogenous fats are absorbed from the intestine as chylomicrons. Endogenous fats are synthesised and secreted by the liver as VLDL, which is metabolised to VLDL remnants through interaction with lipoprotein lipase (LPL). VLDL remnants are then converted to LDL. LDL is the major carrier of cholesterol, transporting up to 70% of the cholesterol to peripheral tissues. LDL is typically cleared from the circulation by hepatic LDL receptors. HDL facilitates the removal of cholesterol from the circulation and from extrahepatic tissues by reverse cholesterol transport, whereby it binds to cell surface receptors via apolipoprotein A, takes up excess cholesterol and re-enters the circulation, only to be cleared by hepatic LDL receptors. Conditions associated with increased LDL levels, either due to increased production or, more commonly, defective clearance mechanisms such as HDL deficiency or malfunction of LDL receptors, result in increased deposition...
of LDL in extrahepatic tissues. Individuals with high LDL and low HDL levels are at an increased risk of atherosclerosis, due to an increased cholesterol deposition in blood vessels, although there may be some gender differences.

The significance of various lipoprotein fractions for the cardiovascular risk may differ in women from that in men. LDL levels in women for example have been shown to be less predictive of cardiovascular risk than in men, possibly because of a higher cholesterol-to-protein ratio in females. Also, the uptake of LDL into the vascular wall may be more efficient in men, so that here relatively lower levels of LDL would be atherogenic. In contrast, HDL levels seem to be the most powerful negative independent predictor of cardiovascular risk in women. Triglycerides appear to be an independent predictor of cardiovascular risk in postmenopausal, but not in premenopausal, women.

LDL particles are without question atherogenic in both men and women, with small, dense LDL-particles being more atherogenic, perhaps due to their susceptibility to oxidisation. All three of the cell types predominant in vascular lesions, endothelial cells, macrophages and vascular smooth muscle cells have the ability to oxidise LDL. Oxidised LDL can be taken up by endothelial cells, macrophages and vascular smooth muscle cells via so called scavenger receptors, which, in contrast to LDL receptors, are not downregulated by an increase in intracellular cholesterol content. This leads to the formation of foam cells, which are involved in atherogenesis.

Lipoprotein (a) (Lp(a)) is a further low density particle which is strongly atherogenic. It has extensive homology with plasminogen, and has been shown to
be an independent risk factor for atherogenesis. Available lipid lowering treatment regimens do not appear to be effective in lowering Lp(a).25

Exogenous female sex steroids in premenopausal women seem to affect lipid profiles adversely, possibly accounting for the substantial increase in cardiovascular risk among combined oral contraceptive pill users. The effect of natural oestrogens however, as used in postmenopausal hormone replacement therapy, seems to produce a largely favourable lipid profile. Large scale studies attribute up to 30-50% of the cardioprotective effect of oestrogen to its action on the lipid profile.17 Oestrogens upregulate the activity of hepatic apoprotein B and E receptors, thus leading to an increased clearance of LDL from the circulation. Also, by upregulating the synthesis of apoprotein A and consequently increasing concentrations of HDL by about 10-20%, oestrogens may enhance reverse cholesterol transport, thus further reducing LDL levels.24,26 LDL levels have been shown to drop by about 10-20% following ERT. There is a small, but significant, reduction in Lp(a) levels following ERT, although this effect has not been extensively studied.27

It is unclear if different routes of administration, types and doses of oestrogen will produce differing lipid profiles, but it has been suggested that oral oestrogen will result in more profound changes due to the hepatic first pass effect. Transdermal oestrogens have been shown to increase HDL levels, albeit to a lesser extent than oral administration routes, but their effect on LDL levels seems negligible. The type and route of oestrogen administration seem to be particularly important for its effect on triglycerides. Conjugated equine oestrogens increase
triglyceride production, whereas oral 17β-oestradiol has little effect. Transdermal oestrogen seems to lower triglyceride levels.28

Although oestrogen stimulates the production of triglycerides and VLDL in the liver, the resulting VLDL particles are large and triglyceride rich. Large VLDL particles seem to be less atherogenic than smaller, denser ones. Further, VLDL clearance is improved with oestrogen.26 Probably most importantly, oestrogen seems to have a potent antioxidant effect, thus rendering potentially harmful lipoproteins less atherogenic.29
1.3.2 Coagulation Factors

It is widely accepted that thrombogenesis as well as atherogenesis are important factors in the development of atherosclerotic vascular disease and related events. It is also known that certain lipid factors will have effect on coagulation and fibrinolysis, such as triglyceride rich lipoproteins and Lp(a), which can impair fibrinolysis.30

Plasma fibrinogen is well established as a cardiovascular risk factor. High fibrinogen levels may lead to an increase in CVD by effects on blood viscosity, increased platelet aggregability, higher amount of fibrin deposited, and possibly effects on atherogenesis. High fibrinogen levels have been linked to CVD, death from cardiovascular causes and fatal and non-fatal stroke. The independent association of fibrinogen with these endpoints is as strong as that of cholesterol, although again there may be a gender difference with the level of association being somewhat weaker in women.4 A further factor linked to CVD is factor VII, a component of the extrinsic coagulation system, which can activate other coagulation factors and thrombin production.31 Antithrombin III (AT III) neutralises thrombin activity and reduces the activity of other coagulation factors. With age AT III levels decline, particularly in men, so that the ability to neutralise pro-coagulant activity is diminished.32
Following cessation of ovarian function, there is a rise in fibrinogen, factor VII and AT III. AT III levels are highest in postmenopausal women, possibly to counteract the generally increased coagulability.\textsuperscript{32} Plasminogen activator inhibitors (PAI 1) levels, on the contrary, have been shown to be elevated in the menopause, thus implying a reduced fibrinolytic potential.\textsuperscript{33} However, there is no evidence of significant changes in fibrin generation or degradation, or thrombin generation.

\textit{Estrogen replacement may reduce fibrinogen levels. In a large study on around 5000 women, Lee et al measured plasma fibrinogen and examined its relationship to oral contraceptive and hormone replacement use.}\textsuperscript{34} They found that women with a history of oral contraceptive use and those on hormone replacement had significantly lower fibrinogen levels than those without \textit{estrogen} exposure.

Antithrombin III activity has been reported to be lowered by \textit{estrogen} or to remain unchanged, depending on the type of \textit{estrogens} used. However, in a study on 1430 subjects from the Framingham Offspring study, age adjusted levels of PAI-1 were significantly lower in \textit{estrogen} users as opposed to non-users.\textsuperscript{33}

It is difficult to accurately gage the clinical significance of the above changes, as some coagulation factors are changed favourably by \textit{estrogen} replacement therapy, and others are modified adversely as far as cardiovascular risk is concerned, and the magnitude of change may depend on type, dose and route of administration of the given \textit{estrogen}. Obviously the balance between clotting and fibrinolysis is redefined. So far, it was presumed to be of little clinical importance, as there was no evidence to support an in-vivo hypercoagulability on \textit{estrogen} replacement. Recently however, relatively large, well designed studies have
demonstrated that thromboembolic risk may be increased with oestrogen replacement.\textsuperscript{35,36} The answer to this problem is far from clear and further, prospective randomised trials with long term follow up are required.

1.3.3 Blood Pressure

Up to 50\% of the female population in the Western world will develop high blood pressure at some time in their lives.\textsuperscript{37} Both systolic and diastolic blood pressure increase with age, but whereas the increase in systolic blood pressure continues into the eighth decade, diastolic blood pressure only rises until the fifth decade, followed by a gradual reduction.\textsuperscript{38} The association of blood pressure changes with the menopause is difficult to assess, as blood pressure is dependent on many other variables, which are themselves related to the menopause and uptake of HRT, such as body mass index and socio-economic class. But we can say that although blood pressure is lower in women than in men in the first half of life, the reverse may be true in higher age groups. Most longitudinal studies into the effect of menopausal status on blood pressure have failed to detect an association,\textsuperscript{39} whereas some cross sectional studies have described a higher systolic and diastolic blood pressure in postmenopausal women.\textsuperscript{40} Cross-sectional studies however, are considered inferior to longitudinal study designs, as they do not allow the accurate assessment of changes over time. On balance, the evidence that menopause per se is associated with an increase in blood pressure is not convincing.
The effect of exogenous female sex steroids on blood pressure is probably even more difficult to evaluate. The controversy surrounding the effect of oestrogen on blood pressure began in the late sixties and early seventies, with investigations into synthetic oestrogen containing oral contraceptive pills. Users of the combined oral contraceptive pill (COCP) were found to have higher systolic and diastolic blood pressure than non-users, even amongst those with blood pressures in the normal range. Women in the perimenopausal age group who used COCP, demonstrated a blood pressure elevation in 5-10% of the cases. From there research moved on to postmenopausal oestrogen replacement. Again, some authors reported a blood pressure elevation in response to treatment, although the sample sizes studied were small. Crane et al for example treated six normotensive women with conjugated equine oestrogens, and found that all six patients developed hypertension within 72 months, which resolved following cessation of treatment. In a study looking at the effects of conjugated equine oestrogens and piperazine oestrone sulphate in 160 postmenopausal women, the authors found that women receiving oral conjugated oestrogens had a higher rate of blood pressure rise and development of hypertension than those receiving the weaker compound. Women receiving piperazine sulphate on the contrary had a higher rate of blood pressure reduction than those receiving the equine hormone. The authors concluded that the more potent effects of conjugated oestrogens on the liver, leading to an increased expression of renin substrate, were possibly responsible for this differential response. In a double blind cross-over study with placebo and conjugated oestrogens another group of investigators demonstrated a significant reduction of systolic and diastolic blood pressure in both treatment arms. Several other studies have failed to detect a significant change in blood pressure with oestrogen treatment in both normotensive and hypertensive women.
1.4 ÖSTROGEN AND VASCULAR FACTORS

Since the effects of oestrogen on lipid and coagulation factors fail to fully explain the reduction in cardiovascular risk in women on HRT, extrahepatic effects of oestrogen have become a focus of interest. It has become apparent, that oestrogens exert significant effects on blood vessels, both structural and functional. These may represent other important ways by which oestrogen exerts cardioprotective effects in postmenopausal women.

1.4.1 Vascular Function

In 1966 Ueland and Parer described an animal experiment in which parenteral administration of oestrogen to sheep produced haemodynamic changes, including reduced peripheral resistance. These experiments have meanwhile been reproduced in humans. Sublingual 17β-oestradiol or placebo was administered to postmenopausal women, and a subsequent increase in forearm blood flow and reduction in forearm vascular resistance was observed in the active treatment group. Coronary arteries of postmenopausal women have been shown to dilate following 17β-oestradiol treatment. Aortic peak flow velocity, which decreases after the menopause, seems to be restored following oestrogen administration,
a fall in pulsatility indices in carotid arteries of postmenopausal women on long-term oestrogen treatment has been demonstrated.\textsuperscript{50}

Cerebrovascular circulation is also affected by oestrogen status. Cerebral blood flow is reduced in oestrogen deficiency states and velocity in the middle cerebral artery is directly related to oestrogen levels.\textsuperscript{51}

The effects of oestrogen treatment on vasoreactivity are partly endothelium dependent, partly endothelium independent. There may be a difference between the mechanisms responsible for acute effects, and those responsible the chronic vasculoprotective effects. They may also vary according to the dose and duration of the oestrogen administration.\textsuperscript{46}

The endothelium dependent vasorelaxant activity of oestrogen is probably mediated by the endothelium-derived relaxing factor (EDRF), now recognised as nitric oxide (NO). NO is generated from L-arginine by the action of nitric oxide synthase (NOS). \textit{O}estrogen can induce NOS, and subsequently increase the amount of vasorelaxant substance in the cell.\textsuperscript{52} Further, NOS can be inhibited by oxidised lipids.\textsuperscript{53} Again, oestrogen can increase the activity of NOS by inhibiting lipid oxidation. \textit{O}estrogen has also been shown to reverse vasoconstrictor stimuli, such as the effect of acetylcholine on atherosclerotic coronary arteries.\textsuperscript{54}

Prostacyclin (PGI\textsubscript{2}) is a prostaglandin generated by endothelial cells, which can induce vasodilatation and attenuate platelet aggregation. Its production may be coupled to NO release.\textsuperscript{55} \textit{O}estrogen has been shown to enhance the production of prostacyclin,\textsuperscript{56} which may be another endothelium dependent vascular effect.
It has been noted that oestrogen mediated vasorelaxation also occurs in endothelium-denuded blood vessels, and therefore an endothelium independent mechanism(s) must be in place.48 There is some pharmacological evidence, that oestrogen may act directly on vascular smooth muscle cells and coronary vascular myocytes. This effect seems to be mediated by a calcium-antagonist mechanism exerted by oestrogen,57 as demonstrated by measurements of cytosolic calcium concentrations and calcium currents.

1.4.2 Vascular Structure

1.4.2.1 Atherosclerosis

Atherosclerosis is an inflammatory and immunological process, encompassing lipid deposition in the intima, recruitment of inflammatory cells such as monocytes and T-lymphocytes, and activation of vascular smooth muscle cells, which then migrate, proliferate, and synthesise extracellular matrix. Lipoproteins in their native form do not appear to lead to an inflammatory reaction such as that necessary for atherogenesis. They have to be first oxidatively modified. Mildly oxidised LDL has undergone minimal modification and its recognition by LDL receptors is preserved,58 but it has the ability to stimulate the production of leukocyte chemoattractant substances by the endothelium, as well as that of colony-
stimulating factors necessary for the differentiation of monocytes to macrophages in the intima.\textsuperscript{49} It can also stimulate procoagulant factors such as tissue factor and PAI-1.\textsuperscript{50} Extensively oxidised LDL is not recognised by bona fide LDL receptors, and is taken up by scavenger receptors, which do not downregulate with increasing intracellular cholesterol concentration. Extensively oxidised LDL may have various proatherogenic effects such as direct cytotoxicity on endothelial cells, foam cell formation due to its uptake by macrophages and vascular smooth muscle cells, and modulation of production of various cytokines and growth factors, which play a role in atherogenesis, by vascular smooth muscle cells and macrophages.\textsuperscript{24}

The effect of oestrogen on atherogenesis has been most extensively studied. One of the effects which is presumed to have considerable impact on the initiation of atherosclerosis, is the antioxidant action of oestrogen. In an animal experiment using hypercholesterolaemic swine, Keany et al demonstrated that oestrogen administration rendered LDL isolated from treated animals markedly resistant to ex vivo oxidation.\textsuperscript{61} This effect is reproducible in humans. In a study on 18 postmenopausal women treated with intraarterial oestradiol, the lag of LDL oxidation increased from baseline during infusion, and returned to baseline after discontinuation of oestrogen administration.\textsuperscript{29}

Probably the oldest experimental studies looking at the effect of sex steroids on atherosclerosis are those utilising the rabbit model. In 1962, a study on the effect of oestrogens and steroids on atherosclerosis in rabbits gave first indications that oestrogens reduce atherosclerosis in an experimental set up.\textsuperscript{62} Several studies followed, using cholesterol fed, oophorectomised and intact rabbits, and it has been repeatedly demonstrated that oestrogen treatment significantly reduced the extent of
atherosclerosis. Furthermore, oestrogen also seems to alter the intra-arterial lipid metabolism, with significantly lower cholesterol ester hydrolysis in the vascular wall in oestrogen treated animals.

The most convincing evidence stems from the USA based Clarkson group, who have studied atherogenesis in the non-human primate model. They designed a series of experiments using the female cynomolgus Macaca fascicularis monkey, which, like humans, has a regular 28-day menstrual cycle, with similar cyclical sex steroid variations. A premenopausal protection from CVD, which is lost after the menopause, has also been described in this species. Clarkson et al have been able to demonstrate a protective effect of female sex steroids on blood vessels, resulting in a reduced incidence, extent and progression of atherosclerotic plaque in oestrogen treated monkeys. They have also demonstrated a significantly lower rate of LDL accumulation in coronary arteries of oestrogen treated as opposed to oestrogen depleted animals.

In humans there is some angiographic evidence that oestrogen replacement may be associated with lower degrees of coronary artery occlusion, however, to date we do not have prospective, human studies looking at the extent of atherosclerosis in relation to past or present exogenous female sex steroid exposure.
1.4.2.2 Intimal hyperplasia

Intimal hyperplasia is widely known as a secondary vascular response to mechanical challenges to blood vessels such as shear stress, and surgical interventions such as endarterectomy, balloon angioplasty and arterialisation of veins in grafts. It remains uncertain whether endothelial cell damage constitutes the sole basis for myointimal proliferation. Interestingly, a whole range of animal models exists in which intimal thickening can be induced without any intravascular manipulations, when some sort of insult, such as electrode stimulation for example, is applied exclusively to the adventitial surface of the blood vessel.\textsuperscript{70}

However, primary intimal hyperplasia in coronary arteries is not unknown, and population of the coronary intima by vascular smooth muscle cells has been described in humans as young as several months.\textsuperscript{71} Intimal hyperplasia has been described as a primary occurrence in stenotic human coronary arteries. In a study looking at directional atherectomy specimens from individuals with primary coronary occlusion, intimal hyperplasia was identified in 44\% of cases, predominantly in younger people.\textsuperscript{72}

Histologically, intimal hyperplasia can be difficult to distinguish from atherosclerosis, and indeed it has been known under the name ‘graft arteriosclerosis’. Following an initiating event a response is mounted, which involves activation of vascular smooth muscle cells in the tunica muscularis media. These cells can migrate from the media to the intimal layer, proliferate, change their phenotype from contractile to synthetic, and commence the production of extracellular matrix and collagen, which is then accumulated in the subendothelial...
space, thus leading to intimal thickening. Intimal infiltration by inflammatory cells can also be seen. Processes such as lipid deposition and intraplaque haemorrhage are usually only seen in atheromata and do not occur in intimal hyperplasia.

There may be an effect of gender on the development of intimal hyperplasia in animals, although it remains controversial whether this is due to a greater resistance of female tissues to the development of an intimal reaction or to a direct or indirect effect of female sex steroids. However, in a recent study on intimal injury in rat carotid arteries it has been demonstrated that the sex difference in myointimal proliferation after vascular damage is oestrogen dependent. This is not surprising, as oestrogen has been shown to inhibit one of the key events in intimal hyperplasia, smooth muscle cell proliferation, in several animal models. Estradiol treatment in rabbits with aortic transplants has been shown to preserve the ultrastructure of endothelial cells and to inhibit macrophage infiltration of the intima, possibly by direct action, as both smooth muscle cells and macrophages possess oestrogen receptors. Further, oestrogen may reduce connective tissue accumulation in the vascular wall, with changes to the collagen-elastin ratio, thus making blood vessels more distensible.
1.5 Vascular Derived Cytokines in Vascular Disease: A Potential Role for Oestrogens?

Recently, interest has risen in the cellular and molecular mechanisms underlying vascular disease processes. Both endothelial cells and vascular smooth muscle cells produce a variety of substances which can affect vascular function and structure, as well as the development and progression of vascular disease, such as atherosclerosis and intimal hyperplasia. Several of these substances may facilitate the adherence of formed elements in blood to vascular endothelium, or induce, and/or maintain, the proliferative response of cellular elements within the vascular wall to injury.

With the exception of Vascular Endothelial Growth Factor, the effect of oestrogens on the secretion of vascular derived cytokines and growth factors has so far not been investigated, although certain mechanisms of oestrogenic action on blood vessels, such as suppression of vascular smooth muscle cell, would suggest that some effects on cytokines may be expected.
1.5.1 Selectins

The selectins are a recently identified family of glycoprotein adhesion molecules, of which some could play a role in atherogenesis. L-selectin was the first to be described for its involvement in the homing of leukocytes. Other members of this family, E-selectin for example, have been detected in endothelial cells overlying atheromatous plaque. The proposed role for E-selectin in atherogenesis is to mediate the transient attachment of leukocytes to the endothelium, a mechanism also termed 'rolling'. This action prolongs the contact between the leukocyte and the vascular wall, thus facilitating leukocyte recruitment into the subendothelial space. The selectins appear to be the initial adhesion molecule at the start of the inflammatory process leading to atherogenesis.

1.5.2 Immunoglobulins

Adhesion molecules belonging to this group contain immunoglobulin like domains. They are expressed by the vascular endothelium and may facilitate the recruitment of leukocytes into the intima. The two most widely researched adhesion molecules from this group are the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The presence of both in atherosclerotic plaques has been described. Whilst ICAM-1 is constitutively expressed on endothelial cells, its expression is significantly upregulated after endothelial activation, when it can also be found on vascular smooth muscle cells.
It is capable of binding both monocytes and lymphocytes, and is therefore a good candidate molecule for recruitment of the inflammatory cells into the subendothelial space. VCAM-1 is expressed by endothelial cells and vascular smooth muscle cells following activation by cytokines such as tumour necrosis factor-α (TNF-α) and interferon, and by lysophosphatidyl-choline, a by-product of lipid oxidation. It has been described to precede the presence of inflammatory cells in the intima and neointima.\textsuperscript{8,3} The presence of both ICAM-1 and VCAM-1 has also been described in the intima of experimentally induced cardiac allograft vasculopathy in animals.\textsuperscript{8,5}

1.5.3 Angiogenic Cytokines

Angiogenic cytokines, or growth factors, such as Fibroblast Growth Factor (FGF), and Vascular Endothelial Growth Factor (VEGF), belong to a family of heparin-binding growth factors, that can be synthesised by vascular smooth muscle cells, and, in the case of FGF, by endothelial cells. Both have potent effects on blood vessel structure and function, and they are angiogenic. The role of FGF in vascular response to injury has been extensively researched, and it has been shown to be expressed after endothelial denudation.\textsuperscript{8,6} Infusion of FGF can increase the extent of intimal hyperplasia, whereas FGF antibodies seem to inhibit its development in animal experiments.\textsuperscript{8,7}

VEGF is a relatively newly described angiogenic cytokine, and its involvement in the development of vascular lesions is yet unknown. It has been described as the prime regulator of physiological and pathological angiogenesis. It
is also a potent mitogen, and a vascular permeability factor. VEGF is constitutively expressed in most adult human tissues, but its expression is markedly upregulated by hypoxia, and downregulated by hyperoxia. It also appears to have some role in vascular maintenance and preservation of endothelial function.
1.6 Plan of Investigations

It is widely recognised that CVD is by no means restricted to men, but it is also obvious that it rarely affects women before the menopause. Since this original observation, oestrogen depletion after the menopause has been strongly implicated in the pathogenesis of CVD. Although controversial, current evidence from epidemiological studies favours the beneficial effects of postmenopausal oestrogen substitution on cardiovascular risk in women.

The mechanisms of this protective effect are not yet fully understood, but it is clear that multiple factors must be involved, including favourable changes in various cardiovascular risk factors observed with oestrogen treatment as well as direct effects on the vasculature. The extensively investigated effects on lipid factors have been shown to contribute up to about 30% to the observed risk reduction, and they will not be subject of this investigation.

The objective of this investigation is to study the effects of 17β-oestradiol on various aspects of the cardiovascular system that have not yet been extensively studied, or have, so far, remained controversial, in an attempt to contribute to the understanding of mechanisms by which female sex steroids may affect the vasculature.
1.6.1 Proposed Investigations

The presented investigations will examine

1. the effect of unopposed postmenopausal oestrogen replacement on ambulatory blood pressure in oophorectomised normotensive women. In a prospective observational study over six months, ninety women were treated with oral or transdermal oestrogen, and changes in their blood pressure were monitored.

2. the genetic background of the blood pressure responses observed in these women. As detailed below, the effect of oestrogen on blood pressure was noted to be variable, and the angiotensinogen genes of these women were examined for a polymorphism implicated in the development of pregnancy-induced hypertension, and hypertension in non-pregnant individuals.

3. the effect of unopposed oral oestrogen treatment over six months on atheromatous plaque dimensions in postmenopausal women with documented stenotic and non-stenotic carotid disease, as measured by ultrasound scanning.

4. the possibility of inducing intimal hyperplasia in blood vessels in vitro, using all human materials. Human ovarian vein segments are cultured in media supplemented with serum from postmenopausal women. The development of such a model would facilitate the study of changes in human blood vessels cultured in controlled, specific conditions.
5. the effect of 17β-oestradiol on the development of intimal hyperplasia in vitro in human blood vessels as described under 4.

6. the effect of 17β-oestradiol on the expression of Ki67 and PCNA as markers of proliferative activity in the vascular wall of cultured human ovarian veins.

7. the effect of 17β-oestradiol on the expression of the adhesion molecules E-Selectin, ICAM-1 and VCAM-1 in the intima of cultured human ovarian veins.

8. the effect of 17β-oestradiol on the expression of the angiogenic cytokine VEGF in the intima of cultured human ovarian veins and its relationship to intimal hyperplasia.

9. the effect of 17β-oestradiol on the secretion of VEGF in vivo, in postmenopausal women treated with transdermal oestrogen replacement.
1.6.2 Methods Employed

Due to the diversity of the methods employed in each chapter, and the inclusion of clinical trials as well as laboratory experiments, a communal materials and methods section will be omitted. Instead, the relevant methodology will be discussed in the context of the appropriate chapters.

In the clinical trials section, the following methods are used:

- ambulatory blood pressure monitoring
- ultrasound measurements of carotid plaque dimensions
- outpatient hysteroscopy

The laboratory experiments will include

- organ culture
- immuno-cytochemistry
- in situ hybridisation
- DNA extraction from whole blood
- polymerase chain reaction
- restriction enzyme digestion
- gel electrophoresis
- ELISA (enzyme linked immuno-sorbent assay)
- light microscopy and image analysis
2. MATERIALS

2.1 ANTIBODIES

**Alpha-Smooth Muscle Actin**, mouse monoclonal, M 0851, DAKO, Denmark

**Anti-digoxigenin alkaline phosphatase Fab fragment antibody**, Boehringer Mannheim Biochemicals

**Endothelial Cell CD 31**, mouse monoclonal, M 0823, DAKO, Denmark

**E-Selectin** (CD 62E), goat polyclonal, BBA 18, R&D Systems, UK

**ICAM-1** (CD 54), goat polyclonal, BBA 17, R&D Systems, UK

**MBI1** mouse monoclonal, NA21-2, Oncogene Science, Cambridge, MA

**PCNA** mouse monoclonal, Novocastra Lab. Ltd, Newcastle

**Rabbit Anti-Mouse** antibody, biotinylated, Dako

**VCAM-1** (CD106), goat polyclonal, BBA 19, R&D Systems, UK

2.2 BUFFERS

**Citrate Buffer**: (10 mM Citrate Buffer pH 6.0). Stock solution: A 0.1 M citric acid (C₆H₈O₇.H₂O) 21.01 g /1000 ml, B 0.1 M sodium citrate (Na₂C₆H₅O₇.2H₂O) 29.41 g /1000 ml. Working solution: A 9 ml, B 41 ml, Aqua dest 450 ml.

**Phosphate Buffered Saline, (0.1M, pH 7.6)**: Sodium chloride (NaCl) 80 g, Disodium hydrogenorthophosphate (Na₂HPO₄) 13.7 g, Potassium dihydrogen orthophosphate (KH₂PO₄) 2 g, Aqua dest to 500 ml, Adjust pH to 7.6, Aqua dest to 1000 ml. Stock remains stable for 6 months at room temperature in Duran bottle.
Make up to 10 litre with distilled water before use. For 10x PBS for molecular biology make up to 1L, treat with DEPC and autoclave for 15 minutes at 15 lb sq.in.-¹ (in pressure cooker).

**Sodium borate buffer 1M, pH 8.5:** Sodium borate 38.11 g, Aqua dest 100 ml. Heat to aid dissolution, then pH to 8.5. DEPC treat and autoclave.

**TB buffer:** Sodium chloride 300 mM, Sodium citrate 30 mM

**TBS (Trisma buffered saline, pH 7.6):** Tris 71 g, NaCl 85 g, Aqua dest to 500 ml, adjust to pH 7.6, MgCl₂ 4 g, Aqua dest to 1l. Stock stable for 6 months at room temperature in Duran bottle. Make up to 10 L with Aqua dest prior to use.

**TBS-BSA:** Bovine Serum Albumin (BSA, Fraction V, Sigma) 1 g, TBS 100 ml.

**Tdt Buffer:** 22.4 g sodium cacodylate + 0.238 g cobalt chloride in 1 litre Aqua dest. pH 7.0 with conc. HCl.

**Multi-Core Buffer:** 25 mM Tris acetate, pH 7.8, 100 mM Kac, 10 mM Mg₂Ac

**TBE electrophoresis buffer:** 10xTBE: 0.9 M Tris-borate, 0.01M EDTA, 108g Tris base, 55 g Boric acid, 20 ml 0.5M EDTA

**Gel loading buffer,** Sigma

### 2.3 ENZYMES

**AmpliTaq DNA polymerase,** 5U/µl, Perkin Elmer N801-0060

**Pepsin (0.4%):** Porcine pepsin 1:2500, Sigma, UK. 0.4 g, Aqua dest, 100ml, 5M Hcl 200 µl

**Proteinase K(10ug/ml)** Boeringer Mannheim Biochemicals, UK. 10 µl stock in 990 µl 0.05 M Tris Hcl pH 7.6
Tth111 restriction endonuclease 10 u/µl, Promega, WI USA, R684/1,2

2.4 FIXATIVES

Formalin 10%, BDH
Paraformaldehyde 0.4%: 4 g in 100 ml 10x PBS.

2.5 KITS

Quantikine Human VEGF Immunoassay, R&D Systems, UK DVE00
5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II, 129964,
Boehringer Mannheim Biochemica, UK.

2.6 SOLUTIONS

Blocking Solution: 3 g BSA in 100 ml unmodified TBS + 100 µl Triton X-100
DEPC (diethyl pyrocarbonate) water: For RNAse free DEPC (diethyl pyrocarbonate) treated solutions or DEPC water add DEPC to the solution to 0.1% (v/v). Shake for 2 hours to dissolve. Leave to stand overnight in a fume hood. Autoclave for 15 minutes at 15 lb sq.in.-1 (in pressure cooker) or boil for 30 minutes. Note: Tris based solutions cannot be DEPC treated in this way and must be made up in treated glassware using autoclaved DEPC water and then autoclaved.
Labeling Solution: 1 µl DIG-11 + 4 µl Terminal Transferase in 500 µl Tdt buffer.
Manganese chloride 10mM*: Manganese chloride 0.198 g, Aqua dest 100 ml
MgCl 1M*: Magnesium chloride 20.331 g, Aqua dest 100 ml
NaCl 5M*: Sodium chloride 29.22 g, Aqua dest 100 ml
Standard saline citrate (SSC 20x)*: Sodium chloride 3 M 175.32 g, Sodium citrate 0.3 M 98.03 g, Aqua dest to 1 litre pH to 7.0.
To treat glassware etc. soak for at least 1 h in 3% Hydrogen Peroxide and dry thoroughly at 37°C.
*Solutions used for molecular biology were all DEPC treated and autoclaved

2.7 STAINS

Millers Elastin, Raymond Lamb, UK
Mayer's Hematoxylin: Hematoxylin 1g, Aluminium potassium sulphate 50 g, Sodium iodate 0.2 g, Citric acid 1g, Aqua dest 1 l.

2.8 SUBSTRATES

Fast red/Naphtol, AS-MX Alkaline Phosphatase Substrate, Sigma Pharmaceuticals, UK
ABC-AP Kit, AK-5000, Vector Laboratories. Reagent A (Avidin), Reagent B (Biotinylated Alkaline Phosphatase). 10 ml buffer + two drops A and two drops B.

2.9 MISCELLANEOUS

A1 minuten pins, Watkins and Doncaster, Cranbrook, Kent
Angiotensinogen Primers, Leicester University
Aquamount aqueous mountant, BDH, UK

DNA ladder, 1kb, Sigma

Forceps, fine ophthalmic

L-Glutamine 2 mmol/l, Seralab, Crawley-Down, Sussex

Microwave oven, 750 Watt, Techolec, UK

17β oestradiol, Sigma, Dorset, UK

17α oestradiol, Sigma, Dorset, UK

Penicillin 50 U/ml, Sigma, Dorset, UK

Pyrex 60x20 mm culture dishes, Corning Ltd, UK

RPMI phenol free medium, Sigma, Dorset, UK

Scissors, fine ophthalmic

Silane (3-Aminopropyl-Triethoxy-Silane), Sigma, Dorset, UK

Streptomycin 50 μg/ml, Sigma, Dorset, UK

Sylgard 184 resin, Dow Corning, Seneffe, Belgium

Thermal cycler, Hybaid, USA

Rabbit Serum, Dako, Denmark

VEGF probe, Leicester University

All other chemicals were obtained from BDH, UK.
3. ÕESTROGEN AND BLOOD PRESSURE

3.1 INTRODUCTION

Over the past two decades, the traditional perception of õestrogen as a hypertensive agent has been the centre of controversy. Studies of normotensive and hypertensive women have failed to detect an adverse effect of postmenopausal õestrogen replacement therapy on blood pressure (BP), and some have reported a BP reduction in response to treatment. We still lack conclusive evidence as to the effect of õestrogen replacement on blood pressure regulation in postmenopausal women, and equally we know very little about the mechanisms regulating the blood pressure response to õestrogen exposure.

3.1.1 Ambulatory Blood Pressure

Apart from problems arising from study designs, we face the difficulty of the choice of methodology as far as the actual blood pressure measurements are concerned. The majority of published studies on the effect of õestrogen on blood pressure in normotensive subjects have used conventional mercury sphygmomanometer measurements. Although the gold standard, such measurements are known to be inaccurate and poorly reproducible, because of the inherent BP variability, observer error, and ‘white-coat-hypertension’. 
An exciting alternative to conventional clinic BP measurements was developed by Dr Maurice Sokolow in the 1950s, who pioneered one of the first ambulatory devices. Ambulatory BP measurements are readings taken during normal daily activities. They can be automated, or patient operated, i.e. the measurements are initiated by the patient. Ambulatory BP monitoring is a more accurate, objective and reproducible way of measuring changes in BP than mercury sphygmomanometer measurements, and has been shown to correlate closely \((r > 0.9)\) with intraarterial BP. Oscillometric devices score particularly in accuracy. These calculate systolic and diastolic BP according to an empirically derived algorithm, the reference point being the point of maximum oscillation during cuff deflation, which corresponds to mean intra-arterial pressure. The oscillatory technique has several advantages over auscultatory methods. The correct placement of the cuff is not as critical, as no transducer needs to be placed over the brachial artery. Further, some problems inherent to auscultatory technique are avoided. The auscultatory gap, arm position during measurement, digit preference, threshold avoidance and rate of cuff deflation are only some of the potential sources of error, not encountered with oscillatory techniques.

Automated ambulatory BP measurements have also been shown to have a greater prognostic value than conventional measurements in predicting target organ damage and subsequent cardiovascular events, due to the increased number of BP measurements, the inclusion of sleep measurements, and the provision of information about BP variability.
3.1.2 Genetic Determinants of Blood Pressure

Blood pressure is a complex quantitative trait which is likely to be the result of multiple genetic and environmental determinants. Several genes, including some encoding components of the renin-angiotensin system, have been suggested to play a role in blood pressure regulation, and the development of its extreme, hypertension. One such candidate gene is that encoding angiotensinogen.

Angiotensinogen (AGT), also known as renin-substrate, is a plasma glycoprotein with a molecular weight of 55-60 kD. It is synthesised by a variety of cells, mainly by hepatocytes, and cleaved by renin to the biologically inactive angiotensin I, which is then further processed by angiotensin-converting enzyme to the active hormone, angiotensin II. AGT expression is modulated by several hormones, including glucocorticoids and oestrogens.

The human AGT gene is located on chromosome 1, and is composed of five exons and four introns. Recently, evidence has been provided from studies on affected siblings for the involvement of the AGT gene in the pathogenesis of essential hypertension, estimating that up to 3-6% of hypertensive individuals may have a predisposing mutation in the AGT locus. Several other studies have linked two mutations in particular to the development of essential hypertension, both within exon 2. One with threonine (T235) rather than methionine (M235) at position 235, i.e. M235T, and one with methionine rather than threonine in position 174 (T174M), with the M235T variant being significantly more frequent.
particularly among female hypertensives. The mechanism by which this mutation may lead to an increase in BP is not clear, but it has been noted that individuals with the above mutation, again females in particular, have significantly higher AGT levels. Raised AGT levels have been described in women receiving òestrogen replacement, and it was widely believed that the idiosyncratic blood pressure elevation observed in about 5% of òestrogen treated women may be explained by this increase in renin-substrate. In the course of this investigation the AGT gene in all women participating in the blood pressure study will be examined for the $M235T$ mutation, to detect a possible link between the blood pressure response to òestrogen and AGT polymorphism.
3.2 DIFFERING RESPONSES TO ORAL AND TRANSDERMAL OESTROGEN IN 24- HOUR AMBULATORY BLOOD PRESSURE.

3.2.1 Methods

The study was conducted in The Menopause Research Unit, Department of Obstetrics and Gynaecology, University of Leicester, with the approval of the local ethics committee.

3.2.1.1 Subjects

Included were ninety healthy women, aged 30-59 years, with a body mass index (BMI) of less than 30. All women underwent automated ambulatory BP monitoring over 24 hours, at least 2 months following a routine hysterectomy and bilateral salpingo-oophorectomy for non-malignant conditions, including menstrual disorders (n=70), uterine leiomyomas (n=16) and endometriosis (n=4).

All subjects were conventionally normotensive, i.e., BP did not exceed 140 mm Hg systolic and 90 mm Hg diastolic on at least two occasions before recruitment, measured in clinic, using a standard mercury sphygmo-manometer. Women on antihypertensive agents, diuretics, systemic steroids, those with a history of recent estrogen replacement or a previous estrogen implant, and women with contraindications to oestrogen treatment were excluded.
The above sample size was chosen to detect a 5 mm Hg difference in systolic blood pressure, with an assumed SD of 6 mm Hg (n=31 per treatment), and a 5 mm Hg difference in diastolic blood pressure, with an assumed SD of 4 mm Hg (n=15 per treatment), with 90% power at a 5% significance level.

3.2.1.2 Estrogens used

The first 40 enrolled women were allocated to transdermal treatment as part of an unrelated clinical trial, the next 50 patients were allocated to oral treatment. Transdermal oestrogen was given as Gynaderm® (Shire Pharmaceutical Development, Andover, UK), a trilaminate hypoallergenic matrix patch delivering 50 µg oestradiol daily. Participants were instructed to change the patch twice weekly. Oral oestrogen was given as Hormonin® (600 µg micronised oestradiol, 270 µg oestril, and 1.4 mg oestrone) (Shire Pharmaceutical Development, Andover, UK), one tablet daily.

3.2.1.3 Blood pressure measurements

Conventional BP was measured using a mercury sphygmomanometer at every visit after a rest period of 10 minutes in a clinic environment. BP was then measured over 24 hours using the validated SpaceLabs 90207 automated oscillometric ambulatory device (SpaceLabs Medical Inc., Redmond, WA) with a cuff of an appropriate size for the subject’s arm circumference. The device was programmed to record half hourly BP measurements over 24 hours (Figure 2). Information on age, parity, body mass index (BMI, kg/m²) and smoking habits was collected on all subjects.
Figure 2: A typical print out of an ambulatory blood pressure recording over 24 hours, showing systolic, diastolic and mean arterial blood pressure, as well as heart rate. The nocturnal reduction in blood pressure is clearly recognisable in this recording.
3.2.1.4 Outcome measures

1. mean conventional clinic systolic and diastolic BP
2. mean day-time and night-time ambulatory systolic and diastolic BP
3. nocturnal decrease in ambulatory systolic and diastolic BP, calculated as the difference between the mean day-time and mean night-time BP after 3 and 6 months oestrogen replacement.

3.2.1.5 Statistical analysis

The two groups were compared with respect to mean age, parity, BMI, mean day-time and night-time ambulatory systolic and diastolic BP, and the nocturnal decrease in both mean ambulatory systolic and diastolic BP at study entry, using two-tailed Student t test for unpaired variables. The proportion of smokers in both groups was examined for statistically significant differences using the \( \chi^2 \) test for non-parametric variables. Changes in mean conventional systolic and diastolic BP, mean day-time and night-time ambulatory systolic and diastolic BP, as well as in the nocturnal decrease in mean ambulatory systolic and diastolic BP, after 3 and 6 months treatment were analysed using two-tailed Student-t test for paired variables. To allow for repeated comparisons, Bonferroni correction was made on all P values relating to repeated measurements analysis.

The correlation between the net change in mm Hg in mean day-time and night-time ambulatory systolic and diastolic BP after 6 months oestrogen treatment and the subjects baseline characteristics including mean age, mean parity, mean
BMI, smoking, and mean ambulatory systolic and diastolic BP at study entry was analysed by multiple regression analysis. All statistical tests were performed using StatView™ 512+ software (Brain Power, Inc, Calabasa, CA).
3.2.2 Results

Seventy-two of the ninety enrolled subjects completed six months of oestrogen replacement, 41 in the oral and 31 in the transdermal treatment group. Fourteen patients were withdrawn before study completion due to treatment side effects (Table 1).

Table 1. Reasons for withdrawal from study.

<table>
<thead>
<tr>
<th>Reason for withdrawal</th>
<th>Oral oestrogen n (%)</th>
<th>Transdermal oestrogen n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin irritation</td>
<td>0</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Breast tenderness</td>
<td>3 (6)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Headaches</td>
<td>2 (4)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td></td>
<td>2 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Poor compliance</td>
<td>1 (2)</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Total withdrawn</td>
<td>9 (18)</td>
<td>9 (22.5)</td>
</tr>
</tbody>
</table>

The remaining four women were not suitable for evaluation, because their 24-hour BP recordings contained less than 20 accurate measurements. These four subjects terminated the recordings within the first 6 hours because they found the BP device too uncomfortable. Both treatment groups were comparable in terms of
mean age, parity, BMI, mean day-time and night-time ambulatory systolic BP, and ambulatory diastolic BP as well as mean nocturnal decrease in BP at study entry (Table 2).

**Table 2: Baseline characteristics of women on oral and transdermal oestrogen.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Oral oestrogen n=41</th>
<th>Transdermal oestrogen n=31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years (range)</td>
<td>46.2 (33-59)</td>
<td>45.3 (30-57)</td>
</tr>
<tr>
<td>Parity, mean (range)</td>
<td>2.2 (0-5)</td>
<td>2.4 (0-6)</td>
</tr>
<tr>
<td>Mean BMI, mean kg/m² (range)</td>
<td>25.2 (21.2-30)</td>
<td>24.3 (19.4-29.8)</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>7 (18)</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Day-time ambulatory systolic BP (mean mm Hg)</td>
<td>120</td>
<td>119</td>
</tr>
<tr>
<td>Night-time ambulatory systolic BP (mean mm Hg)</td>
<td>106</td>
<td>104</td>
</tr>
<tr>
<td>Day-time ambulatory diastolic BP (mean mm Hg)</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Night-time ambulatory diastolic BP (mean mm Hg)</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

None of the differences are statistically significant.
3.2.2.1 Oral oestrogens

Clinic and Day-time: In the oral treatment group (n=41), neither mean clinic systolic nor diastolic BP changed significantly throughout the treatment period (Table 3).

Table 3: Changes in conventional clinic blood pressure over six months unopposed oestrogen treatment.

<table>
<thead>
<tr>
<th>BP / Treatment group</th>
<th>Baseline</th>
<th>3 months’ treatment</th>
<th>6 months’ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP oral oestrogen</td>
<td>131 (125-136)</td>
<td>133 (128-138)</td>
<td>131 (123-139)</td>
</tr>
<tr>
<td>Systolic BP transdermal oestrogen</td>
<td>125 (120-130)</td>
<td>124 (119-129)</td>
<td>124 (119-129)</td>
</tr>
<tr>
<td>Diastolic BP oral oestrogen</td>
<td>78 (73-82)</td>
<td>79 (75-83)</td>
<td>76 (70-83)</td>
</tr>
<tr>
<td>Diastolic BP transdermal oestrogen</td>
<td>80 (77-84)</td>
<td>79 (75-83)</td>
<td>73 (70-77)</td>
</tr>
</tbody>
</table>

Mean mm Hg (95% confidence interval). None of the changes are statistically significant.
Mean day-time ambulatory systolic BP (Figure 3) also did not change significantly from baseline at 3 (-0.9 mm Hg; 95% CI -3.6, 1.7) or 6 months' (-1.2 mm Hg; 95% CI -3.9, 1.6) treatment. There was no significant change from baseline in mean day-time ambulatory diastolic BP (-1.9 mm Hg, 95% CI -4.3, 0.3, after 3 months; -1.45 mm Hg, 95% CI -3.4, 0.5, after 6 months of treatment).

Night-time: Mean night-time ambulatory systolic BP dropped from baseline by -3 mm Hg (95% CI -5.6, -0.5) after 3 months (P = 0.046). After 6 months it was only 1 mm Hg lower than baseline (95% CI -4.4, 2.4, NS). Night-time ambulatory diastolic BP fell significantly by -5 mm Hg (95% CI -9.3, -0.3, P = 0.009) after 3 months of treatment. After 6 months the mean night-time ambulatory diastolic BP remained lower than at study entry (-1.7 mm Hg; 95% CI -4.1, 0.6) but not significantly so.

Nocturnal Dip: The nocturnal dip in mean ambulatory systolic BP increased from 14 mm Hg (95% CI 13, 15) at study entry to 17 mm Hg (95% CI 15, 19) after 3 months of oestrogen replacement, but returned to baseline value after 6 months of treatment (14 mm Hg, 95% CI 13, 15). The nocturnal dip in ambulatory diastolic BP followed a similar pattern, with an initial increase from 13 mm Hg (95% CI 11, 15) at study entry to 14 mm Hg (95% CI 12, 17) after 3 months and a return to baseline value (13 mm Hg, 95% CI 11, 15) after 6 months of oestrogen replacement. None of these changes were statistically significant.
Figure 3: Changes in mean ambulatory blood pressure on oral oestrogen, in mean mm Hg with 95% Confidence intervals.
**Multiple regression analysis:** In the multiple regression analysis the net change (mm Hg) in BP after 6 months' oestrogen replacement did not correlate significantly with age (day-time systolic $r = 0.04$; night-time systolic $r = 0.09$; day-time diastolic $r = 0.08$; night-time diastolic $r = 0.1$), BMI (day-time systolic $r = 0.2$; night-time systolic $r = 0.06$; day-time diastolic $r = 0.3$; night-time diastolic $r = 0.01$), parity (day-time systolic $r = 0.2$; night-time systolic $r = 0.04$; day-time diastolic $r = 0.1$; night-time diastolic $r = 0.002$) or smoking status (day-time systolic $r = 0.3$; night-time systolic $r = 0.06$; day-time diastolic $r = 0.1$; night-time diastolic $r = 0.07$) at study entry.

### 3.2.2.2 Transdermal oestrogens

**Day-time:** In the 31 women on transdermal oestrogen replacement, mean clinic systolic BP did not change significantly throughout the treatment period. There was a reduction in mean clinic diastolic BP by 6 mm Hg after 6 months' oestrogen replacement, which was not statistically significant after Bonferroni correction (Table 3). There was a reduction in mean day-time ambulatory systolic BP from baseline by $-2.6$ mm Hg (95% CI -5.8, 0.5) after 3 months and $-3.6$ mm Hg (95% CI -7.3, 0.2) after 6 months of oestrogen replacement, but this was not statistically significant. There was a significant reduction in mean day-time ambulatory diastolic BP from baseline ($-3.3$ mm Hg; 95% CI -5.5, -0.9; $P = 0.016$) after 3 months. This reduction was maintained after 6 months of oestrogen replacement ($-4$ mm Hg, 95% CI -6.8, -1.2) and was statistically significant ($P = 0.014$).
**Night-time:** Mean night-time ambulatory systolic BP dropped by -3.6 mm Hg (95% CI -7.6, 0.5; NS) after 3 months treatment, and by -4.2 mm Hg (95% CI -7.7, -0.7) after 6 months, which was statistically significant (P = 0.039). Mean night-time ambulatory diastolic BP also fell significantly from baseline after 3 (-3.8 mm Hg; 95% CI -6.6, -0.9; P = 0.027) and 6 (-4.4 mm Hg; 95% CI -7.1, -1.7; P = 0.005) months of oestrogen replacement (Figure 4).

**Nocturnal dip:** The nocturnal dip in ambulatory systolic BP did not change significantly. It was 14 mm Hg (95% CI 11, 17) at study entry, 14 mm Hg (95% CI 11, 17) after 3 months and 15 mm Hg (95% CI 12, 18) after 6 months' treatment. The nocturnal decrease in ambulatory diastolic BP also did not change significantly throughout the treatment period, with 13 mm Hg (95% CI 11, 15) at study entry, 12 mm Hg (95% CI 9, 15) after 3 months, and 13 mm Hg (95% CI 10-15) after 6 months of oestrogen replacement.
Figure 4: Changes in mean ambulatory blood pressure on transdermal oestrogen, in mean mm Hg with 95% confidence intervals.
Multiple regression analysis: The net change in mean day-time and night-time ambulatory systolic BP and ambulatory diastolic BP in mm Hg after 6 months' oestrogen replacement did not correlate significantly with age (day-time systolic $r = 0.04$; night-time systolic $r = 0.08$; day-time diastolic $r = 0.06$; night-time diastolic $r = 0.02$), parity (day-time systolic $r = 0.3$; night-time systolic $r = 0.14$; day-time diastolic $r = 0.1$; night-time diastolic $r = 0.1$) or smoking status (day-time systolic $r = 0.05$; night-time systolic $r = 0.09$; day-time diastolic $r = 0.08$; night-time diastolic $r = 0.07$) in the transdermal oestrogen treatment group. The change in night-time ambulatory diastolic BP in mm Hg over 6 months' treatment correlated significantly but weakly with the BMI ($r = -0.13$, $P = 0.009$). The mean day-time ambulatory systolic BP at study entry correlated significantly with the net change in day-time ambulatory systolic BP ($r = -0.43$, $P = 0.007$) after 6 months oestrogen replacement in the transdermal, but not in the oral oestrogen treatment group ($r = -0.14$). Night-time ambulatory systolic BP at study entry also correlated with the net change in mm Hg observed in night-time ambulatory systolic BP after 6 months of oestrogen replacement ($r = -0.64$, $P = 0.001$), again an effect not observed in the oral treatment arm ($r = -0.3$). There was no significant correlation between day-time ambulatory diastolic BP at baseline and the observed change in day-time ambulatory diastolic BP after 6 months, whereas the mean night-time ambulatory diastolic BP at study entry correlated significantly with the net change in night-time ambulatory diastolic BP after 6 months ($r = -0.6$, $P = 0.004$) in the transdermal, but not the oral treatment arm ($r = -0.14$).
3.2.2.3 Frequency of blood pressure increase

A pronounced difference in the ambulatory BP response to oestrogen replacement within the two treatment groups became apparent. We therefore compared the number of women whose BP increased in response to treatment in either group (Table 4). The proportions of women whose BP rose were comparable between the two groups, with the exception of mean day-time ambulatory diastolic BP, which rose in a significantly higher proportion of women in the oral treatment group ($P = 0.029, \chi^2 = 4.48$). The magnitude of the observed increase in BP in these women at the end of the study period was mostly statistically significant. In women who developed a blood pressure increase while receiving oral treatment, day-time ambulatory systolic BP rose by a mean of 6 mm Hg (95% CI 3.4, 8.6; $P < 0.001$) and night-time ambulatory systolic BP by a mean 8 mm Hg (95% CI 5.3, 10.5; $P < 0.001$).

Table 4. Number of women with increase in mean ambulatory blood pressure on oestrogen replacement

<table>
<thead>
<tr>
<th>Increase in ambulatory blood pressure</th>
<th>Oral oestrogen group n (%)</th>
<th>Transdermal oestrogen group n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-time systolic</td>
<td>14 (34)</td>
<td>11 (35)</td>
<td>N/S</td>
</tr>
<tr>
<td>Night-time systolic</td>
<td>21 (51)</td>
<td>12 (39)</td>
<td>N/S</td>
</tr>
<tr>
<td>Day-time diastolic</td>
<td>16 (39)</td>
<td>5 (16)</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>Night-time diastolic</td>
<td>13 (32)</td>
<td>9 (29)</td>
<td>N/S</td>
</tr>
</tbody>
</table>
Day-time ambulatory diastolic BP increased by a mean of 4.4 mm Hg (95% CI 2.4, 6.4; P < 0.001), night-time diastolic by 7 mm Hg (95% CI 4.5, 9.5; P < 0.001). In women whose blood pressure rose whilst on patches mean day-time ambulatory systolic BP increased by 6.5 mm Hg (95% CI 2, 11; P = 0.02), and mean night-time systolic BP by 5 mm Hg (95% CI 2.7, 7.3; P < 0.002). Mean day-time ambulatory diastolic BP rose by 7.3 mm Hg (95% CI 2.8, 11.8, not significant after Bonferroni correction) and mean night-time ambulatory diastolic BP increased by 4.2 mm Hg (95% CI 2.1, 6.3; P =0.003).

3.2.2.4 Statistical power

As the standard deviations in the above study were somewhat larger than expected from review of ambulatory blood pressure literature, retrospective power calculations were performed to determine the power of the current sample size to detect a difference in BP of 4 mm Hg at a significance level of 5%. In the oral treatment group, the power was 90% for diastolic BP and a minimum of 63% for systolic BP. In the transdermal treatment group, the power was 80% for diastolic BP and a minimum of 60% for systolic BP.
3.3 ANGIOTENSINOGEN GENE POLYMORPHISM AND BLOOD PRESSURE RESPONSE TO OESTROGEN REPLACEMENT.

3.3.1 Methods

3.3.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) originated in California about 11 years ago. Although originally technically difficult and labour intensive, owing to its tremendous sensitivity and wide application it has become an integral part of molecular biological research.

PCR is a method of amplifying nucleic acid sequences of the DNA. The underlying principle is simple, and basically mimics the process of in vivo DNA replication. A double strand of DNA is split into two single strands by heat (denaturation). Two primers, or oppositely orientated oligonucleotide strands, are added, which are designed to mark either side of the region of interest on the gene (annealing). A polymerase enzyme synthesises a new double strand of the marked section, by attaching deoxynucleotide triphosphates in an appropriate sequence to each single strand (extension). It requires rapid temperature changes, which can be easily achieved by using commercially available thermal cyclers.
This process can be repeated endlessly, and with every cycle the number of copies is doubled. PCR has been referred to as “the molecular biologist’s photocopying machine”. There are numerous potential applications for PCR, from sequencing DNA, detection of single point mutations, cloning, site-directed mutagenesis, in situ PCR, detection of infectious agents, amplification and labelling of probes for in situ hybridisation etc.

The main advantages of PCR are its sensitivity, which means that sequences can be detected in extremely small amounts of tissue. Further, by its nature it does not require the DNA sample to be particularly pure or of high quality, and even DNA from ancient samples can be amplified.\textsuperscript{105}

The main problem encountered with PCR is contamination from carry-over of previously amplified PCR products of the same target, which can easily lead to false positive results. This can be avoided by following stringent laboratory procedures and the actual physical separation of the sites and equipment used for the setting up of PCR reactions from those used for the handling of PCR products.

### 3.3.1.2 Sample preparation

Blood samples were collected from the patients participating in the ambulatory BP study as detailed in section 2.2.1. Seven ml of whole blood was collected from each patient into EDTA tubes and stored at -80° C until required.
3.3.1.3 DNA extraction

For DNA extraction blood samples were defrosted at room temperature, and a 0.5 ml aliquot was removed for further processing. Phenol DNA extraction from whole blood was performed using a standard protocol as follows:

1. 0.8 ml SSC (0.15 M NaCl, 0.015 M tri-sodium citrate) was pipetted into an Eppendorf tube.
2. 0.25 ml of whole blood was added.
3. Eppendorfs were microfuged for 2 min.
4. Supernatant was removed and 1.4 ml SSC added.
5. The samples were mixed and spun for 2 min.
6. Supernatant was discarded and the pellet resuspended in 270 μl 0.2M NaAcO (pH 7.0) and 30 μl 10% SDS
7. Samples were mixed gently, 200 μl phenol was added and vortexed for 15 sec. Samples were left to stand for 2 min.
8. 200 μl chloroform was added, vortexed briefly and spun for 5 min.
9. The upper aqueous phase was transferred into a fresh Eppendorf tube, 400 μl chloroform were added, vortexed briefly and spun for 2 min.
10. 0.8 ml of absolute alcohol was added to the upper aqueous phase and mixed gently. A DNA precipitate formed and was spun for 5 min to form pellet.
11. The DNA pellet was washed in 80 % alcohol, spun briefly and air dried after decanting alcohol.
12. The DNA pellet was dissolved in 50 μl of 10 mM Tris (pH 8.5) overnight at 4°C.
3.3.1.4 Protocol for detection of Angiotensinogen Met\textsuperscript{235}\textsuperscript{-Thr}

DNA was amplified using a PCR protocol for the rapid detection of the M235T (T235) allele of the human AGT gene using a custom made primer. Two control tubes were used with every reaction, one placed at the beginning and one at the end of the Eppendorf rack.

**PCR protocol:**

1. A ‘supermix’ was created with sufficient reagents for n+1 reactions with the following:
   - 2.5μl 10x PCR Buffer
   - 0.5μl dNTP (10 mM)
   - 1μl (80 ng) Primer 1 (CAG GGT GCT GTC CAC ACT GGA CCC C)
   - 1μl (80 ng) Primer 2 (CCG TTT GTG CAG GGC CTG GCT CTC T)
   - 19.8μl H\textsubscript{2}O
   - 0.2 μl Taq polymerase (5 u/μl)

2. 25 μl of the ‘supermix’ was added to each Eppendorf tube.

3. 0.5 μl DNA (250-500 ng/μl) was added, replacing this by a volume of water in the control tubes.

4. 20μl mineral oil was added to each tube. The Eppendorf were sealed and placed in a thermal cycler.
5. The following cycling program was commenced:
- 94°C 5 min  1 cycle
- 94°C 1 min  35 cycles
- 70°C 1 min

### 3.3.1.5 Enzyme digestion

Restriction enzyme digestion was performed using Thermus thermophilus (Tth) 111 endonuclease (10 u/μl), with the recognition sites 5'GACN\|NNGTC 3'; 3'CTGNN\|NCAG 5'. 10 units of Tth 111 in Multi-Core Buffer in a dilution of 1:10 were added to 10 μl of PCR product, covered with 20 μl of mineral oil and incubated at 65°C for 4-6 hours.

### 3.3.1.6 Gel electrophoresis

5μl of the digested product was mixed with 8 μl of gel loading buffer (Sigma). 12.5μl of the mixture was loaded onto a 2% Agarose midi-gel with 0.5μl etidium bromide per 10 ml gel, and submerged in electrophoresis buffer (750 ml TBE buffer). The samples were run for 3-4 hours, using a Bio-Rad apparatus.
The resulting bands were visualised under UV light and documented on Polaroid photographs. Samples homozygous for \textit{M235T} (TT) displayed a single band of 165 bp, samples homozygous for \textit{M235} (MM) had a single band of 141 bp and heterozygous samples (TM) had a double band. A 1 kb ladder (Sigma) was used as a positive control.

3.3.1.7 Statistical analysis

The frequency of mean ambulatory blood pressure increase or reduction with any given genotype was examined for statistical significance using the $\chi^2$-test. The correlation of the magnitude of blood pressure change with a given genotype was examined using multiple regression analysis.
3.3.2 Results

3.3.2.1 Allele frequencies

In total, 62 samples were evaluable. The allele frequency of the \textit{M235} allele was 0.59, that of the \textit{T235} allele was 0.43. The homozygous \textit{M235} (MM) genotype was found in 24 samples (39%), the heterozygous (TM) genotype in 27 (43%) and the homozygous \textit{T235} (TT) genotype was present in 11 samples (18%).

3.3.2.2 Blood pressure response by genotype

Table 5 shows the various mean ambulatory blood pressure responses according to genotype. Here the blood pressure response is classified as a category, i.e. either increased, decreased or unchanged. The differences between blood pressure responses in women with different genotypes were examined for statistical significance using the $\chi^2$-test. There were no statistically different responses to oestrrogen treatment within each genotype.

Table 6 shows the mean day and night ambulatory systolic and diastolic blood pressure at baseline and following six months oestrrogen replacement. In the multiple regression analysis, there was no statistically significant correlation of the magnitude of blood pressure change in mm Hg with any given genotype. For changes in day-time systolic BP $R=0.15$ ($y=2.11x -5.9; P=0.27$), for night-time
systolic BP R=0.11 (Y=1.53x -5.7; P=0.43). For changes in day-time diastolic BP R=0.1 (y=1.024x -4.26; P=0.55), for night-time diastolic BP R= 0.11 (y=1.36x -5.5; P=0.42).

Table 5: Changes in mean ambulatory blood pressure after 6 months oestrogen treatment according to genotype.

<table>
<thead>
<tr>
<th>BP change/Genotype</th>
<th>MM (n=24)</th>
<th>TM (n=27)</th>
<th>TT (n=11)</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day SBP $\uparrow$</td>
<td>6 (25%)</td>
<td>13 (48%)</td>
<td>2 (19%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day SBP $\downarrow$</td>
<td>18 (75%)</td>
<td>13 (48%)</td>
<td>9 (81%)</td>
<td>6.32</td>
<td>0.17</td>
</tr>
<tr>
<td>Day SBP $\equiv$</td>
<td>0</td>
<td>1 (4%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night SBP $\uparrow$</td>
<td>12 (50%)</td>
<td>11 (40%)</td>
<td>4 (36%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night SBP $\downarrow$</td>
<td>12 (50%)</td>
<td>16 (60%)</td>
<td>7 (36%)</td>
<td>0.72</td>
<td>0.69</td>
</tr>
<tr>
<td>Night SBP $\equiv$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day DBP $\uparrow$</td>
<td>8 (33%)</td>
<td>9 (33%)</td>
<td>1 (9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day DBP $\downarrow$</td>
<td>15 (63%)</td>
<td>16 (60%)</td>
<td>8 (73%)</td>
<td>3.92</td>
<td>0.42</td>
</tr>
<tr>
<td>Day DBP $\equiv$</td>
<td>1 (4%)</td>
<td>2 (7%)</td>
<td>2 (18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night DBP $\uparrow$</td>
<td>6 (43%)</td>
<td>7 (26%)</td>
<td>3 (27%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night DBP $\downarrow$</td>
<td>18 (57%)</td>
<td>18 (67%)</td>
<td>7 (64%)</td>
<td>2.19</td>
<td>0.701</td>
</tr>
<tr>
<td>Night DBP $\equiv$</td>
<td>0</td>
<td>2 (7%)</td>
<td>1 (9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SBP= Systolic blood pressure, DBP= Diastolic blood pressure

All BP values refer to ambulatory blood pressure measurements.
Table 6: Mean ambulatory blood pressure according to genotype, in mm Hg with standard deviation.

<table>
<thead>
<tr>
<th>Ambulatory BP/Genotype</th>
<th>MM</th>
<th>TM</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day SBP at baseline</td>
<td>129 (13.1)</td>
<td>118 (8.2)</td>
<td>122 (9.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Day SBP at 6 months</td>
<td>117 (12.1)</td>
<td>117 (12.3)</td>
<td>115 (13.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Night SBP at baseline</td>
<td>109 (12.9)</td>
<td>104 (9.2)</td>
<td>108 (10.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Night SBP at 6 months</td>
<td>104 (10.7)</td>
<td>101 (12)</td>
<td>105 (11.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Day DBP at baseline</td>
<td>77 (7.43)</td>
<td>77 (6.6)</td>
<td>78 (7.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Day DBP at 6 months</td>
<td>74 (8.1)</td>
<td>75 (11)</td>
<td>72 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Night DBP at baseline</td>
<td>67 (7.7)</td>
<td>63 (6.6)</td>
<td>68 (12.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Night DBP at 6 months</td>
<td>62 (9.1)</td>
<td>60 (8.8)</td>
<td>63 (8.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

SBP= Systolic blood pressure, DBP= Diastolic blood pressure

All BP values refer to ambulatory blood pressure measurements.
3.4 SUMMARY

To evaluate the effect of postmenopausal oestrogen administration on blood pressure, 90 normotensive oophorectomised women were treated for six months with either oral (Hormonin) or transdermal (Gynaderm) oestrogen. Their ambulatory blood pressure was checked before start of treatment, and then after three and six months on medication, looking at changes in mean day-time and night-time ambulatory blood pressure, and the magnitude of the physiological nocturnal decrease in blood pressure.

In women receiving oral therapy, a transient reduction in mean nocturnal systolic and diastolic blood pressure was demonstrated, however this was not maintained beyond the first three months of therapy. In women receiving transdermal oestrogen a sustained significant reduction in mean day-time diastolic ambulatory blood pressure (-4 mm Hg, 95% CI -6.8, -1.2) was observed (P = 0.014). Further, both mean night-time systolic (-4.2 mm Hg, 95% CI -7.7, -0.7; P=0.039) and diastolic (-4.4 mm Hg; 95% CI -7.1, -1.7; P = 0.005) ambulatory blood pressures were significantly reduced after six months oestrogen replacement.

Multiple regression analysis revealed that there was a positive correlation between the baseline blood pressure and the magnitude of blood pressure reduction in the transdermal treatment group, but not the oral treatment group, making women with high-normal blood pressures more likely to benefit from transdermal oestrogen replacement.
However, the proportion of women whose blood pressure increased on oestrogen replacement, about a third, was comparable in both groups. The observed magnitude of increase in blood pressure was significant, averaging between 4-8 mm Hg depending on the blood pressure modality examined.

To determine whether there is a genetic basis to this differential response to HRT, the genotypes of women participating in this study were examined for the M235T molecular variant of the angiotensinogen gene, which is thought to be associated with essential hypertension. A significant correlation between the angiotensinogen genotype and the blood pressure response to oestrogen treatment could not be demonstrated, however the sample examined was too small to allow a definitive conclusion to be drawn.
4. EFFECT OF ÖSTROGEN REPLACEMENT THERAPY ON VASCULAR LESIONS

4.1 INTRODUCTION

The clinical consequences of atherosclerosis are primarily ischaemic heart disease and myocardial infarction, cerebrovascular disease and stroke and peripheral vascular disease. Atherosclerosis related ischaemic vascular events are the commonest causes of mortality and morbidity in developed countries.¹ Health education and preventative measures have aimed on raising awareness of ways to prevent the development of atherosclerosis, since until relatively recently we have believed that, once an atheroma has developed, it was there to stay, and any measures taken may inhibit its progression, or perhaps inhibit the formation of a thrombus and thus reduce the risk of an ischaemic event. However, there is now evidence that atheroma can regress.

4.1.1 Can Atheroma Regress?

Animal models have provided suggestive evidence that, in a variety of species, atheroma formation can be inhibited or even reversed by diet, lipid lowering drugs, sometimes in combination with other measures, such as increase
ambient oxygen or oestrogen therapy.\textsuperscript{106} The Clarkson group demonstrated a significant reduction in atherosclerosis in cynomolgus macaques fed a high fat diet and subsequently treated with oral contraceptives. They observed a deficit of 50-75\% from the expected plaque extent, rising to 75-85\% when considering the high risk group of animals only, i.e. animals with the most atherogenic lipid profile.\textsuperscript{107} Further, they demonstrated a favourable effect of oestrogen in oophorectomised animals, with smaller plaque areas in oestrogen treated as opposed to untreated animals. Under certain circumstances, i.e. when oestrogen replacement was commenced immediately following oophorectomy, in animals in whom atherosclerosis had been induced, a reduction in the plaque area was observed. This finding is particularly interesting, as the atheroma of cynomolgus monkeys has been found resistant to regression with lipid lowering regimens.\textsuperscript{108}

Evidence that atherosclerosis can regress in humans stems from autopsy findings in starved human populations, and, more recently, from secondary intervention trials. Controlled trials with both clinical and angiographic end points demonstrated that treatments leading to lower LDL cholesterol levels and higher HDL cholesterol levels favour regression of coronary atherosclerosis, retard the progression of lesions and reduce the incidence of coronary events.\textsuperscript{109}
4.1.2 Imaging of Vascular Lesions

A major drawback in the study of atherosclerotic lesions is the difficulty of imaging noninvasively and with reasonable reproducibility, to allow adequate follow up of progress. The methods currently employed for quantification of atheromata in humans are fraught with difficulties and have serious limitations. Serial coronary angiography is the diagnostic technique used in most studies published to date. Several studies have shown that coronary angiography may underestimate the severity of atherosclerotic lesions, and may not assess the extent of atherosclerosis adequately, due to vascular remodelling by which lesions are displaced outwards, so that the luminal diameter may be preserved up to a point of moderately severe lesions. Similarly, if atherosclerosis is diffuse, thus narrowing the entire lumen, it will not be recognised by angiography. Furthermore, as atheromatous lesions regress, endothelial function may improve and therefore the vascular tone may not be the same in repeat imaging sessions. Other techniques employed for the quantification of atherosclerosis include femoral angiography, intra-vascular ultrasonography and magnetic resonance imaging. They also have drawbacks in that they are either invasive, such as the intravascular sonography and angiography, or expensive, as in the case of magnetic resonance imaging.

Carotid sonography also can be used to assess the extent of atherosclerosis and its response to treatment. It is non-invasive, reproducible, and allows repeat measurements at reasonable cost, and the method has been validated for use in the evaluation of carotid atherosclerosis. Furthermore, atherosclerotic lesions are highly prevalent, and can be demonstrated in up to 25% of adult population.
The ultrasound technique usually employed is Duplex imaging, which includes real-time and Doppler studies. The real-time component provides information on the degree of lumen stenosis, particularly in stenoses of less than 50%, and plaque length and thickness can be seen in images parallel to the axis of the vessel. Further, information on vascular wall structure and plaque morphology and characteristics can be obtained. The Doppler component provides information on flow velocities and wave form, which is particularly helpful in assessing stenoses of greater than 50%.
4.2 Regression of carotid atheromata following unopposed oestrogen treatment: A pilot study.

4.2.1 Methods

The study was conducted in the Menopause Research Unit, Department of Obstetrics and Gynaecology, in collaboration with the Vascular Studies Unit, Department of Surgery, Leicester University School of Medicine, with the approval of the local Ethics Committee.

4.2.1.1 Subjects

Over a period of 18 months, thirty five women were screened for entry into the study. Inclusion criteria were:

- postmenopausal status, at least 5 years
- no previous exposure to exogenous sex steroids
- no absolute contraindications to postmenopausal oestrogen therapy
- no history of postmenopausal bleeding
- known carotid disease, not requiring surgical intervention at point of contact
- ability to understand and comply with study requirements
Twenty postmenopausal women with carotid disease were subsequently recruited into the study. Their mean age was 68 years (+/- SD 6), and mean time since menopause was 14 years (5-20 years). Two women were recruited from the Menopause Clinic, because they were found to be at high risk for carotid atherosclerosis, and on clinical examination had bilateral carotid bruits. Thus the presence of carotid disease was suspected, and subsequently confirmed with ultrasound. The remaining patients were recruited from the patient database of the Vascular Studies Unit.

Two women were oophorectomised, but the remaining eighteen had undergone a natural menopause. Sixteen of the participating patients had an intact uterus, and the remaining patients were hysterectomised.

4.2.1.2 Õestrogens used

All participating women were treated with continuous oral unopposed œstrogen for six months. Õestrogen replacement was given in the form of Hormonin (600 µg micronised 17β-œstradiol, 270 µg œstriol and 1.4 mg œstrone), 1 tablet daily.
4.2.1.3 Concomitant medications

Two of the patients were taking concomitant Simvastatin, one a calcium antagonist (Nifedipine) and twelve were taking Aspirin. All patients had been receiving their concomitant medication for at least 6 months.

4.2.1.4 Assessment of plaque size

All patients underwent ultrasonographic assessment of their carotid arteries, with a Diasonics VST Masters Duplex Ultrasound scanner (Diasonics Ultrasound Inc., Milpitas, CA, USA) using a 5 MHz linear array probe. Real time sonography was performed to identify the presence of plaque and measure their dimensions. The internal carotid artery was imaged parallel to its axis, and the length and thickness of existing plaque was recorded. The thickness of the intima was also measured. The measurements were performed before oestrogen therapy was commenced, and subsequently following three and six months treatment.

All analysed scans were performed by a single operator, who was blinded to previous measurements. Intra-observer variability was assessed in six serial measurements of IMT, PT and PL in six carotid arteries (4 patients), i.e. 108 serial measurements. The operator was blinded to all measurements by covering the measurement box on the screen during the validation session. The Coefficient of Variation was 8% for IMT, 8% for PL and 10% for PT.
4.2.1.5 Outcome measures

The following outcome measures were evaluated:

1. Intimal thickness (IMT)
2. Plaque length (PL)
3. Plaque thickness (PT)

prior to treatment and following three and six months of oestrogen replacement therapy.

4.2.1.6 Endometrial safety

Non-hysterectomised women underwent standard outpatient hysteroscopy under local anaesthetic. The patient was placed in lithotomy position and after a bimanual examination the cervix was visualised with a Cusco speculum.

Intracervical block was performed using 4 ml 1% lignocaine. After grasping the anterior lip with a Vulsellum clamp, the cervix was dilated to Hegar no.4 and a rigid Wolf hysteroscope (4 mm) was passed into the uterine cavity. The cavity was distended using CO$_2$ through a hysteroflator with a pressure limit of 100 mm Hg. A fibre-optic cold light source provided adequate illumination and the hysteroscopic image was transmitted through a closed circuit television system with printing and video facility. The uterine cavity was examined and endometrial biopsy was taken at the end of the procedure for routine histopathology in all patients, using Pipelle de Cornier. No serious complications of the procedure were observed and no overnight hospital admissions were required.
4.2.1.7 Statistical analysis

All results are reported in mean mm with standard deviation, the net changes are reported in mean mm change with 95% Confidence Intervals. To avoid error through repeated comparisons, all P values relating to repeat measurements were Bonferroni corrected. Measurements of IMT, PT and PL were analysed using the two-tailed Student-t test for paired variables, comparing the measurements obtained at baseline with those at three and six months.

Because regression studies published to date have predominantly involved coronary or femoral angiography, the results available in the literature relate to degrees of stenosis or plaque surface area, whereas this study was concerned with absolute measurements of plaque length and thickness. Consequently, it was not possible to adequately estimate the required sample size from available published data. Therefore an arbitrary sample size was chosen, and the study is to be seen in a context of a small pilot trial, the results of which can be utilised to calculate the sample size for a definitive trial.
4.2.2 Results

Eighteen out of the twenty patients completed six month oestrogen treatment, with a total of twenty four atheromatous plaques. One patient’s carotid disease became symptomatic within one week of embarking into the study, when she suffered a transient ischaemic attack and amaurosis fugax. She was referred back to the vascular surgery team, and subsequently underwent carotid endarterectomy. A second patient developed heavy vaginal bleeding after four weeks of oestrogen replacement. Hysteroscopy and endometrial sampling revealed simple hyperplasia. She was withdrawn from the study and treated with 10 mg Medroxyprogesterone acetate daily for six weeks, which resulted in regression of the endometrial hyperplasia as confirmed by a repeat hysteroscopy and endometrial biopsy. Several plaques lost their clear outline in the course of their study, and/or became increasingly echolucent. The total number of evaluable plaques was therefore reduced from twenty four to eighteen at the end of the study period.

4.2.2.1 Changes in plaque dimensions

The mean Intima -Media thickness was 0.9 mm (+/- 0.5) at baseline, 0.80 mm (+/- 0.4; P=0.074) at three months and 0.8 mm (+/- 0.2; P=0.161) at six months follow up. There was no statistically significant difference. The mean plaque length fell from 13.7 mm (+/- 4.6) at baseline to 11.8 mm (+/- 4.7; P=0.006) after three months, and 12.3 mm (+/- 4.3; P=0.021) after six months oestrogen replacement. These changes were statistically significant at both three and
six months follow up. The mean plaque thickness was also reduced from 4.4 mm (+/- 5.8) at baseline to 3.8 mm (+/- 6.1; P=0.6) at three months, and to 3.7 mm (+/- 5.9; P=0.026) after six months œstrogen replacement. The change at six months was statistically significant.

The net change in plaque length was by -1.8 mm (95% Confidence Interval -2.6, -1.0; P=0.006) after three and by -1.4 mm (95% CI -1.3, -1.1; P=0.021) after six months. Plaque thickness was reduced by -0.6 mm (95% CI -1.5, 0.3; P=0.6) after three months treatment, and by -0.6 mm (95% CI -1.1, -0.1; P=0.026) after six months treatment (see Figure 5).
Figure 5: Changes in carotid plaque dimensions over six months of unopposed continuous oestrogen replacement, in mm from baseline with 95% Confidence intervals.
4.2.2.2 Hysteroscopy findings

One patient was withdrawn after 1 month of treatment as previously described. A further three patients complained of breakthrough bleeding, and their treatment was modified to a cyclical regime, i.e. they were instructed to take one tablet of Hormonin daily for 21 days out of every 28 days. They underwent hysteroscopic examination and endometrial sampling before the end of the study period, which revealed inactive endometrium in one case, and weakly proliferative endometrium in the other two cases. All three completed the study.

The fifteen patients with intact uteri, who had completed the study, underwent hysteroscopic evaluation and endometrial sampling after six months of estrogen treatment (Table 7). Two patients were found to have simple endometrial hyperplasia. Neither of them had complained of vaginal bleeding during the study period. Their endometrium reverted to a non hyperplastic picture following a course of Medroxyprogesterone acetate. This was confirmed by repeat hysteroscopic assessment of the uterine cavity and endometrial sampling.
Table 7: Endometrial histology of non-hysterectomised subjects after six months of unopposed oestrogen treatment.

<table>
<thead>
<tr>
<th>Endometrial histology</th>
<th>No of patients</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>atrophic</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>weakly proliferative</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>simple hyperplasia</td>
<td>2(3)*</td>
<td>13(19)*</td>
</tr>
</tbody>
</table>

* numbers in brackets are inclusive of the patient who withdrew after one month of treatment.
4.3 SUMMARY

To examine the effect of unopposed oestrogen replacement on atheromatous plaque in vivo, 20 postmenopausal women with known carotid disease were treated with continuous oral oestrogen (Hormonin) for six months. Their carotid arteries were examined ultrasonographically, by a single observer, before and after 3 and 6 months oestrogen replacement. Intima-media thickness, plaque length and plaque thickness were taken as outcome measures. Women with an intact uterus underwent outpatient hysteroscopy with endometrial sampling for endometrial safety.

Oral unopposed oestrogen replacement over a period of six months was associated with a significant reduction of ultrasonographically assessed carotid plaque size. Both plaque thickness ( -0.61 mm, 95% CI -1.1, -0.12; P=0.026) and plaque length ( -1.36 mm, 95% CI -1.25, -1.07; P=0.021) were significantly reduced, however a significant change in intima-media thickness could not be demonstrated.

Endometrial hyperplasia was observed in 19% of treated women who had an intact uterus, with spontaneous regression occurring in all cases, following withdrawal of oestrogen and progestogen treatment, as confirmed by repeat outpatient hysteroscopy and endometrial biopsy.
5. EFFECT OF ÒESTROGEN ON INTIMAL HYPERPLASIA

5.1 INTRODUCTION

Although some evidence exists from animal models that oestradiol may inhibit myointimal proliferation (see also General Background), to date we lack conclusive data relating to the response of human female blood vessels to events leading to intimal hyperplasia in vivo or in vitro, and to the effect of 17β oestradiol on this process.

The objective of this experimental work was to find an in vitro vascular model that would facilitate the study of the effect of sex steroids on blood vessels under controlled conditions, using all human material. In the following a model is introduced for induction of intimal hyperplasia in human ovarian veins cultured with the addition of human postmenopausal serum, and its potential modulation by sex steroid exposure. The model is adapted from a similar experiment developed to investigate mechanisms of the development of intimal hyperplasia and its inhibition in human saphenous veins.
5.1.1 Tissue culture

5.1.1.1 Cell Culture versus Organ Culture

Tissue culture techniques may date as far back as the 19th century, although, as may be expected, in those early days the experiments were fraught with numerous difficulties. These were due to factors such as lack of adequate tissue culture media, lack of knowledge of appropriate culture conditions, and infection of the cultured material. In the early years of the 20th century the techniques were improved in so far that longer term cultures became possible, principally due to the development of aseptic tissue culture techniques. Methods continued to be improved and it became possible to culture increasing numbers of different tissue types.

The two principal tissue culture techniques are cell culture and organ culture. The main aims of any tissue culture technique are:

- generation of adequate numbers of viable cells by continuous proliferation.
- preservation in vitro of the cell or tissue specific function of the material under study.

Cell culture utilises cells or cell lines, and their continuous cultivation, potentially over several generations, by proliferation, thus providing very homogeneous material for study. The principal advantages of cell culture are precisely this homogeneity, and the ease of control of the culture environment, such
as temperature, humidity, pondus Hydrogenii and tension of both O₂ and CO₂. The main disadvantages are both technical and functional. Firstly, stringent laboratory techniques must be employed to avoid contamination and bacterial or fungal overgrowth. Most laboratories will routinely add antibiotics to the culture media, but in spite of this infection occurs. Secondly, the cultured cells are removed from their physiological context, and there certainly seem to be differences in behaviour between cultured cell isolates and the same cell types in vivo. These differences probably arise because of the loss of specific cell interaction with different cell types in the three dimensional, organ specific arrangement, and the absence of systemic influences on the cultured cells, which would have modulated their function in vivo.

To overcome this latter disadvantage, organ culture was introduced. Here the cells are cultured in the physiological, three dimensional arrangement found in vivo, as organs, or parts of organs. This technique dates back to 1956, when Trowell first described vascular culture, and concluded that flat tissues are particularly suited for this particular scenario, as a problem with adequate diffusion of nutrients does not arise. Subsequently, several studies of human vein cultures were published, and the development of a neointimal layer was described.

The main advantage of culturing whole vein segments as opposed to endothelial cells is that the intact vascular wall architecture in culture allows the preservation of specific cellular interactions, as well as of individual components of extracellular matter, known to play a role in smooth muscle cell proliferation. The development of vein culture can therefore be viewed as a major advance in vascular research.
5.1.1.2 Arteries versus Veins

*In vivo* intimal hyperplasia occurs in both arteries and veins. Although it is widely known as a pathological process, it may have physiological, adaptive function to stress. A certain degree of intimal thickening is present in most veins, where it is related to factors such as age, venous pressure, local haemodynamic effects, and maybe hypoxia. It is particularly prominent in dependent, unsupported veins such as the saphenous vein. This is probably attributable to the higher intraluminal pressures at these sites, although it is also observed, albeit to a lesser degree, in well supported sites such as axillary veins.\(^{17}\)

Intimal thickening in arteries may occur at a very young age, again with considerable regional variations. In a histopathological study comparing internal thoracic arteries and anterior descending coronary arteries in 293 necropsies, Sims et al\(^{71}\) described intimal hyperplasia in coronary arteries at birth, with progressive increase with years. These changes were absent in the internal thoracic artery. In this particular study, the authors found that the presence of a thickened intima corresponded with a deficient lamina elastica interna. Coronary arteries have a relatively weak lamina elastica interna compared to other arteries, which may particularly predispose them to myointimal hyperplasia, as it facilitates the migration of smooth muscle cells from the media to the intima. This particular characteristic would also explain the frequency of intimal thickening in veins, as in most veins the lamina elastica interna is deficient or even absent.
Although several animal models have used arteries for culture, and a model using human mammary artery has also been described,\textsuperscript{118} this particular characteristic makes veins particularly suitable for use in models of induction of intimal hyperplasia, and therefore in the subsequent model for study of intimal hyperplasia ovarian veins were used.
5.2 INDUCTION OF INTIMAL HYPERPLASIA: IN VITRO
MODEL USING HUMAN OVARIAN VEINS AND
POSTMENOPAUSAL SERUM.

5.2.1 Methods

5.2.1.1 Adaptation of the saphenous vein model

In 1990 A.C Newby and his group won the European Society for Vascular Surgery Prize for the development of the organ culture of the human saphenous vein. This enabled the study of intimal hyperplasia in human veins, whilst preserving the anatomical relationship of the various vascular wall layers (see also 5.1.1.1). The model involved surgical preparation of the saphenous vein, and its subsequent culture in standard culture medium with added fetal calf serum. The experimental conditions of the saphenous vein model have been further modified by several research groups, including Porter et al. in the Department of Surgery at Leicester University School of Medicine.

For the purposes of this study, the human ovarian vein were used instead of the human saphenous vein. The reason for this choice was the fact that ovarian veins completely lack a lamina elastica interna, thus in theory being particularly susceptible to intimal hyperplasia (see 5.1.1.2). To minimise the use of non-human
components in this experiment, fetal calf serum was replaced by pooled human postmenopausal serum.

5.2.1.2 Collection and preparation of serum

80 ml of blood each was collected from 25 healthy, postmenopausal women who had given verbal consent. They were all non smokers with a mean age of 50.5 years (range 45-58), and were not on any concurrent medication. All had at least 6 months amenorrhoea. Menopausal status was confirmed biochemically, with a serum FSH of > 30 iU and serum oestradiol of <70 pmol/l. The clotted blood samples were centrifuged at 2000g for 30 minutes. The serum was then filtered through a 0.2 µm bacterial filter and, to inactivate growth factors, incubated for 30 minutes at 56° C in a water-bath. Following heat-inactivation the sera were pooled, aliquoted, and stored at -20° C until required.

5.2.1.3 Collection and preparation of veins

Fifteen salpingo-oophorectomy specimens were obtained from a routine gynaecological theatre list. All operations were performed for benign conditions, mainly menstrual disorders. Ovaries with the attached segment of infundibulo-pelvic ligament were immersed in sterile normal saline solution and transported to the laboratory at ambient temperature. All specimens were processed within 4 hrs of surgery.

The infundibulo-pelvic ligaments were dissected using fine ophthalmic forceps and scissors under sterile conditions, and the ovarian vein plexus were
identified. Vein segments of about 0.5 cm length were excised with a scalpel blade. The adventitia was cleaned from excess fatty tissue, the vein segments were opened longitudinally and pinned into culture dishes containing Sylgard resin with the endothelium uppermost (Figure 6). One segment from each vein (n=15) was fixed in 10% Formalin uncultured to serve as a paired control. Adjacent segments (n=15) were cultured as described below.

5.2.1.4 Vein culture

The vein segments were immersed into a culture medium containing phenol free RPMI, N-Glutamine, 50 μg/ml Penicillin and 50 μg/ml Streptomycin (all by Sigma). Instead of using the in the literature described fetal calf serum to supplement the culture media, heat inactivated pooled postmenopausal serum was used, constituting 20% of the medium. The culture medium was changed every 48 hours. The vein segments were cultured for 14 days in a humidified carbon dioxide chamber at 37° C. On day 15 the culture medium was discarded, the tissue was covered with 10% formalin and fixed for 48 hours.
Figure 6: Blood vessel segment in a culture dish, opened and pinned endothelium uppermost into Sylgard resin (size 200%).
5.2.1.5 Demonstration of vascular wall structure

5.2.1.5.1 Immunocytochemistry

Immunocytochemistry utilises the specific binding of an antibody to its antigen, and visualising the site of its binding by attaching a microscopically dense marker to the antigen-antibody complex. Historically, the first markers were fluorescent probes. Peroxidase and alkaline phosphatase probes followed on, and are the two probably most widely used markers in routine immunocytochemistry.

Both monoclonal and polyclonal antibodies can be used for immunocytochemistry. The ideal antibody should be highly specific, have a high amount of binding activity (titre), and a high affinity (stereochemical fit) and avidity (total binding strength).

Antigen, antibody and the microscopically dense marker are linked together in successive steps. One-, two- or three-step techniques exist. In one-step techniques the antibody is directly coupled with the microscopically dense marker. This technique is feasible in the routine running of the same immunocytochemical test, where an abundance of antibody is present. In the two-step method, the antigen is incubated with the specific antibody, followed by the dense probe. The most widely used technique is probably the three-step, or indirect method. Here a secondary antibody is utilised between the primary antibody and the dense marker. The three-step technique is highly sensitive.
Immunocytochemical methods have several inherent problems. Firstly, non-immunological binding of antibodies and microscopically dense probes can occur. This can be counteracted by using blocking reagents such as bovine serum albumin, or by the use of surfactants and chemical reagents, within the ICC protocol. Because it is possible to mistake background staining with immuno-staining, controls are absolutely essential. Positive controls test the reactivity of the antibodies and markers used, and negative controls will provide information on methodological non-specificity of the technique employed.

5.2.1.5.1.1 Frozen sections vs formalin

Some antibodies are principally suited for use in frozen section. However, frozen sections have inherent disadvantages. In studies involving investigation of structural vascular changes, frozen sections are suboptimal, as the required degree of preservation of vascular wall integrity may not be easy to achieve. Apart from problems with morphology, frozen sections are less suitable for high magnifications because of poor resolution. Further, only limited retrospective work can be carried out, and there is a residual health hazard associated with the handling of unfixed tissues. Formalin fixation preserves morphology, however, it alters molecular configurations of antigen sites, thus reducing their binding of specific antibodies. With the development of antigen unmasking techniques such as enzyme digestion, microwave treatment and superheating, it has been possible to obtain satisfactory results using Formalin-fixed, paraffin-embedded sections.
5.2.1.5.1.2 **Antigen unmasking**

- *Enzyme pre-treatment* of formalin fixed tissue unmasks antigenic sites, which allows binding of the specific antibody to these sites in the immunohistochemical staining process. The most commonly used enzymes are pepsin and trypsin.

- Heating of the tissues to be stained is another useful unmasking technique for use with formalin fixed tissue, either in a microwave oven or under pressure in an autoclave (pressure cooker). The sections have to be immersed in an antigen unmasking agent before heating.

5.2.1.5.2 **Immunocytochemical stains**

The vein segments were paraffin embedded. Sections of 5 μm thickness were cut using the Leica RM2035 microtome and mounted on glass slides. All slides used in the immunocytochemical and molecular biology work were pre-treated as follows:

- The slides to be coated were stored in black plastic racks and soaked in 1% lipsol for 30 minutes.
- Next the slides were washed in running tap water and then soaked 2 changes of in Ultra Pure water for 10 minutes each.
- Each slide rack was taken through 2 changes of 95% methylated spirit and then air-dried.
- Each slide rack was submerged in 3% silane in methanol for 5 seconds, then
washed briefly in 2 changes of Ultra Pure water.

- Slides were dried at 40°C overnight.

To confirm the presence of intact endothelium on every vein segment, the sections were stained with mouse monoclonal CD 31 endothelial antibody following a standard immunocytochemistry protocol, using the indirect Streptavidin-Biotin Technique.

**Anti-CD 31 protocol:**

1. Slides were dewaxed in Xylene, then taken to water through an alcohol gradient (3 min in each 99% Alcohol, 95% Alcohol, 70% Alcohol, tap water).
2. Slides were submerged in Aqua dest for 3 min.
3. Sections were incubated with 100 μl Pepsin (50 ml Aqua dest, 0.2 g Pepsin, 100 μl Hcl) for 15 min at 37° C, then washed with tap water followed by Aqua dest.
4. Slides were again submerged in TBS (Tris buffered saline, pH 7.6) for 5 min.
5. Sections were dried; Rabbit Serum 1:10 in TBS, 100 μl per section was applied and incubated 10 min at room temperature.
6. Sections were dried; primary antibody anti-PECAM-1 or anti-CD 31 in a concentration of 1:200 in TBS, 100 μl per section was applied and incubated 2 hrs at 37° C or overnight in fridge at 4° C.
7. Slides were washed in TBS for 20 min.
8. Sections were dried; Rabbit anti-Mouse secondary antibody (1:400 in TBS) was applied and incubated for 30 min at room temperature.

9. Slides were washed in TBS for 20 min.

10. Sections were dried; ABC Reagent (5 ml Phosphate buffered saline pH 7.6, PBS + 2 drops A+2 drops B), 100 μl per section, was applied and incubated for 30 min at room temperature.

11. Slides were washed in PBS for 30 min.

12. Colour reagent Fast Red TR/Naphtol AS-MX (10 ml Aqua dest, TRIS buffer & Fast red/Naphtol tablet), was applied and incubated approximately 10 min, then washed with tap water.

13. Cover slips were mounted on slides using an aqueous mounting solution such as Aquamount.

   To demonstrate vascular wall structure, the sections were then stained with anti-α Smooth Muscle Actin, again following a standard immunocytochemistry protocol as detailed above, using mouse monoclonal anti-α Smooth Muscle Actin antibody (Dako) at a concentration of 1:500; subsequently they were counterstained in Miller’s Elastin stain (Raymond Lamb).

   **Anti-α Smooth Muscle Actin & Miller protocol:**

   Steps detailed in anti-CD 31 protocol were followed, however, enzyme digestion (step 3) was omitted. Following step 12, sections were taken to alcohol, then submerged in Miller’s Elastin stain for 10 min, rinsed in absolute alcohol and washed with running tap water for 5 minutes.
5.2.1.6 Measurement of intimal thickness

Intimal thickness was measured using light microscopy and image analysis. The equipment used included an Axioplan light microscope (Carl Zeiss, Herefordshire UK), a colour video camera (Sony DXC-151P) with a camera adapter (Sony CMA-151P), a RasterOps 24STV graphics display board and an Apple Macintosh computer (Centris 650), with an image analysis system for Macintosh (Image 1.52), at 20x10 magnification. The image analysis display was calibrated using a 200x1 μm scale, with 20 pixels being equivalent to 1 μm. Intima-media thickness was measured 4x in 5 high-power fields per slide (20 measurements per blood vessel) by two observers blinded to the culture conditions.

The vascular wall in fresh ovarian vein specimens consists of four distinct layers. On the luminal side of the vessel there is a layer of endothelial cells, followed by three muscular layers. The first layer consists of loosely scattered, longitudinally orientated muscle, embedded in variable amount of extracellular matrix. Ovarian veins do not possess a lamina elastica interna, therefore this layer is in a continuum with the subendothelial extracellular matter. The next layer consists of more densely packed, circular muscle layer, followed by a further coat of longitudinal muscle. The measurement was performed from the luminal endothelial margin to the consistent, circular layer of medial muscle.
5.2.1.7 Validation

The intra-observer variability was assessed by obtaining two series of 10 measurements each of 5 sections of cultured veins, i.e. 100 measurements, by a single observer. The measurements were taken at a magnification of 20x10 in constant lighting conditions, from the endothelial margin to the intima-circular medial muscle layer interface. The observer was blinded to the measurement values by obscuring the measurements box on the monitor screen. The coefficient of variability was 10%.

The inter-observer variability of the intimal thickness measurement was assessed on 15 sections of cultured veins. The vein segment was centralised in the visual field of the microscope at a magnification of 20x10 in constant lighting conditions. Each observer captured the image on the image analysis program after focusing, and measured the distance from the endothelial margin to the intima-circular medial muscle layer interface 10 times. A Bland-Altman plot for the inter-observer error was generated (Figure 7). The limits of agreement between observers were -1.6μm to 1.8μm, which is a variability of about 11% for a mean intimal thickness of 15.7μm.
Figure 7: Bland Altman plot of inter-observer error in the measurement of intimal thickness.
5.2.1.8 Statistical analysis

Intimal thickness of each blood vessel was obtained from the mean of both observers. Differences in mean intimal thickness between uncultured and cultured vein segments were analysed for statistical significance using Student-\(t\) test for paired variables. The relationship between the baseline intimal thickness in uncultured vessels and the thickness of the neointima following culture was examined using simple regression analysis.
5.2.2 Results

5.2.2.1 Endothelial coverage

All fresh and cultured specimens had largely intact endothelial coverage as demonstrable with CD 31 staining (Figure 8). Any endothelial coverage defects occurred near the cut edges of the vein segments and did not involve more than approximately 25% of the endothelial surface in any of the specimens.

5.2.2.2 Structure of fresh ovarian veins

The structure of the ovarian vein is described in 5.2.1.6. (Figure 9a). In a proportion of fresh veins, a degree of pre-existent intimal thickening was observed, both focal and generalised thickening. This was most prominent in veins from specimens where large uterine fibroids had been present.

5.2.2.3 Cultured veins

Intimal hyperplasia, consisting of thickening of the intima-media layer, accumulation of actin positive cells and extracellular matrix, was induced to a varying degree in all cultured blood vessels (Figure 9b). A layer of loosely stratified cells was observed overlying the endothelial margin of the blood vessel in 8 of the 15 cultured blood vessels (Figure 10). This layer will be referred to as the 'proliferative layer'.
Figure 8: Ovarian vein section stained with the endothelial marker CD 31. Both the luminal endothelium (large arrow) and that of the vasa vasorum (small arrow) are staining positive (x100).
Figure 9a & 9b: Section of a human ovarian vein, stained with α-Smooth Muscle Actin and Millers Elastin, demonstrating its layers. 9a: native vein (x200), 9b: cultured vein (x400). L: Lumen. 1: The intima including the first muscle coat. 2: The second, circular muscle coat. 3: The outer muscle coat.
Figures 10a & 10b: Cultured human ovarian vein, with the new, loose, multilayered stratum the "proliferative layer" (P), overlying the luminal endothelium (10a, x1000), and extending around the cut edges of the section (10b, x 100). L: lumen. C: cut edge. A: adventitial edge of vein.
The proliferative cell layer was negative for CD 31. Some cells within this layer were weakly positive for α-smooth muscle actin. Where a proliferative layer has formed, it extended over the cut edges of the vein section, and in some instances included the adventitial side.

The mean intimal thickness increased from 8.25 μm (95% Confidence Interval 6.3, 10.2) in fresh veins to 15.7 μm (95% Confidence interval 13.6, 17.8) in cultured specimens (Figure 11). This difference was statistically significant (P=0.0001). There was no significant correlation between the baseline intimal thickness of the fresh vein, and the degree of subsequent neointimal development in culture (R=0.21, P=0.45) (Figure 12).
Figure 11: Mean intimal thickness in ovarian veins before and after culture, in mean μm with 95% Confidence Intervals.
Figure 12: Correlation of intimal thickness in cultured and fresh ovarian veins.
5.3 Effect of 17β-oestradiol on the development of intimal hyperplasia.

5.3.1 Methods

For specimen collection and preparation as well as culture technique see 4.2.1. Ten vein segments were cultured in a standard culture medium supplemented with heat-inactivated human postmenopausal serum as described previously. Ten paired vein segments were cultured with added 17β-oestradiol in physiological concentration (10^{-6}). A further ten segments from the same veins were cultured with the addition of the biologically inert 17α-oestradiol (10^{-6}), to serve as controls. Five vein segments each were cultured with 17β-oestradiol in a dose response curve, with concentrations ranging from physiological through to pharmacological levels (10^{-7}-10^{-9}). The oestrogen supplement was added throughout the fourteen day culture period, with routine change of culture medium every 48 hours.

5.3.2 Results

The addition of 17β-oestradiol in a pharmacological concentration (10^{-6}) was associated with a reduction in intimal thickness from 15.1 μm (95%
Confidence Interval 11.9, 18.3) in veins cultured in postmenopausal serum without sex steroid addition, to 8.9 μm (95% Confidence Interval 6.9, 10.8). This difference was statistically significant (P=0.008). Although the overall thickness of the intima was reduced, the addition of oestrogen did not seem to affect the development of a proliferative layer, as again it was present in more than half of the blood vessels.

In veins cultured with the addition of the inert 17α-oestradiol mean intimal thickness was 14.3 μm (95% Confidence Interval 11.4, 17.2). This was not significantly different from veins cultured without the addition of sex steroids (P=0.23) (Figure 13). There was no statistical difference in intimal thickness between the various doses of oestradiol, but only the pharmacological dose produced a significantly thinner intima when compared to veins cultured without sex steroid addition (see Table 8).
Table 8: Mean intimal thickness with 95% Confidence intervals in blood vessels under varying culture conditions.

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Mean Intimal Thickness in µm</th>
<th>95% Confidence Interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>no sex steroid</td>
<td>15.1</td>
<td>11.9, 18.3</td>
<td>N/A</td>
</tr>
<tr>
<td>17 β-ōestradiol 10^6</td>
<td>08.3</td>
<td>06.0, 10.6</td>
<td>P=0.008</td>
</tr>
<tr>
<td>17 β-ōestradiol 10^7</td>
<td>11.9</td>
<td>08.4, 15.4</td>
<td>P=0.19</td>
</tr>
<tr>
<td>17 β-ōestradiol 10^8</td>
<td>10.1</td>
<td>07.8, 12.5</td>
<td>P=0.06</td>
</tr>
<tr>
<td>17 β-ōestradiol 10^9</td>
<td>10.9</td>
<td>08.8, 12.7</td>
<td>P=0.16</td>
</tr>
<tr>
<td>17 α-ōestradiol 10^6</td>
<td>14.3</td>
<td>11.4, 17.3</td>
<td>P=0.23</td>
</tr>
</tbody>
</table>
Figure 13: Intimal thickness in ovarian veins cultured with 17\(\alpha\) and 17\(\beta\) oestradiol, in mean \(\mu m\) with 95\% Confidence intervals.
5.4 Expression of proliferation markers in cultured human ovarian veins.

5.4.1 Methods

5.4.1.1 Proliferation markers

One of the main characteristics of intimal hyperplasia is the increase in proliferation, particularly that of vascular smooth muscle cells. An increase in proliferative activity has been shown to precede the development of neointima in animal vascular cultures. Studies on human saphenous veins have confirmed that smooth muscle cell proliferation increases in culture, with the maximum proliferation occurring at around two weeks of culture. The extent of pre-existing proliferation in the human vascular intima is unclear, but ex vivo work on segments of fresh human arteries suggests this to be very low, with a proliferation index somewhere in the region of 0.1%. It has been suggested that inhibiting smooth muscle cell proliferation may contribute to a reduction in vascular disease, and one of the presumed mechanisms of the protective action oestrogens exert on the vascular system is indeed an anti-proliferative effect.

Several methods are available for the assessment of proliferative activity in tissues, including labelling with $[^3]$H-thymidine or its analogue, 5-Bromodeoxyuridine (BrdU). Relatively recently immunocytochemical techniques have been introduced utilising antibodies which identify nuclear antigens in different
phases of the cell cycle, such as Ki67 and PCNA (proliferating cell nuclear antigen).

Sophisticated methods for the assessment of proliferation in tissues such as DNA flow cytometry and [3H]-thymidine labelling require expensive equipment, and a relatively high level of expertise, which makes them less suitable for routine use. However, BrdU can be used instead of [3H]-thymidine, and is available for use in kit form. BrdU is incorporated into the DNA during the S (synthetic) phase of the cell cycle, and can then be detected by monoclonal antibodies following DNA denaturation. This technique produces a good demonstration of cells in S phase, but its main disadvantage is the need for in vitro incubation of fresh tissue. In fact, it has been shown to be toxic to blood vessels in vitro, thus potentially limiting its usefulness in vascular culture models.

The Ki67 antigen is a non-histone protein component of the nuclear matrix, which is expressed in all phases of the cell cycle except G₀ and early G₁. Its immunoreactivity has been shown to correlate well with other indices of cell proliferation such as [3H]-thymidine and BrdU incorporation, and flow cytometry. The antibody to Ki67 is available as a mouse monoclonal antibody. Its use for the assessment of proliferation indices of human blood vessels has been successfully performed by several authors. Unfortunately, the Ki67 antigen is sensitive to fixation, and for best results frozen sections should be used. For use in paraffin embedded tissues, as used in this work, antigen retrieval, in this case microwaving, is necessary. This has been described using monoclonal antibodies to Mib 1, which is a recombinant part of the Ki67 antigen.
PCNA is another cell cycle related antigen, which functions as an auxiliary protein in DNA synthesis. It is present in low levels in quiescent cells, but reaches its maximum in the S phase of the cell cycle. The anti-PCNA works well in Formalin fixed tissues, however its immunoreactivity declines with fixation time. Its detection is greatly reduced after 48 hours fixation, and almost non-existent after 72 hours. However, by its nature it works better in freshly obtained tissues. As with Ki67, PCNA has been used in blood vessels, in both fresh and Formalin fixed sections.

5.4.1.2 Basic stain

To allow the assessment of proliferation indices, the nuclei were stained using with Mayer’s Haematoxylin. For this purpose the sections were dewaxed and taken to water, then stained by immersion in Mayers’ Haematoxylin solution for 10 minutes, washed and mounted.

5.4.1.3 Bromodeoxyuridine

A BrdU labelling was performed using a commercial kit (Boehringer Mannheim Biochemicals). BrdU was added to the culture medium in a 1:1000 dilution from day 12 to day 14. Following culture the veins were fixed and embedded in paraffin. BrdU uptake was demonstrated with anti-BrdU antibody supplied in the kit, according to the manufacturer’s protocol.
5.4.1.4 Anti-Mib-1

A standard ICC protocol using an indirect Streptavidin-Biotin-Alkaline Phosphatase technique was followed as detailed in 4.2.1.4, except:

- Following step 2, submerge slides in Citrate Buffer 10 mM pH 6.0, and microwave for 15 min, cool down to room temperature, wash with Aqua dest and proceed to step 3.
- Primary antibody anti-Mib1 is applied in a concentration of 1:25 in TBS and incubated overnight at room temperature.

5.4.1.5 Anti-PCNA

A standard ICC protocol using an indirect Streptavidin-Biotin technique was followed as detailed in 4.2.1.4, with the primary antibody anti-PCNA applied in a concentration of 1:50.

5.4.1.6 Light microscopy

Nuclei were counted using light microscopy (Axioplan microscope, Zeiss, Germany) with an image analysis program (Image 1.52) for Macintosh. The image of the intimal layer at a magnification of 100x10 was captured and visualised on the monitor. The number of positive nuclei per high power field was counted on 15 high power fields per slide. The nuclei were counted in the intimal layer, excluding the loose neointimal layer, as this was not present in all specimens.
5.4.2 Results

5.4.2.1 Intimal Nuclear Counts

The mean number of nuclei per high power field in the intimal layer of uncultured veins was 10.9 (95% Confidence Interval 9.5, 12.5). This rose to an mean of 14.1 (95% Confidence Interval 12.5, 15.6) nuclei per high power field in cultured veins. This increase was statistically significant (P=0.001). In the intima of veins segments cultured with the addition of 17β-oestradiol in pharmacological concentration (10⁻⁶) the nuclear count per high power field was significantly lower than that of veins cultured without the addition of sex steroids (11.6 nuclei per high power field; 95% Confidence Interval 10, 13.3) (P=0.045). The addition of 17α-oestradiol did not lead to a significant change in intimal nuclear count, when compared to veins cultured without steroid addition. The mean number of nuclei per high power field in these control segments was 12.9 (95% Confidence Interval 11.8, 13.9).

5.4.2.2 Intimal proliferation

None of the three proliferation markers used has been successful. The BrdU labelled slides had an unacceptably high level of non-specific background staining. Despite attempts to reduce non-specific binding by blocking with undiluted rabbit serum and the introduction of extra washing steps, the degree of background staining could not be reduced to a level which would have allowed an adequate visualisation of BrdU positive nuclei.
The Mib 1 antibody protocol required a 15 minute period of microwaving to unmask the Ki67 antigen in Formalin-fixed sections. This led to disruption of the vascular wall morphology, and partial or complete detachment of the sections from the glass slides, although the slides were silane coated for better adhesiveness. Attempts to reduce microwaving time, to microwave in pulses of 5 minutes each, or to substitute microwaving by other antigen retrieval techniques such as enzyme digestion and superheating, were unsuccessful.

As with BrdU, the degree of background staining made it impossible to adequately visualise PCNA positive nuclei. Using varying dilutions, neat rabbit serum blocking and additional wash steps has proved futile, and therefore adequate assessment of proliferative activity in cultured ovarian veins could not be made.
5.5 Summary

The cultured human ovarian veins maintained viability throughout the observation period of 14 days, in a standard culture medium supplemented with heat-inactivated pooled human postmenopausal serum. Intimal hyperplasia was observed following culture. The mean intimal thickness increased significantly from 8.3µm (95% Confidence Interval 6.3, 10.2) in fresh veins to 15.7 µm (95% Confidence interval 13.6, 17.8) in cultured specimens (P=0.0001).

The addition of 17β oestradiol in pharmacological doses (10⁻⁶) resulted in a reduction of mean intimal thickness by 58% when compared to that of veins cultured without oestrogen, or with the inert 17α oestradiol. However, this effect was only observed with pharmacological doses of oestrogen. There was no significant reduction in intimal thickness in veins cultured in a dose response curve experiment using 17β oestradiol concentrations of 10⁻⁷-10⁻⁹.

In some veins a new loose, multi-layered stratum was observed overlying the luminal endothelium. The cells in this layer did not stain positive for CD31, therefore it is unlikely that they are of endothelial origin. The development of this proliferative layer was not affected by the addition of sex steroids and its origin and significance are yet to be determined.
An attempt was made to assess proliferation in the neointima with the markers BrdU, Ki67 (Mib 1) and PCNA, however this was unsuccessful. The lack of success was partly due to the intolerance of formalin fixed ovarian veins to microwaving (as an antigen retrieval technique), and excessive background stain, which precluded reproducible evaluation of the results. However, nuclear counts in the neointima increased significantly from 10.9 (95% Confidence Interval 9.5, 12.5) in fresh veins to an average of 14.1 (95% Confidence Interval 12.5, 15.6) nuclei per high power field in cultured veins (P=0.001). The addition of 17β oestradiol again led to a significant reduction in nuclear counts. This suggests that regulation of proliferation is involved in the process of neointimal development.
6. EFFECT OF ÕESTROGEN ON VASCULAR DERIVED CYTOKINES

6.1 INTRODUCTION

Cell to cell communication involves the release of soluble substances by certain cells, and their recognition by others. The messenger substances exchanged are called cytokines. The actual exchange of information between cells may involve numerous autocrine, paracrine and juxtacrine pathways. In an autocrine loop, the cell producing a certain cytokine possesses a receptor for the same substance, whereas in paracrine loops, a particular type of cells releases a substance that will then act on a different cell type. The juxtacrine pathway is similar, except here a direct cell-to-cell contact is required, as the cytokine is bound to the membrane of the producing cell.126

Cytokine production does not require a particularly specialised tissue, in fact any given cytokine may be produced by a variety of cell types, but they are recognised by specialised cell membrane receptors on their target cells. Their binding to the receptor induces an intracellular cascade of events which eventually leads to nuclear activity, via a second messenger, resulting in the induction of a specific process such as proliferation, differentiation or other cell function.
Cytokines fulfil a wide range of physiological and pathological functions, and with the development of sophisticated molecular biology and immunocytochemistry techniques it has become possible to investigate the roles various cytokines play in health and disease.

Although this process is not unique to blood vessels, vascular derived cytokines have generated much interest because of their role in tumour growth and cardiovascular disease. Numerous vascular derived cytokines have been described to date, including PDGF (platelet derived growth factor), FGF (fibroblast growth factor, TGF-β (transforming growth factor-β), interleukins, colony stimulating factors, endothelins etc. In the following this work will focus on a selection of adhesion molecules and growth factors, that have recently been implicated in the pathogenesis of vascular disease and endothelial dysfunction. They include the adhesion molecules ICAM-1, VCAM-1 and E-Selectin, and the angiogenic cytokine VEGF. So far, these four substances have been implicated in the pathogenesis of vascular damage (see General Background), however, to date findings on the effect of sex steroids on their expression are yet to be published. That is with the exception of VEGF, which has been widely researched in the context of reproductive medicine and endometriosis, where its expression in the endometrium seems to be upregulated by œstrogen.
6.1.1 Vascular endothelial growth factor

VEGF, also known as VPF (vascular permeability factor), is a potent, multifunctional, heparin binding cytokine, originally described as a tumour secreted protein. It is a highly conserved 45 kD glycoprotein, with significant, although not extensive, homology with PDGF and placental growth factor. The expression of four isoforms of VEGF by human cells has been described to date, arising from alternative splicing of a single gene. They comprise polypeptides of 206, 189, 165 and 121 amino acids. VEGF\textsubscript{165} is the predominantly expressed isoform. The four isoforms have similar biological activities, however the two smaller polypeptides are secreted in soluble form and appear in the serum, whereas the two longer chains are cell bound.

VEGF binds to high affinity tyrosine kinase receptors Flt-1, Flt-4 and Flk-1/KDR. These receptors are present only on endothelial cells, and consequently, in contrast to other vascular derived growth factors, such as acidic and basic FGF, which may act as a mitogen for vascular smooth muscle cells and fibroblasts, the mitogenic action of VEGF is endothelial cell specific. However, VEGF may act via certain low affinity binding sites on other cell types, so it has been shown for example to exert chemotactic effects on mononuclear phagocytes.

Several function have been linked to VEGF so far. VEGF plays an important role in vasculogenesis, and in both physiological and pathological
angiogenesis. It is overexpressed in most malignant tumours, healing wounds, placenta and fetal tissues, endometrium and corpus luteum.\textsuperscript{131}

VEGF is a potent endothelial cell mitogen and has been shown to stimulate endothelial cell migration. It has been linked to endothelial dysfunction by upregulating the expression of factors affecting both coagulation (tissue factor) and fibrinolysis (PAI-1).\textsuperscript{132} Further, VEGF increases microvascular hyperpermeability significantly.\textsuperscript{133} Its permeabilising activity is quite striking, with about 50 000x the potency of histamine, a feature which may be linked to its angiogenic potential.

The expression of VEGF seems to be regulated by a number of cytokines and growth factors, particularly those stimulating vascular smooth muscle cell proliferation.\textsuperscript{134} It is possible that the stimulation of VEGF expression by these growth factors is tissue specific, i.e. different growth factors are required to stimulate VEGF expression in various cell types. Its expression is also upregulated by tissue hypoxia, and interestingly, VEGF shares sequences with erythropoietin, another oxygen-sensitive gene. The increased VEGF expression in response to a hypoxic stimulus has been demonstrated in studies on compensatory angiogenesis in underperfused tumours, and on cultured endothelial cells.\textsuperscript{135}

Recently, the application of VEGF to damaged endothelium in rat carotid arteries has been shown to lead to a reduction in the extent of intimal hyperplasia on these sites.\textsuperscript{90} As in this investigation it has been possible to demonstrate a reduced degree of intimal hyperplasia in òøstrogen treated blood vessels, it was of interest to see whether this is associated with changes in VEGF expression. In the investigation described below, changes in the expression of secreted VEGF in vivo were examined in the serum of postmenopausal women receiving òøstrogen
replacement, using a commercially available ELISA kit. The expression of VEGF in vitro by ovarian veins, as well as its modulation by sex steroids, was examined in the vascular culture model introduced earlier in this thesis, using in situ hybridisation for VEGF mRNA.

### 6.1.2 E-Selectin

The selectins are glycoproteins with a molecular mass of 90-140 kD. They are expressed on the surface of blood cells and endothelial cells. E-selectin (CD62E) was first described as ELAM-1 (Endothelial-leukocyte adhesion molecule-1) in 1987. E-selectin core protein has a molecular weight of 64 kD, with 11 N-glycosylation sites. Like other selectins, it possess a lectin-like binding domain, and an Epidermal growth factor-like domain. Its ligand is sialyl Lewis X (CD15s), a tetrasaccharide with fucose and sialic acid linkage, heavily expressed on neutrophils, natural killer cells and monocytes.

In contrast to other selectins, E-selectin is not expressed constitutively on endothelial cells. It is synthesised de novo after endothelial cell activation, with a 4-6 hour time lag. Like ICAM-1 and VCAM-1, its expression is induced by a variety of cytokines, including Interleukin-1 and TNF-α. E-selectin expression has been demonstrated in human atherosclerotic coronary arteries, where it appears to follow the distribution of CD 31, and is expressed consistently on intimal endothelium. Its expression on activated endothelial cells is thought to facilitate the adhesion of neutrophils, T-cells and monocytes, and possibly their recruitment into the intima.
However, widespread, uniform expression of E-selectin has been observed in the intimal endothelium of atherosclerotic coronary arteries, even in plaque devoid areas, suggesting that leukocyte recruitment and the progression of focal lesions may not be the only role of E-selectin in intimal disease.\textsuperscript{137}

Data on the expression of E-selectin in blood vessels with myointimal proliferation or intimal hyperplasia is not yet available. However, as the vascular wall in the ovarian vein culture model has clearly shown a response to activation by surgical preparation and culture, it was of interest to investigate the range of adhesion molecules expressed in vitro as a result of this stimulus, and this included E-selectin, mainly because of its well described role in vascular inflammatory response.

### 6.1.3 ICAM-1 and VCAM-1

Like E-selectin, the expression of the immunoglobulins ICAM-1 and VCAM-1 can be upregulated by a variety of cytokines released in the course of blood vessel inflammation. Whilst ICAM-1 is constitutively expressed at a low, basal level on vascular endothelium, VCAM-1 is only synthesised following vascular insults. Human ICAM-1 is located on chromosome 19. It has a 55 kD core protein with five Ig-like domains, with a binding site for LFA-1. VCAM-1 is a 110 kD protein with seven Ig-like domains.
Both molecules are thought to be involved in leukocyte recruitment into the intima in diseased blood vessels, and the presence of both has been demonstrated in atherosclerotic human plaques. However, several studies have identified ICAM-1 and VCAM-1 expression by subsets of vascular smooth muscle cells in vascular lesions, and it has been suggested that both molecules could serve as markers of vascular smooth muscle cell activation, thus defining it more accurately than the empirical division into 'contractile' and synthetic' phenotypes.

Interestingly, the expression of both VCAM-1 and ICAM-1 has been shown to be particularly high around sites of neovascularisation within atheromatous plaques. This has led some authors to suggest that this is due to increased leukocyte recruitment via the new blood vessels, however it may simply be a reflection of stimulation of ICAM-1 and VCAM-1 expression generated by increased levels of angiogenic growth factors.

In animal experiments, myointimal lesion in cardiac allograft vasculopathy were shown to involve increased expression of both ICAM-1 and VCAM-1. To investigate the pattern of expression of these adhesion molecules in human blood vessels, human ovarian veins cultured both with and without the addition of sex steroids, were examined, using immunocytochemistry, in experiments described in the following sections.
6.2 EFFECT OF TRANSDERMAL OESTROGEN ON SERUM VEGF LEVELS IN OOPHORECTOMISED WOMEN.

6.2.1 Methods

This was a longitudinal, prospective observational study over 6 months, performed in the Menopause Research Unit, based at the Leicester Royal Infirmary, with the approval of the local Ethics committee.

6.2.1.1 Subjects

Forty hysterectomised women were enrolled into this study. Their mean age was 47.8 years (range 35-65 years), and their mean Body Mass Index was 25.5 (range 21.4-32.8). They had all undergone hysterectomy and bilateral salpingo-oophorectomy at least 3 months prior to study entry, with postmenopausal values for serum FSH (>30 IU/l).

6.2.1.2 Oestrogen replacement

The participating women were treated with continuous, unopposed transdermal oestrogen for six months. This was administered in the form of Gynaderm patch (Shire Pharmaceuticals, UK), a new hypoallergenic matrix patch.
releasing 50 μg 17β-oestradiol daily. The participants were instructed to change the
patch twice weekly.

6.2.1.3 Specimen collection

Serum samples were collected prior to and following six months of oestrogen treatment, aliquoted, and stored at -80° C until required. To determine serum VEGF levels, samples were defrosted to room temperature and assayed for VEGF using a commercially available enzyme linked immuno-sorbent assay kit (Human VEGF Quantikine™ ELISA, R&D Systems).

6.2.1.4 VEGF ELISA

The principle of the assay is that of quantitative sandwich enzyme immunoassay. Standards and samples are pipetted into wells of a microtiter plate, which is pre-coated with VEGF specific monoclonal antibody. Any present VEGF is bound by the immobilised antibody. An enzyme linked VEGF specific polyclonal antibody is then added, followed by a substrate solution. Colour develops in proportion to the amount of VEGF bound in the initial step and the amount of VEGF can be calculated following measurement of light extinction in a photometer.
6.2.1.4.1 Kit contents

- 12 8-well Microtiter Plate strips
- VEGF Conjugate (polyclonal antibody against VEGF conjugated to horseradish peroxidase)
- VEGF Standard (recombinant human VEGF)
- Assay Diluent (buffered protein base)
- Calibrator Diluent (animal serum)
- Wash Buffer Concentrate (buffered surfactant)
- Colour Reagent A (hydrogen peroxide)
- Colour Reagent B (tetramethylbenzidine)
- Stop Solution (2N sulfuric acid)
- Adhesive Plate Cover strips

6.2.1.4.2 Assay protocol

1. 100 µl assay diluent was added to each well.
2. 100µl serum was added and incubated for 2 hrs at room temperature.
3. The wells were aspirated and washed 3 times.
4. 200 µl Conjugate was added and incubated 2 hrs at room temperature.
5. The samples were again aspirated and washed 3 times.
6. 200 µl Substrate solution was added and incubated 25 min at room temperature.
7. 50 μl Stop Solution was added and samples were read at 450 nm within 30 min.

6.2.1.5 Statistical analysis

The difference in the mean serum VEGF level before and after oestrogen treatment was examined for statistical significance using Student-t test for paired variables.
6.2.2 Results

Thirty-five women completed the study. Five women withdrew because of side effects associated with the treatment (breast tenderness \( n=2 \), skin reaction \( n=2 \), headaches \( n=1 \)).

Prior to commencement of oestrogen treatment the mean serum VEGF level was 328 ng/ml (95% Confidence Interval 272, 238). This dropped to 285 ng/ml (95% Confidence Interval 242, 237) after six months oestrogen treatment (Figure 14). The mean difference over six months was -43.4 ng/ml (95% Confidence Interval -82, -4). This was statistically significant (\( P=0.0299 \)).
Figure 14: Serum VEGF levels before and after oestrogen therapy, in mean ng/ml with 95% Confidence intervals.
6.3 Effect of oestrogen on VEGF expression in cultured ovarian veins.

6.3.1 Methods

6.3.1.1 Culture technique

Five segments of ovarian veins were cultured as described in 5.2.1. Five specimens were cultured without the addition of sex steroids, five with 17\(\beta\)-oestradiol and five with 17\(\alpha\)-oestradiol at a concentration of 10\(^{-6}\), for 14 days. Following culture, the sections were fixed in 10% formalin and paraffin embedded. Sections of 5 \(\mu\)m thickness were cut and mounted on silane coated glass slides.

6.3.1.2 In situ hybridisation protocol for VEGF

In situ hybridisation (ISH) is a molecular biology methodology which locates DNA or RNA nucleic acid sequences in biological material, be it in cytoplasm, nucleus or organelles. In a similar way to polymerase chain reaction, a probe is used to identify a nucleic acid sequence, but unless in situ PCR is performed, the material is not amplified. A detectable marker is attached to the probe, so that the hybridisation site is visualised.
The tissue is then permeabilised to facilitate access for probe and detection reagents. This is usually achieved by enzymatic digestion with pepsin/Hcl or Proteinase K. The tissue is then fixed and desiccated to minimise diffusion of RNA outside the cell.

A probe with the desired sequence is then labelled by enzymatic or chemical methods. Labels can be radioactive or non-radioactive. A commonly used non-radioactive label is Digoxigenin, a plant steroid which can be enzymatically incorporated into DNA or RNA, using the nucleotide derivative Digoxigenin-11-UTP.

DNA probes and their target sequences need to be denatured prior to hybridisation, to form single stranded nucleic acid chains. RNA is single stranded, but longer strands can sometime duplicate on themselves within similar regions in their sequence, and they too benefit from denaturation.

The probe is then applied to the tissues and left to hybridise overnight. To minimise non-specific background binding, blocking solutions containing formamide, inert polymers, sodium dodecyl sulphate or bovine serum albumin can be used. Following post-hybridisation washing, a detection system for the label is used to enable visualisation of the hybridisation site. The detection systems used will depend on the type of label, i.e. radioactive or non-radioactive. Non-radioactive reporter systems include methods similar to those described for immunocytochemistry, i.e. Streptavidin-biotin, alkaline phosphatase, and peroxidase systems.
The resulting stain is evaluated by light microscopy with or without the use of an image analysis system. Again, the use of positive and negative control tissues is essential to determine the specificity of probe hybridisation.

The in situ hybridisation for VEGF mRNA was performed using an oligonucleotide probe cocktail for VEGF generated in house in the department of Medicine, University of Leicester. Digoxigenin labelling and an alkaline phosphatase detection system was used.

1. Sections were dewaxed in a series of Xylene and alcohol (as for immunocytochemistry) and rehydrated in DEPC (diethyl pyrocarbonate) water.
2. Sections were immersed in 2x Standard Saline Citrate at 70°C for 10 minutes, then washed for 5 minutes in DEPC water at room temperature.
3. Proteinase K was applied to sections at a concentration of 5 μg/ml, (diluted in 0.05 M Tris-HCl, pH 7.6, DEPC) and incubated at 37°C for 1 hour.
4. The reaction was stopped by washing for 10 minutes at 4°C, in two changes of DEPC water.
5. Slides were transferred to 0.4% paraformaldehyde solution and incubated at 4°C for 20 minutes.
6. Sections were washed for 5 minutes at room temperature in DEPC water, then covered with 100 μl of pre-hybridisation solution.
7. Slides were dried and incubated at 37°C for 1 hour.
8. 50 μl of a VEGF oligonucleotide probe cocktail was loaded onto the sections, which were then covered and left overnight at 37°C.
9. Sections were washed twice for 10 minutes each in a solution of 2x SSC/30% Formamide at 37°C, then transferred to blocking solution for 10 minutes at room temperature.

10. 100 μl of anti-digoxygenin alkaline phosphatase Fab fragment antibody (Boehringer Mannheim Biochemicals), was applied (1/600 dilution in blocking solution), and then incubated for 30 minutes at room temperature.

11. Sections were washed in two changes of TBS over 10 minutes, then rinsed in sterile aqua dest and transferred to Tris HCl buffer (pH 9.5) for 5 minutes.

12. Alkaline phosphatase substrate was added to the sections, covered with glass coverslips and incubated in the dark overnight.

13. Sections were washed in running tap water for 5 minutes and mounted using Aqua mount.

6.3.1.3 Assessment of staining intensity

The probe hybridisation sites were visualised using light microscopy and image analysis as in section 5.2.1.5. The staining intensity of the section was graded from 0 (negative) through + (weakly positive), ++ (positive) to +++ (strongly positive). The grading was performed by two independent observers blinded to the culture conditions of the tissues examined.
6.3.2 Results

The intensity of staining for VEGF was weakly positive in most blood vessels cultured without sex steroid addition, and in the 17α-oestradiol controls.

The ovarian veins cultured with the addition of 17β-oestradiol were all positive for VEGF (Figure 15), with varying degrees of intensity (see Table 9).

**Table 9:** Intensity of staining for VEGF mRNA in human ovarian veins. Grading of intensity by two independent observers for each culture condition.
Figure 15: Human ovarian vein section cultured with the addition of 17β oestradiol, staining positive for VEGF following in situ hybridisation (x1000, oil immersion). L: lumen. Arrows: examples of positive cells in the proliferative layer.
6.4 Effect of 17-β oestradiol on the expression of Inter-Cellular-Adhesion-Molecule-1 (ICAM-1), Vascular-Cell-Adhesion-Molecule-1 (VCAM-1) and E-Selectin in human ovarian veins.

6.4.1 Methods

6.4.1.1 Culture technique

Five segments of ovarian veins were cultured as described in 5.2.1. Five specimens were cultured without the addition of sex steroids, five with 17β-oestradiol and five with 17α-oestradiol at a concentration of $10^6$, for 14 days. Following culture, the sections were fixed in 10% formalin and paraffin embedded. Sections of 5 μm thickness were cut and mounted on silane coated glass slides.

6.4.1.2 Anti-ICAM, anti-VCAM and anti-E-Selectin Protocol

All three antibodies were goat polyclonal antibodies (R&D systems, UK). Routine immunocytochemical protocols used as follows:
The protocol is the same as in 5.2.1.4 except:

- Following step 2, sections were incubated with 100 μl Pepsin (50 ml Aqua dest, 0.2 g Pepsin, 100 μl Hcl) for 15 min at 37°C, then washed with tapwater and Aqua dest.

- Slides were submerged in Citrate Buffer 10 mM pH 6.0, and heated in a waterbath for 2 hours at 65°C, cooled down to room temperature, washed with Aqua dest. The next step was step 3 of the above protocol.

- Primary Antibody anti-ICAM-1, anti-VCAM-1 or anti-E-Selectin was applied in a concentration of 1:200 in TBS, and incubated for 30 min at room temperature.

- As a secondary antibody Rabbit anti-Goat (1:400 in TBS) was used, which was incubated for 30 min at room temperature.
6.4.2 Results

The application of the anti-E-selectin antibody was unsuccessful. Again, microwave antigen retrieval led to destruction of the blood vessel section and its detachment from the slide, although the control tissue (human kidney sections) stained positively. Other antigen retrieval methods such as enzyme digestion and superheating were unsuccessful, in that neither the investigated tissue nor the positive controls stained positively for E-Selectin.

The same problem was encountered with anti-VCAM-1. Again, judging by the positive control tissue, 30 minutes of microwaving at full power was required to unmask the appropriate antigen, and this could not be achieved with the ovarian vein sections without destruction of morphology.

Anti-ICAM-1 antibodies did stain the ovarian vein endothelium in most sections, although a marked degree of background staining was present (Figure 17). Staining was achieved by the above protocol, including enzyme digestion and heating in a waterbath. In all veins cultured in heat inactivated postmenopausal serum the endothelium stained positive for ICAM-1. Some patchy, weak staining of intimal smooth muscle cells was observed, but there was little staining in the media.

The immunohistochemical expression of ICAM-1 in the intima of blood vessels cultured without the addition of sex steroids ranged from weakly positive (+) to strongly positive (+++). This pattern of ICAM-1 expression did not differ
qualitatively from the pattern of expression in blood vessels cultured after the addition of 17β-estradiol or 17α-estradiol. The pattern of expression seemed to be maintained by any one blood vessels regardless of culture conditions. However, in view of the marked background staining and the relatively poor agreement between observers (see Table 10), these results have to be viewed with caution. The results are summarised in Table 10.

**Table 10**: Staining intensity with anti-ICAM-1 in ovarian veins. The grading of staining by both observers for all culture conditions is shown.
In the first part of the above study, to investigate the effect of postmenopausal oestrogen replacement on VEGF secretion in vivo, forty healthy, oophorectomised women were treated with transdermal oestrogen continuously for six months. Serum VEGF levels were measured before oestrogen administration and following six months of treatment, using a commercially available VEGF ELISA kit.

The mean serum VEGF level before oestrogen treatment was 328 ng/ml (95% Confidence Interval 272, 238). This dropped to 285 ng/ml (95% Confidence Interval 242, 237) after six months oestrogen treatment, and this change was statistically significant (P=0.0299).

In the second part of this study, the expression of VEGF at vascular level and its changes in response to 17β oestradiol were examined, using in situ hybridisation with an in-house generated probe. Human ovarian veins were cultured under controlled conditions, with and without the addition of oestrogen in pharmacological concentrations. In blood vessels exposed to oestrogen the staining intensity for VEGF was considerably higher than in those cultured as controls.

The expression of a further three adhesion molecules, known to play a role in vascular responses to injury, was examined in the same specimens, using immunocytochemistry. These were ICAM-1, VCAM-1 and E-Selectin. However,
the methodology used proved unsuccessful in the case of VCAM-1 and E-Selectin, due to the intolerance of the ovarian vein sections to heat mediated antigen retrieval, particularly by microwaving.

ICAM-1 was demonstrable in the human ovarian vein sections, albeit with marked background staining. Its expression was variable and appeared unrelated to varying culture conditions and the addition of sex steroids. However, due to the marked background staining, these results have to be viewed with caution.
7. DISCUSSION

The last two decades have seen a growing interest of the public in the benefits of postmenopausal hormone replacement, fuelled by both scientific and lay press. Between 1980 and 1990, the uptake of HRT amongst British women has more than trebled, with approximately 1:10 women classified as current users by the end of 1990. This figure has probably increased even further, with an estimated 1:6 women using HRT in the mid-nineties.  

Although many women embark on oestrogen replacement to improve their quality of life and combat menopausal symptoms, a growing proportion of patients presents with the question whether they should be on HRT as a preventative measure, to reduce their risk of osteoporosis and cardiovascular disease. However, in spite of the extensive research into the effects of oestrogen on longterm health, we are still not in the position to confidently say, that the cardiovascular protection associated with oestrogen replacement has been statistically proven. Although there are numerous trials with cardiovascular endpoints, involving large numbers of patients, it has been argued that the majority of these studies are flawed, particularly with selection bias.

In an attempt to resolve the controversy, several meta-analyses have been published to date, reviewing in excess of 30 published studies each. Overall, their results indicated that there is a cardioprotective effect to be gained from oestrogen
treatment. However, adding further fuel to the controversy, the most recent meta-analysis published in the BMJ in 1997, having reviewed 22 clinical trials, found that the relative risk of cardiovascular events increased to 1.4.139

This illustrates the need to conduct further research into the effects of oestrogen on the female cardiovascular system. That applies both to studies with clinical endpoints, such as the MRC long term randomised controlled trial of hormone replacement therapy, which is currently underway and will hopefully recruit sufficient numbers of patients to obtain meaningful results, as well as studies aimed at understanding the mechanisms of oestrogenic actions on elements of the cardiovascular system.

The present study contains elements of both clinical studies and basic science work, which were believed to be of value for the continuing effort to uncover further aspects of HRT, and gain in depth understanding of the underlying mechanisms involved in the observed outcomes. In the following, results of the individual studies are discussed in more detail.

7.1 OESTROGEN AND BLOOD PRESSURE

The present study on ambulatory blood pressure indicates that oral oestrogen replacement does not alter mean ambulatory BP in the short term, whereas transdermal oestrogen treatment is associated with a significant reduction in mean night-time ambulatory systolic and diastolic BP as well as in day-time ambulatory diastolic BP. The relative importance of day-time versus night-time and
systolic versus diastolic BP remains the subject of debate, but most authors agree that an association exists between nocturnal BP and the severity of target organ damage, particularly in relation to the development of left ventricular hypertrophy. Mean day-time ambulatory diastolic BP is also of importance, since there is evidence that the level of diastolic BP during waking hours correlates with relative left ventricular wall thickness. As a consequence, a reduction in both night-time ambulatory systolic and diastolic BP and day-time diastolic BP of 4-5 mm Hg, as observed in our study, can be considered beneficial, particularly if this effect can be reproduced in hypertensive women. Our data suggest that this may well be the case, because women with a higher baseline level of both ambulatory systolic and diastolic BP were more likely to have a BP-lowering response to transdermal ERT than women with a low-normal pre treatment BP. Regression analysis has shown that at least 20-50% of the BP reduction was accounted for by the level of pre treatment BP, with the regression gradient ranging from -0.4 to -0.7.

To our knowledge, this is the first study of automated 24-hour BP data in normotensive women receiving postmenopausal ERT, and so we can only compare our data to previously reported conventional BP measurements. A differential response of BP to oral and non oral oestrogen has previously been described, and non-oral ERT has been shown not to affect BP to the same extent as oral treatment. One proposed mechanism is that oral oestrogens induce increased hepatic expression of angiotensinogen, resulting in increased levels of the potent vasopressor, angiotensin II. The hepatic effect of transdermally delivered oestrogen is less pronounced than that of oral ERT, and a significant increase in renin-substrate levels has not been observed. In addition a variety of
direct and indirect effects on blood vessels have been reported, amongst others an increase in prostacycline production\textsuperscript{,55} calcium channel blockade\textsuperscript{145} and an endothelium independent vasorelaxation\textsuperscript{,48} so that the resulting conventional BP in most women seems to remain at least stable, if not lower than pre-treatment levels.

Although these phenomena may help to explain the difference in response that we observed between the oral and transdermal treatment groups, they do not assist our understanding of a further finding that has emerged from our ambulatory BP data. Although the mean ambulatory BP of the group as a whole remained unchanged on oral ERT and was reduced in the transdermal group, in both treatment groups there was a relatively high proportion of women whose BP increased. With the exception of day-time diastolic BP, the proportion of women whose BP rose was similar in both groups. A BP elevation was observed in more than one-third and up to one-half of subjects on either treatment, which is considerably higher than the 5-12\% reported in the literature on conventional BP measurement. Indeed, one would have expected this proportion to be lower in women receiving transdermal oestrogen treatment, given the mechanisms of oestrogen effect on BP discussed. Moreover, the observed magnitude of BP elevation was considerable and statistically significant, which posed the question as to which other factors may be involved in blood pressure modulation in response to oestrogen treatment. One further factor evaluated in the course of this investigation was the possible link between AGT polymorphism and the increase in blood pressure.

The frequency of both the T235 and M235 alleles, and that of the individual genotypes in the study population were comparable to those reported in
the literature, but a significant association between the alleles or genotypes on one hand and variations in blood pressure response on the other, could not be demonstrated. Although it has been presumed that the differences in blood pressure response in postmenopausal women are probably a result of an accentuated rise in AGT production in women who will become hypertensive on oestrogen, when AGT levels in women who remained normotensive on oestrogen therapy were compared to those of women whose blood pressure rose, no significant difference was detected. Further, it has been suggested that certain individuals may express an alternative form of AGT in response to sex steroid stimulation, which may have a higher affinity to renin and result in higher angiotensin II levels, thus leading to a rise in blood pressure. This hypothesis has not been confirmed, and subsequent investigations revealed this high molecular AGT to consist of angiotensinogen that is covalently linked to another serum protein. Its relevance, if any, is unknown, and no connection has been made to link a high molecular weight moiety of AGT to variations in the genetic AGT locus. Therefore, other factors may be responsible for the differing responses of blood pressure to oestrogen treatment.

The study sample size employed provided sufficient power to detect 4 mm Hg changes in diastolic BP, although the power to detect a similar change in systolic BP was somewhat lower. Nevertheless, a significant impact of estrogen treatment on BP in oophorectomized women using ambulatory BP monitoring has been demonstrated. In light of these findings, conventional office BP measurement may not provide enough detail to greatly contribute to our understanding of BP changes in women on ERT, and the application of ambulatory 24-hour measurement may be more appropriate. The above results do not suggest a causal
link between angiotensinogen gene polymorphism and a differential blood pressure response to oestrogen. However, we must bear in mind that the sample size employed was chosen to provide sufficient statistical power to detect changes in blood pressure in response to oestrogen treatment, and was unlikely to be adequate to detect an association of a multifactorial phenomenon with a particular genotype.

7.2 Oestrogen and Atherosclerotic Plaques

The data presented in this study suggest a beneficial effect of oestrogen on atherosclerotic lesions. This is the first study to date looking at the effect of oestrogen treatment on actual plaque dimensions, rather than at changes to the vascular lumen diameter, i.e. stenosis, or indeed at Doppler studies. Measurements of lumen diameter are largely dependent on the vascular tone, whereas Doppler flow pattern and velocity can be influenced by a variety of stimuli such as lighting, level of environmental noise, environmental temperature, quantity and timing of last food intake etc. Measurements of absolute plaque dimensions are not affected by these factors, and are reproducible with acceptable levels of intra-observer variability.

Further, ultrasonography is a simple, non-invasive, pain- and risk-free technique, and it is also relatively inexpensive. However, it has become apparent in the course of this study that not all plaques are suitable for sonographic measurement. Plaques that are ill-defined, irregular or echoluent may not be measurable with the degree of accuracy required for follow up in the assessment of treatment effects, and in fact we have not been able to complete follow up on four plaques because their morphology had changed during the treatment period.
As early as 1927, several reports had indicated that atherosclerosis and its clinical manifestations could be ameliorated after periods of starvation. However, most these reports were flawed, and it was not possible to differentiate between the effect on structural changes in atherosclerotic lesions and on reduction in the rate of clinical events. During the past two decades evidence from experimental animal models has increasingly supported those early findings, and it is now clear that substantial regression of atherosclerotic plaques in humans is possible with diet, lifestyle changes and cholesterol-lowering drugs, translating into a reduction of the clinical dangers of such lesions.

Several mechanisms of plaque regression have been suggested. The amount of lipid in the vessel wall decreases, possibly quite rapidly. During plaque regression two main elements, which play an important role in plaque progression, are reversed. The first is the increasing endothelial damage, and the second is the excessive proliferation of vascular smooth muscle cells, which in turn leads to increased collagen, elastin and proteoglycane elaboration and storage in the vascular wall. Endothelial dysfunction plays a critical role in complications of atherosclerosis (sick-vessel-syndrome). Endothelium-dependent relaxation is usually impaired, even if the endothelium is structurally intact. This may be partly caused by free radicals and an increased break down of nitric oxide in the atherosclerotic vascular wall. Interestingly, endothelial dysfunction can involve microcirculation, even when atheromata are confined to larger vessels. During regression, vasodilator responses are restored, although the maximum vasodilator capacity may not be reached due to residual vascular fibrosis. Abnormal vasoconstrictor responses to stimuli such as activated leukocytes, serotonin, ADP
and acetylcholine, which prevail in dysfunctional atherosclerotic blood vessels, are improved.\textsuperscript{153} The decrease in cell proliferation also plays an important part in regression of atherosclerosis, although this has so far been only demonstrated in swine.\textsuperscript{154}

As discussed in Chapter 1, oestrogen can potentially perform all the above tasks. It is capable of attenuating reverse cholesterol transport by increasing HDL-cholesterol, thus facilitating lipid removal from plaques. Also, it can improve both endothelium-dependent and independent vasoreactivity, and increase both nitric oxide and prostacyclin production. Further, it has been shown to reduce smooth muscle cell proliferation, and reduce connective tissue accumulation in the vascular wall. The time scale of the events leading to plaque regression in humans is not entirely clear, since the vast majority of the data relating to regression of atherosclerosis was obtained from animal studies. It is quite possible, given the duration of the above study, that the reduction of the actual plaque dimensions observed is mainly the consequence of lipid depletion of these plaques, possibly with a condensation of the fibrous elements in the lesions, but obviously as it was not possible to obtain pathological specimens, this is merely a speculation. Interestingly, the regression of plaque length preceded that of plaque thickness, which would perhaps support this theory in so far as the plaque margins would have smaller lipid deposits, which could be disposed of faster than the thick, central areas of the lesion.

Although plaque regression has been observed with lipid lowering agents, the plaques of the two patients taking Simvastatin in this study in fact did not reduce in size. Calcium antagonists and Aspirin may act synergistically or
inhibit atherogenesis, but there is no evidence that they contribute to plaques regression. Although the results of the pilot study presented here should be viewed in the context of a small, uncontrolled, non randomised, observational study, they are encouraging. There may be a role for oestrogen treatment of existing atherosclerosis, and this therapeutic possibility should be explored in a larger, adequately designed, definitive trial.

### 7.3 OESTROGEN AND INTIMAL HYPERPLASIA

The development of intimal hyperplasia in humans is difficult to study, as early changes are asymptomatic, and at that stage usually clinically not detectable. Apart from necropsy material, we hence have to rely on in vitro work in the understanding of myointimal disease. The above described vascular culture model has proved successful, as it was possible to maintain, over a fourteen day period, viability in human ovarian veins in culture. Further, it was possible to induce a significant degree of intimal hyperplasia, using culture media supplemented with 20% human serum. Although it will remain difficult to deduce from in vitro experiments as to mechanisms in place in vivo, using all human materials can be in vascular culture work eliminates the necessity to extrapolate data across species.

The absence of the lamina elastica interna in human ovarian veins represents both the main advantage and difficulty of this model. Although on one hand this characteristic facilitates the induction of intimal hyperplasia, it makes a clear, functional as well as histological definition of an intima problematic. As described
in the results section, the lack of microanatomical separation between intima and media results in the confluence of the subendothelial ground substance with the extracellular matrix in which the first loose muscular layer is embedded. In a study looking at the fine structure of the human ovarian vein, Stones et al described this first muscle layer as part of the media. However, as the amount of both extracellular matrix and muscle in this layer was variable, and it was impossible to determine the exact transition of intima into media on light microscopy, the intimal thickness in the above vascular culture study was taken to include this layer. The subsequent, circular muscle layer was consistent and clearly demarcated, allowing accurate measurement as reflected by a low inter-observer variability. Functionally, it makes sense to consider both confluent layers as the “intima”, as they are likely to be affected to the same degree by environmental changes. Any increase in extracellular matrix production by vascular smooth muscle cells can be expected to appear as a thickening of the already present ground substance within this layer. Further, as it is not the absolute thickness of this layer, but changes in intimal thickness of paired specimens, measurements taken as described above would not lack validity.

A pre-existing degree of focal or diffuse intimal thickening was observed in several ovarian veins. This is in keeping with the observation of Charles and Gresham, who examined human veins from different sites of the body, and found focal intimal thickening in native veins, possibly related to venous pressure and local haemodynamic effects. In the case of human ovarian veins we have to take into account the increased circulatory demands of female reproductive life, with increase in venous pressure in pregnancy and in association with large uterine fibroids. The extent of increased haemodynamic load and its duration necessary to
induce intimal changes remains uncertain, but the prominent musculature of the ovarian vein is a clear reflection the degree of stress it is designed to cope with.

The results of this study also demonstrate that oestrogen in pharmacological doses may reduce the extent of intimal thickening in cultured human ovarian veins. The mechanism of this effect is not readily apparent. Although both intimal thickness and cellularity of the intima, as reflected in the mean number of nuclei present, were clearly decreased in oestrogen treated veins, the addition of 17β-oestradiol did not affect the development of a superimposed neointimal layer. As this is the first experimental study looking at intimal hyperplasia in human ovarian veins, it is not possible to compare the above results with other authors. However, although several authors have described an inhibitory effect of oestrogen on myointimal proliferation in animal experiments both in vivo and in vitro. Oestrogen has been shown to suppress proliferation in vascular smooth muscle cells, but migration of the vascular smooth muscle cells from the media to the intima may occur as an independent event. In a study on inhibition of myointimal hyperplasia in rabbit aorta allografts, the authors described the presence of vascular smooth muscle cells in the intima of grafts harvested from oestrogen treated animals. The smooth muscle cells were morphologically different from those seen in untreated animals, demonstrating little or no vacuolisation. This may suggest that, although migration still occurred, the vascular smooth muscle cells did not undergo the transformation from contractile to synthetic phenotype, which seems to be associated with the increased matrix elaboration seen in myointimal disorders.
The 'proliferative' layer, the covering of loose cells over the endothelium, may originate from these migrating smooth muscle cells. Particularly in ovarian veins, where the lamina elastica interna is grossly deficient or absent, migration of smooth muscle cells is a likely mechanism. In this study, the new layers of cells lining the luminal surface were not recognised by the endothelial cell marker anti-CD 31, which would make it appear unlikely that these cells originate from the endothelium. Further, they were not only seen along the endothelial surface, but also along the cut edges and the adventitial side. In a culture of human saphenous veins Slomp et al also described this phenomenon.\textsuperscript{15} They observed that the cellular overgrowth in fact started over the cut edges of the specimens, and then advanced over the endothelial surface. It formed in vein segments that had not been properly pinned down into the resin, as well as in those left tubular rather than opened. The new proliferative layer in their work was equally not recognised by endothelial markers, however, some cells within this layer stained positive for a fibroblast marker, the 5B5 antibody. Interestingly, no cells in the vascular wall had stained positive for the fibroblast marker prior to culture.

If, as deduced by Slomp et al, these cells originated over the cut edges, i.e. in the medial/adventitial layer, it would appear that the proliferative layer, as observed in the present work, would develop independently from bona fide intimal hyperplasia. This would also explain the observed discordance between the effect of oestrogen on the intima and its cellularity on one hand, and its effect -or rather the lack of it- on the formation of this new proliferative, loose covering on the other.

The protective effect of oestrogen in pharmacological concentrations was not reproduced in the specimens cultured with a reducing dose of oestrogen. As only
five specimens per dose were cultured in the dose response experiment, it is possible that the lack of effect is simply a reflection of the small number of blood vessels examined. This is certainly an aspect of this study which will need further work.

Also, we lack conclusive data on the proliferation status of the cultured specimens, as the methodology employed in this study did not generate adequate results. In view of the importance assigned to modulation of cell proliferation as a means to inhibiting intimal hyperplasia by most authorities on the subject, further work will be necessary, possibly employing other proliferation markers, or alternatively frozen specimens, to detect any antiproliferative effect exerted by oestrogen. BrdU could be employed again, perhaps using a different antibody with less non-specific binding. A further alternative would be the use of in situ hybridisation for Histone proteins.

In conclusion, human ovarian veins are suitable as a model for the study of induction and modulation of intimal hyperplasia, and viability can be maintained using culture medium supplemented with human postmenopausal serum. Evidently, oestrogen in pharmacological doses reduces the degree of intimal hyperplasia observed. A dose response effect to oestrogen will have to be re-examined in a larger number of specimens to allow adequate assessment of its effect in more physiological doses.
7.4 **Estrogen and Vascular Endothelial Growth Factor**

VEGF was originally described as a factor with a primarily detrimental effect on blood vessels. However, more recently the perception of VEGF as mediator or marker of endothelial dysfunction has been questioned. Through its prominent role in angiogenesis, VEGF was shown to be involved in vascular 'salvage' actions. Induction of myocardial ischaemia for example, both in vitro and in vivo, has been shown to result in increased VEGF expression by porcine myocytes, thus, by its angiogenic impulse, providing the neovascularisation essential for myocardial survival. In a further study, looking at retinopathy of prematurity, a neovascularising disease which leads to blindness, the authors were able to demonstrate that VEGF downregulation by hyperoxia preceded the regression of blood vessels, the hallmark of this condition. This led to the conclusion that VEGF is in some way important for vascular survival or maintenance. Recently, evidence has emerged from animal models looking at hindlimb ischaemia in rabbits, that VEGF has a favourable impact on endothelium-dependent vasoreactivity. As oestrogen also modulates vascular function, it was of interest to see whether this effect is mediated by VEGF secretion.

In this study, the in vivo effect of oestrogen treatment on VEGF secretion was examined by determining serum VEGF levels in oophorectomised women before and after exposure to oestrogen. Oestrogen administration was associated with a significant decline in serum VEGF levels following six months of continuous unopposed oestrogen replacement. This is somewhat surprising, as in vitro work
has conclusively shown that oestrogen upregulates tissue expression of VEGF. However, data on the effect of oestrogen on VEGF secretion in vivo are limited to peritoneal fluid assays in patients with endometriosis, which limits their suitability for further comparison. Results from studies on serum VEGF levels in other conditions associated with endothelial dysfunction, such as pre-eclampsia for example, are not particularly helpful. One is reluctant to compare results obtained from pregnant subjects, albeit with dysfunctional endothelium, with those in menopausal women. Reports on VEGF levels in pre-eclampsia are particularly controversial, with conflicting results, possibly due to the increased production of VEGF-binding proteins in pregnancy, as well as the cross-reaction of different VEGF isoforms when some kit-based assays are used.

However, a reduction in VEGF secretion in response to oestrogen administration becomes perhaps more plausible, if one considers the underlying mechanisms of both their effects on endothelial function. The effect of oestrogen on endothelium-dependent vasoreactivity is well researched, and it is widely recognised that it is largely mediated by increased release of nitric oxide by the vascular endothelium. VEGF in turn has also been shown to exert its effects on endothelium by increasing nitric oxide production. In a study looking at VEGF induced vasorelaxation in canine coronary artery, the authors reported an inhibitory effect of pre-treatment with a nitric oxide synthase inhibitor, L-NAME. Subsequently, experimental work has established that -in vitro- VEGF causes a dose dependent increase in NO production in vascular endothelium of normal human veins and arteries. Not only does NO mediate VEGF effect on endothelial function, but it also appears to contribute to its angiogenic properties. Taking this model a step further, evidence has emerged that in vitro VEGF expression in injured
blood vessels can actually be downregulated by nitric oxide donors such as sodium nitroprusside. Treatment with L-NAME in vivo on the other hand increases VEGF expression. This suggests a reciprocal relationship between VEGF and NO. The mechanism of this modulation of VEGF expression by nitric oxide is possibly by downregulating VEGF promoter activity.  

In the light of these findings, the decline in serum VEGF levels following oestrogen treatment in this study may in fact confirm that this mechanism is functional in humans in vivo. We could therefore conclude that oestrogen replacement, by increasing nitric oxide production, directly inhibits VEGF expression and secretion. However, before this conclusion is made, it would be of interest to examine the effect of surgical menopause on VEGF expression. Although all women participating in this study had their hysterectomy and bilateral salpingo-oophorectomy three or more months before study entry, we do not have information on the magnitude or duration of increased VEGF production associated with tissue trauma and healing response. There is therefore the possibility that the observed decline in VEGF levels is in fact a return to baseline after a period of increased expression. This question is probably best answered in a controlled study, where pre-operative as well as post-operative serum VEGF levels are determined, and their evolution without HRT is observed in comparison with that in women receiving oestrogen.

The in vitro arm of this study examined VEGF expression in human ovarian veins. Characteristically, a degree of baseline expression in most cultured ovarian veins was demonstrated. This is in agreement with a recently published study looking at the expression of VEGF in normal arteries and veins, as well as in
atheromatous blood vessels. Moderate to strong immunostaining for VEGF was demonstrated in 80% of the native saphenous vein specimens examined.165

The in vitro effect of oestrogen on VEGF expression has been the subject of numerous publications, and appears to be considerably more consistent than reports on measured serum VEGF levels. Most of the work published on the effect of oestrogen on VEGF expression relates to the female reproductive tract, with focus on ovarian steroid regulation of VEGF expression in the endometrium during the menstrual cycle, and in the pathogenesis of endometriosis.159-160 In the light of the available data, the results of the in vitro arm of this study, looking at the effect of oestrogen on VEGF expression in human ovarian veins, are in keeping with the published evidence that oestrogen upregulates tissue expression of VEGF. The staining for VEGF was markedly stronger in veins cultured with the addition of 17β oestradiol, compared to that of veins cultured in standard culture medium without sex steroids, or those cultured with 17α oestradiol.

The question remaining to be answered is whether the reduction in intimal thickness, demonstrated in oestrogen treated veins, is in anyway linked to the increased VEGF expression. Over the past decade considerable amount of research has been carried out into ways of preventing or inhibiting intimal hyperplasia, with vascular grafts and post-angioplasty restenoses in mind. It has emerged that the key point in prevention of neointimal thickening is maintaining -or re-establishing- endothelial integrity following any manipulation, and that endothelial cell regeneration is one of the most potent inhibitors of smooth muscle cell proliferation.102 The Isner group in Boston, MA, have been actively researching the
involvement of VEGF in restoring endothelial integrity. In a study looking at the
development of intimal hyperplasia in balloon-injured rat carotid arteries, they
demonstrated that VEGF application to the denuded site in vivo promoted
reendothelialisation, and thereby indirectly reduced the extent of neointimal
thickening to a significant degree. However, blood vessels with intact
endothelium also derive some protection from VEGF. Laitinen et al studied the
effect of VEGF gene transfer on intimal thickening in collared rabbit carotid arteries,
where intimal hyperplasia is induced without intravascular manipulation. They
demonstrated a significantly reduced intimal thickness in blood vessels transfected
with the VEGF gene when compared to control animals, transfected with β-
galactosidase only. In an attempt to elucidate the mechanism of this protective
effect, they again used L-NAME to inhibit nitric oxide synthase. Interestingly, in
animals fed L-NAME, the difference in intimal thickness between VEGF and β-
galactosidase transfected arteries was abolished. This strongly suggests that VEGF
inhibits vascular smooth muscle cell proliferation via a mechanism that involves
VEGF-induced release of nitric oxide from endothelial cells.

Although this model is convincing, it remains difficult to be certain as to
whether the inhibition of intimal thickening by Óestrogen demonstrated in the present
study is actually mediated by VEGF, particularly as Óestrogen is also capable of
upregulating nitric oxide synthesis, and could theoretically suppress smooth muscle
cell proliferation without VEGF involvement. To answer this question conclusively,
the experiments would need to be extended to include VEGF neutralising antibodies
on one hand, and Óestrogen receptor blockers on the other hand, to eliminate each
agent in turn.
The results of the in vivo and in vitro arms of this study appear to yield conflicting results, in that in vivo, VEGF secretion appears to be reduced by oestrogen therapy, whereas in vitro, VEGF expression is upregulated. It is important to recognise that in vitro observations may not be fully reproducible in vivo, however, the most important factor in the present investigation is likely to be the difference between a model with intact endothelium, and that with significant endothelial trauma. Treating postmenopausal women with oestrogen amounts to sending an endothelial benefactor into a more or less intact system, where no additional stimulus to VEGF secretion is given. On the other hand, the in vitro model involves surgical trauma and a considerable amount of handling of the blood vessels, resulting in endothelial and smooth muscle cell activation. The increased VEGF expression has to be seen partly as a healing response to these injuries, and a downregulation of involved factors, if at all desirable, is likely to meet with some difficulty.

In conclusion, it appears that some of the vascular effects of oestrogen, exerted both in vitro and in vivo, may involve modulation of VEGF expression. However, a direct causal link remains to be proven, and more structured, controlled experiments to this effect are required.
7.5 **OESTROGEN AND ADHESION MOLECULES**

In contrast to E-Selectin and VCAM-1, ICAM-1 is expressed in low levels in healthy blood vessels, but its expression is markedly upregulated following vascular insults. There is a strong association between circulating soluble ICAM-1 levels and the development of ischaemic heart disease in humans. Further, ICAM-1 expression is increased in chronic graft vascular disease and in reperfusion injury, when its production seems to be related to hyperoxia.

Limited data on the effect of oestrogen on ICAM-1 secretion and expression in vivo and in vitro have been published. In one study soluble ICAM-1 levels were examined in pre- and postmenopausal subjects, however no difference was found. The authors inferred from these results that endogenous oestrogen does not appear to affect ICAM-1 secretion. Estrogen treatment in rats has been shown to blunt myocardial ICAM-1 expression following myocardial ischaemia, and to reduce ICAM-1 expression in the aorta in splanchnic ischaemia-reperfusion experiments. Again, this effect may be mediated via the release of nitric oxide, as nitric oxide inhibitors accentuate, whereas nitric oxide donors attenuate the in vitro upregulation of ICAM-1 expression.

In the present investigation a baseline level of ICAM-1 expression was observed in all specimens, but there was no clear effect of added oestrogen. However, although the use of the ICAM-1 antibody appeared to produce positive results, the background staining led to difficult interpretation and marked inter-observer disagreement. The results of this experiment should therefore be
interpreted with caution, and certainly require confirmation once the methodology has been developed to a more satisfactory degree.

It would also have been of considerable interest to examine the expression of the two adhesion molecules E-Selectin and VCAM-1 in the intimal hyperplasia model, as they are not constitutively expressed in normal blood vessels, and their presence is taken to denote activated endothelial cells. E-Selectin in particular may have provided some indication as to the regenerative effect of oestrogen on the endothelium.

Regrettably, in the present investigation, the demonstration of the pattern of expression of the adhesion molecules E-Selectin and VCAM-1 was not successful. The main problems encountered were methodological, as both anti-E-Selectin and anti-VCAM-1 antibodies require heat-mediated antigen retrieval techniques when used in formalin fixed, paraffin embedded tissues. Because of the nature of the tissue used in this experiment, heat treatment in the microwave or superheating in a pressure cooker, which was required to achieve adequate staining in the positive control sections of human kidney, led to partial or complete disruption of the integrity of the ovarian vein wall, thus making the sections unevaluable. To obtain good quality results, the experiments detailed in section 6.4 will need to be performed on frozen rather than formalin fixed specimens. Alternatively, the continuous development of new antibodies may provide products that are better suited for use in paraffin sections.
7.6 Future Research

In the course of the present investigation on the effect of oestrogen on the female cardiovascular system, several important points have been demonstrated, both in clinical studies and experimental work. As expected, a whole range of new questions has been generated, and there is a considerable scope for further research in this field. The following is a limited selection of specific questions inviting further investigation:

7.6.1 Blood pressure

The clinical study investigating the effect of two forms of oestrogen replacement on blood pressure in postmenopausal women has demonstrated a lowering effect of transdermal HRT on BP. However, in a significant proportion of women BP rose on oestrogen treatment. This did not seem to be associated with a higher prevalence of the M235T genotype.

Suggestion for future research include

- the effect of (surgical) menopause on ambulatory blood pressure
- the effect of cyclical and continuous combined HRT on ambulatory blood pressure
- the effect of selective oestrogen receptor modulators on blood pressure
• prevalence of M235T genotype in relation to BP in a larger sample of women
• other genetic determinants of hypertension, such as nitric oxide synthase, in women with oestrogenic hypertension

7.6.2 Atheromatous plaque

Unopposed continuous oral oestrogen was associated with a reduction in the size of carotid atheromata. Further investigations may include:

• a definitive, controlled study with sufficient statistical power, using the same methodology
• a similar study using combined HRT
• assessment of plaque size using other methods such as MRI scanning
• effect of oestrogen treatment on intermittent claudication as assessed by a treadmill test

7.6.3 Intimal hyperplasia, angiogenic cytokines and adhesion molecules
The in vitro experiments in the present investigation have demonstrated that human ovarian veins develop intimal hyperplasia in culture. The addition of oestradiol to the culture medium was associated with a reduced intimal thickness. In vitro, VEGF expression was upregulated in response to oestrogen, whereas in vivo the serum levels of secreted VEGF were reduced following six months of oestrogen therapy. Regrettably, because of methodological difficulties, the expression of the adhesion molecules ICAM-1, VCAM-1 and E-Selectin could not be adequately assessed in this investigation.

Some of the following points probably warrant further study:

- the effect of varying doses of oestrogen on intimal hyperplasia in human veins, using a larger number of samples
- time effect of oestrogen on the development of intimal hyperplasia in human veins
- the effect of oestrogen antagonists on the development of intimal hyperplasia in human veins
- the effect of progestogens and oestrogen/progestogen combinations on the development of intimal hyperplasia in human veins
- expression of nitric oxide synthase in human veins under varying culture conditions
- assessment of proliferation markers in human veins in culture and with added oestrogen
- assessment of adhesion molecules ICAM-1, VCAM-1 and E-Selectin in frozen specimens of human veins, with and without oestradiol addition
The list of possible topics, taking the findings of the present investigation a step further, is very long indeed, and the above represents only a small sample. However, with the continuing increase in the numbers of women taking HRT, this field of research remains a focus of interest, with a great potential for further development.
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9. AUTHORS PUBLICATIONS

Based on research contained in this thesis.

9.1 PEER REVIEWED PUBLICATIONS


9.2 JOURNAL ABSTRACTS


Blair-Bell Abstract


9.3 Conference Abstracts
