MICROVASCULAR FUNCTION IN NORMAL AND COMPLICATED PREGNANCY

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Declaration

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

This thesis has been composed by myself, and I have been responsible for patient recruitment, clinical investigation, and data analysis, unless otherwise acknowledged.

Jane Ramsay

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Abstract

Microvascular dysfunction has been proposed as a link between clinical risk factors and cardiovascular disease. Many of these risk factors have also been proposed to predispose to complications of pregnancy such as pre-eclampsia and intrauterine growth restriction. My aims therefore, were to consider cardiovascular risk factors in relation to microvascular function in normal and complicated pregnancy using the novel, non-invasive technique, laser Doppler perfusion imaging in an attempt to examine potential mechanisms of such complications.

In chapter two I describe the development of our methodology for in-vivo examination of skin microvascular function, using iontophoresis of acetylcholine (ACh), an endothelium dependent vasodilator, and sodium nitroprusside (SNP), an endothelium independent vasodilator, in conjunction with a laser Doppler perfusion imager. A novel feature involved monitoring voltage across the iontophoresis chambers during current application. Both drugs elicited vasodilatation but with differing magnitudes and time-courses. During drug delivery a 3-4 fold difference in calculated skin resistance was observed between subjects, with higher resistance being associated with lower dilator responses to both drugs. This was corrected in individual subjects by multiplying individual perfusion values by the resistance.time integral, which reduced response variability. Cyclooxygenase inhibition by aspirin apparently attenuated ACH and SNP vasodilator responses but after correcting for skin resistance this effect was no longer observed. We propose that monitoring voltage across the iontophoretic

circuit is critical as the circuit resistance, which can be corrected for, influences effective drug delivery in individual subjects.

Iontophoretic assessment of skin microvascular function is complicated by the occurrence of electrically induced hyperaemia, especially at the cathode. Therefore in chapter three I examined the possible mechanisms of this effect in an attempt to reduce or eliminate this artefact. I noted voltage across chambers containing drug and salt solutions were significantly lower than the voltage profile of H_2O alone and eliminated this artefact. Voltage.time integral rather than charge was the prime determinant of electrically induced hyperaemic responses. Hyperaemic responses appeared to be prostaglandin dependent as they were ablated by cyclooxygenase inhibition. Therefore, use of a low resistance vehicle combined with larger chamber sizes and lower currents can prevent such artefacts, increasing the robust nature of this methodology for clinical assessment of endothelial function.

In chapter four I considered the effects of the increasingly prevalent cardiovascular risk factor, obesity, on maternal metabolic and vascular function. I examined healthy women in the third trimester of pregnancy and categorised the participants into two groups of lean and obese. A detailed panel of metabolic and inflammatory parameters was measured and an in-vivo assessment of endothelial dependent and independent microvascular function made using laser Doppler imaging. As in non-pregnant obese individuals, a significant dyslipidaemia and pro-inflammatory phenotype was demonstrated in obese women with leptin and

fasting insulin levels more than double that seen in controls. Both endothelial-dependent and independent vasodilatory responses were reduced in obese women. CRP (r=0.289, P =0.049) and insulin (r=0.339, P =0.02) were related inversely to endothelial-dependent function. Such factors may contribute to maternal complications in obese women and as a result potentially influence fetal programming of adult vascular disease.

In chapter five, I examined pregnant women with type 1 diabetes. Our aim was to compare vascular function, metabolic and inflammatory risk factors prospectively, in the antenatal and postpartum periods. Microvascular responses in both controls and diabetic women were better during pregnancy compared with postpartum. Moreover those women with worst ACH response postpartum showed most significant improvements during pregnancy. Responses in women with diabetes were less than controls during both periods despite similar lipoprotein profiles. The difference in vascular responsivity between cases and controls was attenuated by adjustment for HbA1c, but not CRP concentrations in the two groups. I concluded that pregnancy enhances microvascular function, but in women with diabetes such improvements are insufficient to attain responses seen in healthy non-pregnant women. This suggests a persistent vascular defect in women with type I diabetes that may contribute to adverse pregnancy outcome. Our data suggest a role for the chronic effects of hyperglycaemia in the impaired vascular responsiveness in such women.

In chapter six I examined cutaneous microvascular function in pre-eclamptic women. I also considered circulating markers of endothelial damage and simultaneously measured lipid and cytokine levels. I considered pre-eclamptic women in relation to their first trimester BMI and as pre-eclampsia and intra-uterine growth restriction (IUGR) are proposed to arise from similarly impaired placentation, I also examined women with IUGR. To the extent of our knowledge, most work concerning endothelial function in PET has not considered or controlled for the effects of body mass index (BMI). I demonstrated elevated concentrations of both fasting leptin and triglyceride in pre-eclamptic women, independent of BMI. Women with IUGR had smaller 1st trimester BMI (p=0.044) and 3rd trimester LDL-C concentration (P=0.021) compared with controls. I noted a stepwise increase from lean to obese to pre-eclamptic in the concentration of IL-6, VCAM-1 and E-selectin. I demonstrated reduced endothelial dependent vasodilatation in pre-eclamptic as compared with lean women and responses to ACH correlated inversely with the inflammatory cytokine IL-6 ($r = -0.705$, $P = 0.023$). However, obese healthy women showed smaller endothelial dependent responses than pre-eclamptics. I propose that in-vivo endothelial dysfunction as demonstrated by this technique, does not represent the entire pathophysiological mechanism in PET. Increased capillary permeability, perhaps secondary to elevated placentally derived leptin, may contribute to symptoms of pre-eclampsia. Therefore, in-vivo methods of microvascular function assessment are not sensitive or specific for the prediction of pre-eclampsia.

Epidemiological studies have recently demonstrated a relationship between pre-eclampsia and an increased risk of maternal coronary heart disease in later life. Common risk factors, either genotypic or phenotypic, may underlie both pre-eclampsia and coronary heart disease. Therefore in chapter seven I aimed to test the hypothesis that insulin resistance, inflammation, and hyperlipidaemia would persist in women with a history of pre-eclampsia and secondly that within this group there would exist demonstrable endothelial dysfunction. I demonstrated that women with a past history of pre-eclampsia have increased plasma concentrations of the inflammatory markers, VCAM-1 and ICAM-1 and impaired endothelial dependent vasodilatation, 15 years or more after the index pregnancy. In conclusion therefore, these data may suggest that the phenotype associated with pre-eclampsia is linked to novel mechanisms underlying coronary heart disease.

Therefore, I have developed a robust mechanism for the in-vivo assessment of cutaneous microvascular function. This has been used to demonstrate impaired microvascular responses in pregnant women with an elevated risk of cardiovascular disease in pregnancy and in later life. I have also demonstrated impaired microvascular function in women with a past history of pre-eclampsia. These observations have been closely related to both traditional (metabolic) and novel (inflammatory) cardiovascular risk factors. However, using this technique I have demonstrated that in-vivo endothelial dysfunction in women with pre-eclampsia may be secondary to differences in BMI. Also women with IUGR tend to be thinner and have less of an atherogenic metabolic disruption, perhaps

suggesting a systemic cardio-protective role for "leanness" in this group. Leptin concentrations are elevated in pre-eclampsia, independent of BMI, and I propose roles for leptin both as a placental response to ischaemia and in the pathophysiology of pre-eclampsia.

Chapter One

INTRODUCTION

Endothelial cell dysfunction:

implications for women's health.

A review of evidence examining microvascular dysfunction as a link between shared clinical risk factors and cardiovascular disease in pregnancy and later life.

"It will not be foreign to the subject if I here show further, from certain familiar reasonings, that the circulation is matter both of convenience and necessity. In the first place, since death is a corruption which takes place through deficiency of heat, and since all living things are warm, all dying things cold, there must be a particular seat and fountain, a kind of home and hearth, where the cherisher of nature, the original of the native fire, is stored and preserved; from which heat and life are dispensed to all parts as from a fountain head; from which sustenance may be derived; and upon which concoction and nutrition, and all vegetative energy may depend. Now, that the heart is this place, that the heart is the principle of life, and that all passes in the manner just mentioned, I trust no one will deny.----------Unless the heart were truly that fountain where life and heat are restored to the refrigerated fluid, and whence new blood, warm, imbued with spirits, being sent out by the arteries, that which has become cooled and effete is forced on, and all the particles recover their heat which was failing, and their vital stimulus wellnigh exhausted."

On The Motion Of The Heart And Blood In Animals, 1628

William Harvey (1578-1657)

Introduction

Developments in cardiovascular research over the last two decades have established the vascular tree to be more than an inanimate conduit for blood, but instead a highly complex endocrine and paracrine organ. The vascular endothelium is proposed as the mediator of these physiological functions and recently an accumulation of evidence has suggested that activation and damage of endothelial cells may be the first step in the development of cardiovascular disease.

In the healthy vascular tree, smooth muscle maintains a continual state of activation secondary to interactions between many chemical and physical factors. In 1980 Furchgott and Zawadzki observed that isolated arteries would demonstrate relaxation in response to acetylcholine, but only in the presence of an intact endothelium. [1] They postulated that endothelial cells were able to produce a diffusible vasodilator, which became known as endothelial derived relaxant factor (EDRF). Ignarro and Palmer have since, independently, identified this compound as Nitric oxide (NO). [2 3] This is derived from the terminal guanidino atom of L-arginine, catalysed by the Ca^{2+} dependent stimulation of enzyme nitric oxide synthase (NOS), resulting in production of NO plus L-citruline. **(Figure 1)** A number of other vasodilators, such as bradykinin and substance P, have also been shown to require an intact vascular endothelium for their function. Following diffusion into the adjacent smooth muscle, nitric oxide (NO) activates soluble

guanylate cyclase, which increases cyclic guanosine 5-monophosphate (cGMP) resulting in enhanced sarcolemelle outward transport of calcium and consequent relaxation of vascular smooth muscle. **(Figure 1)**

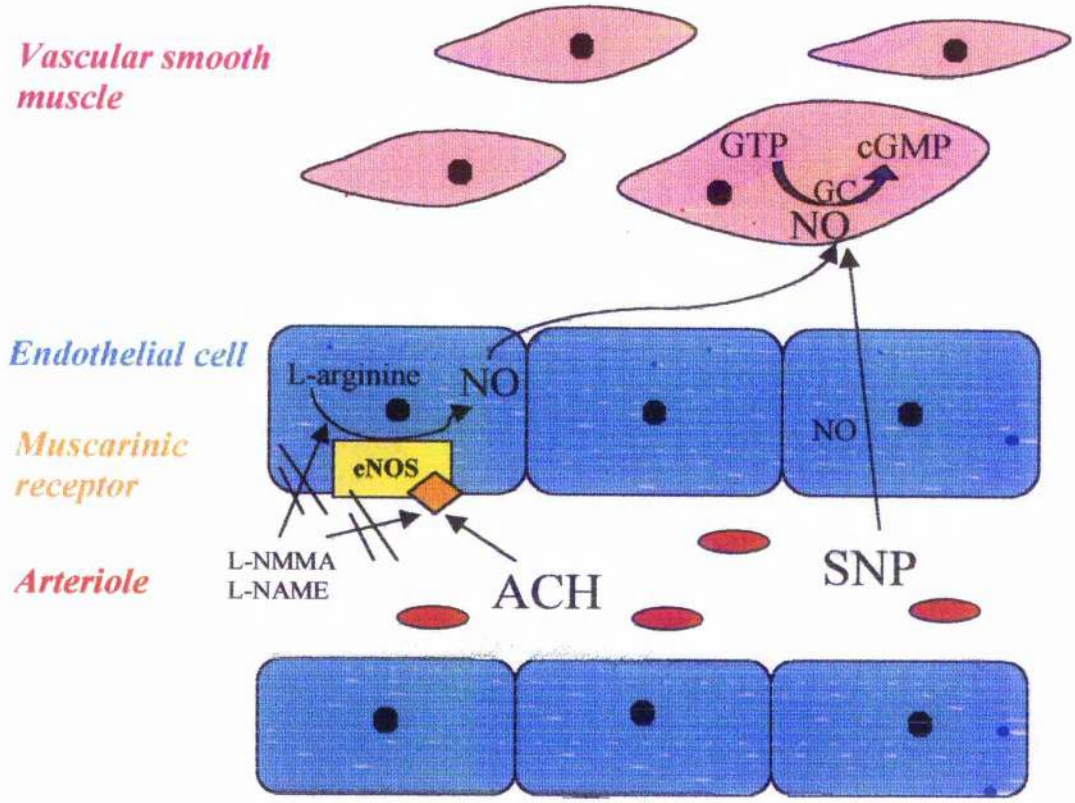


Figure 1: Mechanism of action of endothelial dependent and independent vasodilation via nitric oxide system, illustrating mode of action of pharmacological inhibitors of NO production. (L-NMMA, L-NAME) ACH=acetylcholine, SNP=sodium nitroprusside, NO=nitricoxide, eNOS=endothelial nitric oxide sythase, GC=guanylate cyclase, cGMP=cyclic guanosine 5-monophosphate L-NAME=NG-nitro L-arginine methyl ester, L-NMMA=NG-monomethyl L-arginine

However, NO is not only a potent vasodilator but also inhibits platelet aggregation, smooth muscle cell migration and proliferation, adhesion molecule expression and consequently monocyte adhesion. Thus NO provides the vessel

wall with protection against the development of thrombosis and atheromatous plaques. [4] Other vascular smooth muscle relaxants produced by the vascular endothelium include the eicosanoids, particularly prostacyclin, and the still elusive endothelial derived hyperpolarising factor, as well as the vasoconstrictor endothelin. Prostacyclin (PGI_2) also inhibits platelet aggregation by stimulation of platelet adenyl cyclase leading to an increase in platelet cyclic AMP. Moncada and Vane demonstrated that disease states such as atherosclerosis and diabetes are associated with reduced PGI_2 production and their observations led to the introduction of PGI_2 therapy in conditions associated with increased platelet aggregation such as haemodialysis and peripheral vascular disease. [5]

Many clinical and epidemiological studies have provided evidence that certain risk factors, both traditional and novel, can be useful predictors of coronary heart disease. Such traditional risk factors include hypercholesterolaemia, particularly elevated plasma concentrations of low-density lipoprotein cholesterol (LDL-C), hypertriglyceridaemia, diabetes mellitus, insulin resistance and obesity. [6 7 8 9 10] Novel risk factors encompass the theory of inflammation and include elevation in concentration of inflammatory cytokines, soluble adhesion molecules and other inflammatory proteins released in response to inflammatory stimulus such as C reactive protein (CRP) [11 12 13]. Each risk factor appears to be inextricably linked and may provide an insight into the mechanism by which an alteration in vascular function is produced. Of interest, these cardiovascular risk factors are also highly significant with regards to the development of some complications of pregnancy,

particularly pre-eclampsia. Therefore one may hypothesise that in some individuals, the metabolic stress of pregnancy may "unmask" those women who are predisposed to develop cardiovascular disease in later life. [14] This predisposition may be genetically inherited or acquired via in-utero fetal programming as has been suggested in Barkers fetal origins hypothesis. [15]

Consequently, in this thesis I will attempt to consider these risk factors with regards to vascular function in pregnancy and their influence on such cardiovascular complications as pre-eclampsia and intra-uterine growth restriction.

Lipoproteins and cardiovascular reactivity

Hypercholesterolaemia is now well established to be a predictor of coronary heart disease and more significantly, cholesterol-lowering therapy can reduce the incidence of events associated with coronary artery disease. [16 17] Many studies have demonstrated impaired vascular function in association with hypercholesterolaemia. Endothelial dependent vasodilatation, in response to administration of acetylcholine (ACH), has been demonstrated to be significantly impaired in forearm resistance vessels [18] and non-atherosclerotic coronary arteries in hypercholesterolaemic patients [19]. Other groups have suggested an impairment of endothelial independent vasodilatation as demonstrated by the administration

of sodium nitroprusside (SNP). Forearm blood flow responses to SNP were reduced in hypercholesterolaemic patients compared with controls [20] and similar results have been demonstrated in isolated resistance vessel preparations from hypercholesterolaemic patients [21]. Of interest pathophysiologically, this vascular dysfunction can be reversed by the restoration of serum cholesterol levels to normal or by treatment with anti-oxidant therapy. [22 23]

It has been proposed that short term changes in LDL-Cholesterol levels and probably more importantly the extent of oxidation of LDL-C modifies both vasodilator and vasoconstrictor responses. In animal models of hypercholesterolaemia, there is an observed increase in the production of NO suggesting a diminished effect rather than reduced production [24]. In 1991 Galle et al [25] demonstrated that NO liberated from endothelial cells was inactivated by oxidised LDL-C and also Schmidt et al [26] observed that NO stimulation of smooth muscle guanylate cyclase was impaired in a dose dependent fashion by oxidised LDL. These observations provide mechanistic evidence for the role of oxidised LDL in impairment of endothelial dependent and independent vasodilatation. Additionally, enhanced NO inactivation may be provoked by oxidised LDL stimulated superoxide formation. [27] **(Figure 2)**

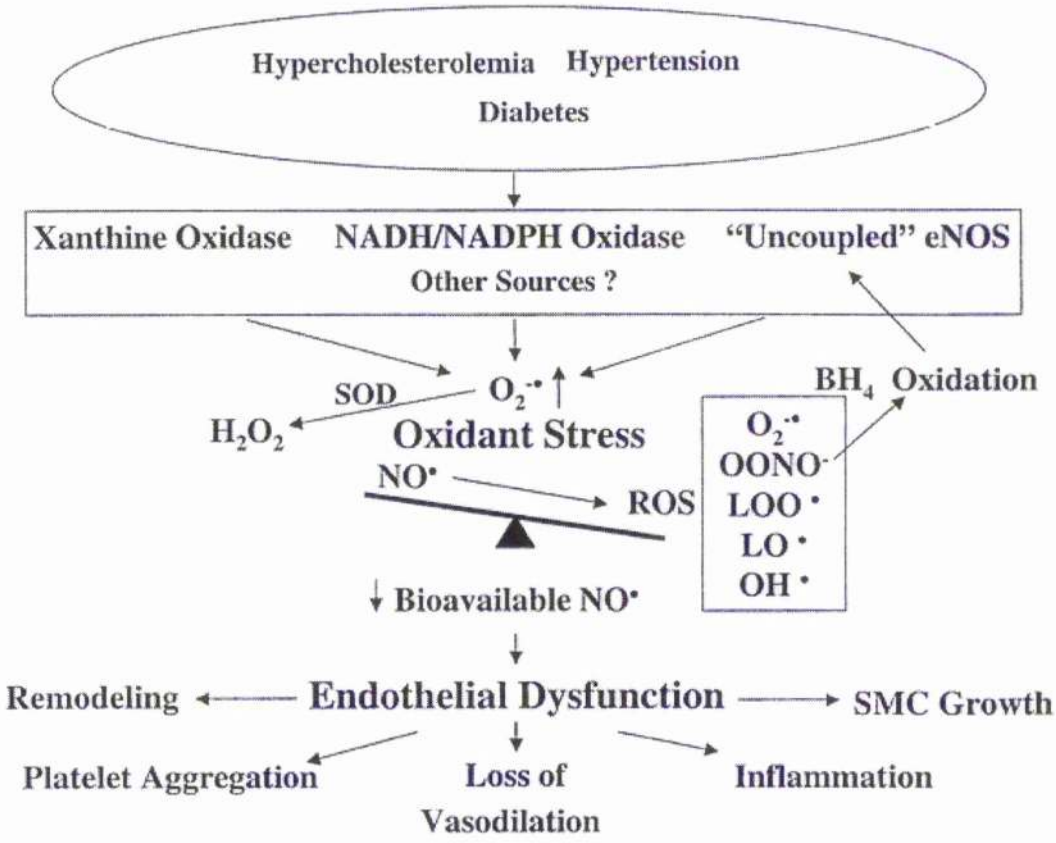


Figure 2. Mechanisms for oxidant stress-induced endothelial dysfunction in cardiovascular diseases (from [28])

Of interest, high-density lipoprotein (HDL) cholesterol has been demonstrated to protect against abnormal responses to ACH infusion in coronary arteries [29], and in isolated strips of rabbit aorta, HDL reversed LDL induced endothelial dysfunction [30].

In the publication of the eight-year follow up data from the PROCAM study, serum triglyceride has now been demonstrated to have a significant and independent relationship with the incidence of major coronary events. [6] In a large meta-analysis of eight population-based prospective studies, a 1mmol/l increase in

serum triglyceride concentration was equivalent to a 14% and 37% increased relative risk of coronary heart disease in men and women respectively. [31] The mechanism by which triglycerides exert their influence on the development of atherogenesis may be variable, including effects on endothelial cell function, macrophage loading, thrombogenesis and determination of LDL size, i.e. defining the smaller more dense atherogenic particle size. [7] Of interest, hypertriglyceridaemia is strongly associated with non-insulin dependent diabetes (NIDDM) consistent with the key role of insulin resistance in both conditions.

Impaired insulin sensitivity and microvascular function

Micro- and macro-vascular complications are problematic in both non-insulin dependent (NIDDM) and insulin dependent diabetes (IDDM). In NIDDM, insulin resistance and resultant hyperinsulinaemia is proposed as a mechanism by which cardiovascular complications such as hypertension arise. [32] Another theory suggests increased blood glucose concentrations and the resulting increase in glycation products quench NO and therefore impair vascular reactivity. [33] However in animal models, impaired vascular reactivity was only demonstrated in association with prolonged supra-physiological glucose levels [34]. In humans, at least one group has demonstrated that a sustained moderate hyperglycaemia of 10-15 mmol was not associated with impaired vascular function [35] and Morris et al observed no relationship between serum HbA1c levels and vascular reactivity in NIDDM. [36]

An alternative hypothesis suggests impaired micro-vascular endothelial function in the metabolically important capillary beds, as opposed to large vessels, may be the precursor to the development of insulin resistance [37]. Reduced endothelium dependent vasodilatation may result in a decrease in capillary density resulting in an increased diffusion distance for insulin and glucose from capillary to muscle cells. [38] As a consequence, decreased skeletal muscle capillary recruitment may increase total peripheral resistance and therefore blood pressure. Capillary endothelial dysfunction may play an important role in the development of the atherogenic lipid profile commonly associated with the insulin resistant individual, through impaired action of endothelial-bound lipoprotein lipase (LPL) resulting in elevated levels of triglyceride and reduced high density lipoprotein (HDL). [37] In support of these hypotheses, impaired endothelium dependent vasodilatation has been established to be significantly associated with insulin resistance and hypertension. [39 40 41]

Serne et al [42] demonstrated an inverse relationship between blood pressure and insulin sensitivity as well as a strong positive relationship between microvascular function and insulin sensitivity. Of interest, waist hip ratio i.e. central body fat distribution also shows a strong association with insulin sensitivity, blood pressure and microvascular function. Consequently, this group proposes abdominal fat to be a source of free fatty acids (FFA) and cytokines, which are secreted into the circulation. These circulating factors would promote vascular inflammation and endothelial dysfunction, and result in insulin resistance and

hypertension as described above. (**Figure 3**) The deleterious effect of FFA and cytokines on endothelial function has been demonstrated previously. [11 43]

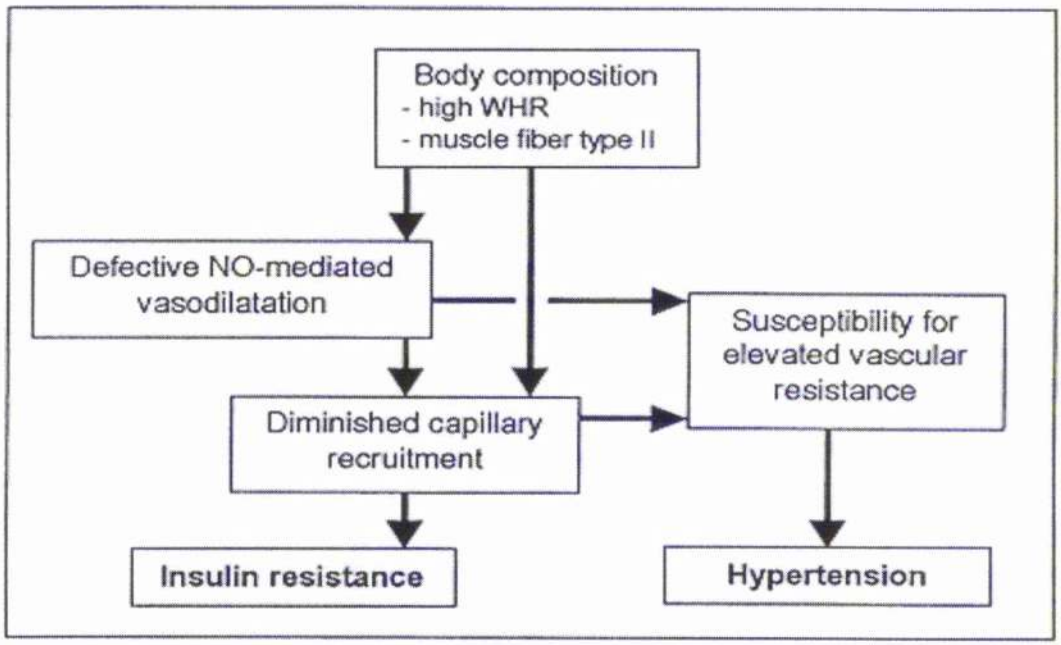


Figure 3. Postulated relations linking insulin resistance and hypertension. Perturbed microvascular vasodilator capacity may serve as a common antecedent determining both insulin sensitivity and blood pressure. (From [42])

Insulin dependent diabetes and microvascular function.

Micro- and macro-vascular disease, as seen in complicated insulin dependent diabetes (IDDM), has been suggested to arise from a subtly different mechanism from that observed in NIDDM. The anatomical distribution of vascular complications also varies with peripheral resitance vessel disease predominating in NIDDM. Hypertension for example, occurs in as many as 40% of affected

individuals, [44] whereas small vessel complications of organs such as the kidney, brain and retina are more prevalent in IDDM. A vast array of ex- and in-vivo, animal and human work has been performed to assess the changes in vascular reactivity associated with IDDM. From this work, it appears that endothelial dysfunction could arise from a variety of mechanisms such as decreased production or enhanced deactivation of NO, impaired diffusion of NO into smooth muscle cells or impaired responsiveness of smooth muscle to NO. Confusion exists due to the comparison of small and large vessel experiments and the assessment of different vascular beds, where endothelial cells may exhibit different metabolic and structural properties and may be variably influenced by hyperglycaemia. In many studies hyperglycaemia is proposed as the mediator of vascular complications. Hyperglycaemia is believed to induce multiple changes in intracellular metabolism such as activation of the polyol pathway, protein kinase C or increasing oxidative stress. Also long term changes in structure and function of macromolecules secondary to nonenzymatic glycation and oxidation of proteins, results in the formation of advanced glycation end products (AGEs) and may produce alterations in vascular function. (Reviewed in [45]) Advanced glycation end products (AGEs), form under diverse circumstances such as aging, diabetes, and kidney failure. Recent studies suggested that AGEs may form in inflamed foci, driven by oxidation. A principal means by which AGEs alter cellular properties is through interaction with their signal-transduction receptor RAGE. These receptors have been described in association with increased

concentrations of cellular adhesion molecules and therefore may prime proinflammatory mechanisms in endothelial cells [46].

In the early stages of IDDM some groups have suggested that increased micro-vascular flow and consequent increased capillary pressure can be demonstrated. (Reviewed in [47]) Up-regulated tissue perfusion results in further shear stress applied to the vessel wall and consequently the vascular endothelium manufactures extra-vascular matrix proteins as an injury response. The resulting effect is of micro-vascular sclerosis with a consequent impairment in vasodilatory reserve and loss of autoregulation. The endpoint of this process would be demonstrated in the micro-angiopathic complications commonly associated with IDDM. One possible mechanism supporting this hypothesis is demonstrated by work performed by Graier et al [48]. This group demonstrated that cultured endothelial cells exposed to supra-physiological concentrations of D-Glucose increase their production of EDRF. Elevated glucose concentrations may also result in increased generation of reactive oxygen species. These exert oxidative damage to the endothelium with consequent accumulation of glycation end products and remodelling of the basement membrane. (Reviewed in [47])

Obesity and microvascular function

An elevated incidence of atherosclerotic heart disease is a well-recognised association in obese individuals [49] and obesity in isolation is established as an independent cardiovascular risk factor. [10] Moreover, the distribution of body fat, that is abdominal or central adiposity, has been proposed to be a strong predictor of morbidity and mortality and correlates with hyperinsulinaemia, hypertriglyceridaemia, reduced HDL concentrations and hypertension. [50] Steinberg et al employed femoral artery infusion of metacholine and subsequent measurement of leg blood flow to demonstrate a 40% reduction in endothelial dependent vasodilatation in obese (BMI>28) compared with lean (BMI<28) control subjects. [39] More recently Arcaro et al corroborated these findings utilising Doppler ultrasound assessment of femoral artery diameter variation in response to distal post-ischaemic leg hyperaemia in similarly defined subjects. [51] A variety of possible mechanisms have been proposed in support of these observations including dyslipidaemia, particularly elevated small dense LDL and elevated free fatty acids, and relative insulin resistance resulting in hyperinsulinaemia. (discussed above)

Inflammation and micro-vascular function.

One potential unifying hypothesis for the effect of lipids, hyperinsulinaemia and obesity with regards to endothelial function, involves the theory of "inflammation". Arteriosclerosis is now believed to be a disease of inflammation

[52] and the serum concentrations of inflammatory markers have been demonstrated to be predictive of coronary events. [53]

There are several sources of production for inflammatory markers, such as the liver, the heart, and circulating cells like macrophages. The inflammatory markers, C-reactive protein (CRP) and fibrinogen are derived from the liver under the influence of systemic cytokines. CRP and fibrinogen have been demonstrated to be independent predictors of cardiovascular events [54, 55] (**Figure 4**) and Cleland et al have recently demonstrated that CRP concentrations, even within the normal range, strongly correlate with endothelial function as assessed by venous plethysmography in a group of healthy subjects [56].

A

1. Determine TC:HDLC Quintile
2. Determine hs-CRP Quintile
3. Assess Cardiovascular Relative Risk

B

Quintile	hs-CRP (mg/L)	TC:HDLC (Women)	TC:HDLC (Men)
1	0.1 - 0.7	< 3.4	< 3.4
2	0.7 - 1.1	3.4 - 4.1	3.4 - 4.0
3	1.2 - 1.9	4.1 - 4.7	4.0 - 4.7
4	2.0 - 3.8	4.7 - 5.8	4.7 - 5.5
5	3.9 - 15.0	> 5.8	> 5.5

C

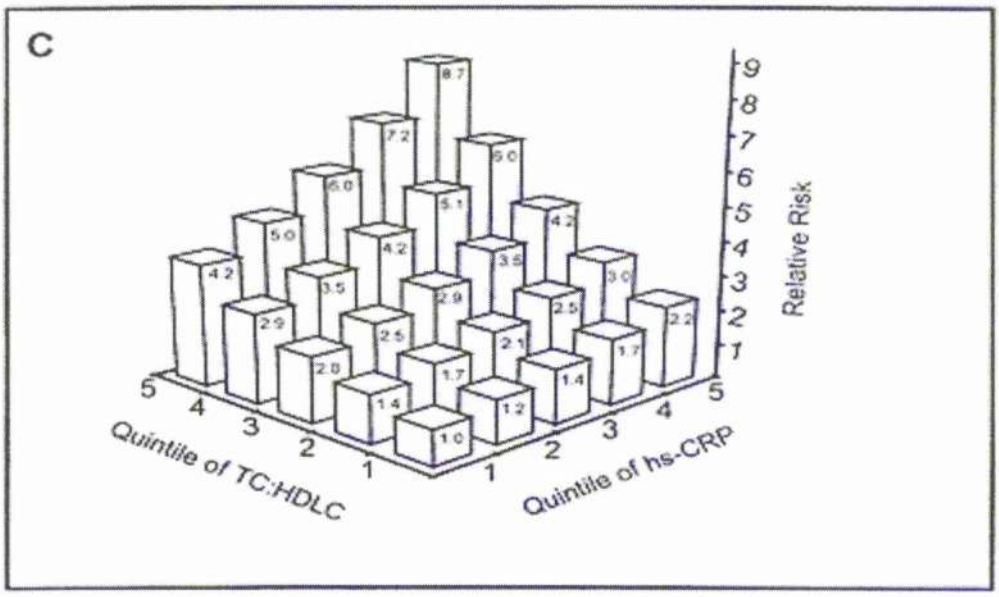


Figure 4: High-sensitivity C-reactive protein: a novel and promising marker of coronary heart disease [54]

The vessel wall itself produces cell adhesion molecules such as VCAM-1 and ICAM-1. These cell surface molecules are expressed and up regulated in response to stimulation by cytokines and their soluble component can be measured in the blood. Their purpose is the recruitment of circulating inflammatory cells and the eventual transmigration of the cells through the endothelium, a step believed to be the initiating step in the development of atheromatous deposits. Ridker et al have

demonstrated soluble ICAM-1 concentrations also to be an independent predictor of cardiovascular events [12]

Specific cytokines such as interleukin-1β, (IL-1β) interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) are largely responsible for the increased expression of inflammatory markers by the liver and the endothelium and in a recent study, IL-6 was predictive of the risk of myocardial infarction despite adjustment for CRP concentrations. [57] In cardiovascular disease, a potential source for the production of such inflammatory cytokines may be the myocardium or the atherosclerotic plaque itself. However, adipose tissue is increasingly recognised as a source of cytokines such as IL-1β, IL-6 and TNFα. IL-6 has been proposed as a key element in the pathogenesis of cardiovascular disease through a variety of mechanisms involving the stimulation of peripheral inflammation as discussed above. [58 59] Also IL-6 concentrations are strongly associated with central adiposity, hypertension and insulin resistance, that is the features of the dysmetabolic state. [11] **(Figure 5)**

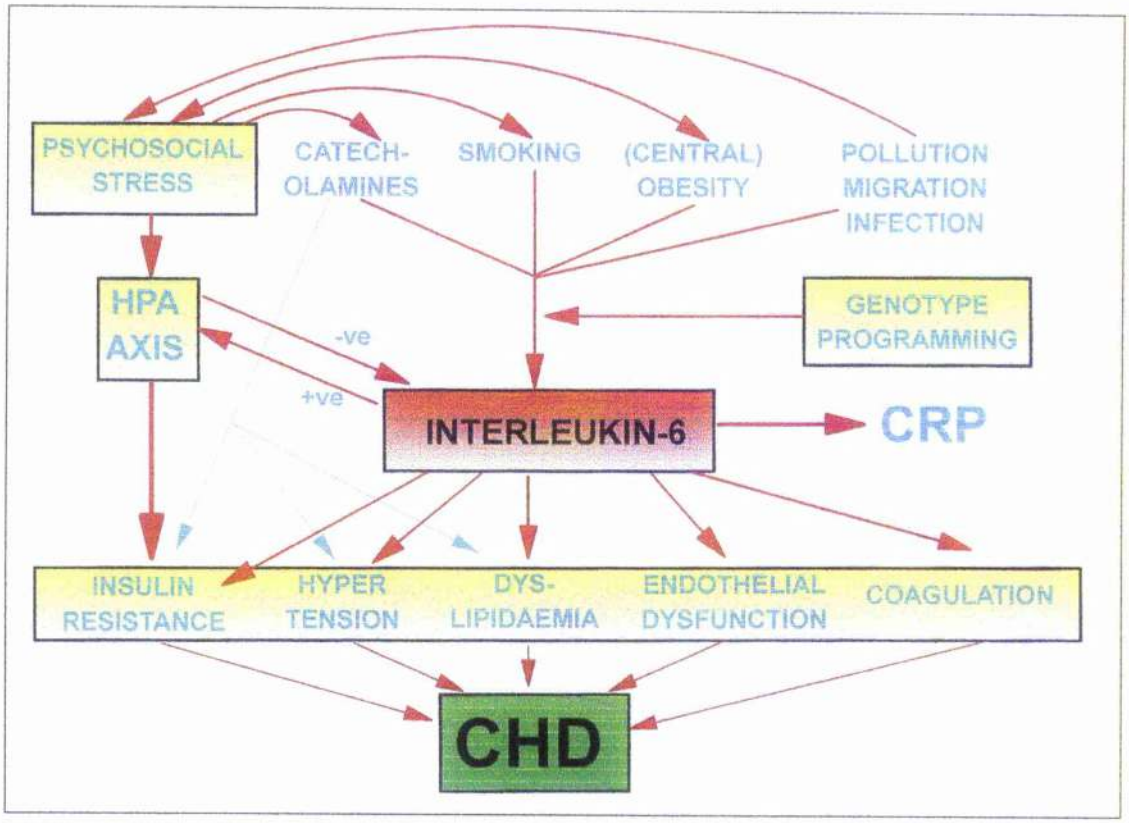


Figure 5. The role and regulation of IL-6. (from [11])

MATERNAL CARDIOVASCULAR AND METABOLIC ADAPTATIONS ASSOCIATED WITH UNCOMPLICATED PREGNANCY

On achievement of conception, the developing embryo implants into the maternal decidua and commences an outstanding cascade of events designed to beneficially alter the maternal environment in order that the developing fetus may be optimally maintained and nurtured. These physiological changes are imperative for the achievement of a successful and uncomplicated pregnancy and failure can

result in detrimental and life threatening effects not only for the fetus, but also for the mother.

The Maternal Vasculature in uncomplicated pregnancy

Of particular interest are the maternal systemic cardiovascular adaptations. Maternal systemic vascular resistance falls early in pregnancy and therefore, despite a 40% increase in cardiac output (20% within the first eight weeks of pregnancy) and a 50% rise in circulating blood volume, maternal blood pressure begins to decrease towards the end of the first trimester. The nadir of systolic and diastolic pressure is attained by around 22-24 weeks gestation and thereafter a steady rise in blood pressure is observed, until pre-pregnancy levels are achieved by term. [60]

Gant et al first demonstrated the resistance of the maternal cardiovasculature to the effects of the vasoconstrictor agent, angiotensin II (AgII) [61] and it is now generally accepted that pregnancy results in a reduced pressor response to many physiological vasoconstrictors such as adrenaline, noradrenaline and the eicosanoids. This reduced sensitivity to pressor agents must contribute to the overall vasodilatation of normal pregnancy. In support of this, Everett et al [62] demonstrated that in normotensive women, responsivity to AgII can be restored by prostaglandin synthetase inhibitors such as Apirin and Indomethacin. Broughton Pipkin et al [63] also observed that simultaneous infusion of AgII with

prostaglandin E2, blunted the pressor effect associated with AgII, suggesting a role for prostaglandins in the mechanism of this effect. Everett also described a restoration of the refractory nature of the maternal cardiovascular system to the pressor effects of AgII, with intravenous infusion of the progesterone metabolite, 5alpha-pregnan-3,20-dione. [64]

As a consequence of the ethical considerations involved in research concerning human pregnancy, most of the work performed in this field has been either animal based or ex-vivo human experiments. These techniques represent either an indirect assessment of vascular function such as demonstration of circulating serum markers of vascular activation or direct, such as isolated vessel wire myography.

Most groups now accept that peripheral vasodilatation associated with healthy pregnancy is a result of up-regulation of an endothelial-derived vasodilator such as prostacyclin (PGI_2), EDHF or nitric oxide (NO). (See above) A significant increase in the synthesis of PGI_2 during pregnancy has been demonstrated [65] and patients who develop pregnancy-induced hypertension exhibit a lesser increment in prostacyclin biosynthesis than healthy pregnant subjects [66]. Animal experiments have suggested an increase in NO synthesis in healthy pregnancy. Weiner et al demonstrated an up-regulation of calcium dependent NO synthase (NOS) activity in a variety of tissues in pregnant guinea pigs, [67] while other

groups have observed increased nitrate excretion in the pregnant rat. [68] In the human, increased excretion of cGMP, the second messenger responsible for mediating NO–induced vasodilatation, has been reported in healthy pregnant women [69], as has increased nitrate turnover in women with a carefully controlled nitrate intake. [70] Both of these observations indirectly suggest the stimulation of NO synthesis in association with pregnancy.

The literature concerning human studies of endothelial function in pregnancy is somewhat contradictory. McCarthy et al were unable to demonstrate a difference in ACH responsiveness and therefore endothelial function between subcutaneous arterioles obtained at Caesarean section and from vessels obtained from non-pregnant women during routine surgery. [71] This observation has since been reproduced by Pascoal et al using omental vessels from pregnant and non-pregnant pre-menopausal women exposed to ACH and bradykinin. [72] In an experiment using isolated arterioles from subcutaneous fat, no difference was seen in the vasoconstrictor response to norepinephrine between those samples from pregnant and non-pregnant individuals, before and after denuding the vessels of endothelium. However, vessels from normotensive pregnant women demonstrated a significantly greater relaxation when exposed to bradykinin than those vessels from non-pregnant women. These effects were felt to be dependent of endothelium as denuding the vessels obliterated the relaxation. Also indomethacin, a cyclo-oxygenase inhibitor, had no effect on vessel relaxation and therefore the effect was felt to be NO, but not prostacyclin dependent. [73] Cockell

et al demonstrated a significant difference ($p<0.01$) in a study of isolated subcutaneous vessels from healthy pregnant and non-pregnant women exposed to increase of flow and therefore shear stress. Vessels from pregnant women demonstrated a greater relaxation to shear stress and this was attenuated by the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) suggesting a possible mechanism for the fall in peripheral vascular resistance in healthy pregnancy. [74] In a study of cultured, isolated hand vein endothelial cells, intracellular calcium response to adenosine triphosphate (ATP) administration was measured in pregnant and non-pregnant women. The endothelial cell response to ATP was significantly greater in the pregnant as compared with the non-pregnant group. These data suggested a difference in the responsiveness of venous endothelial cells in pregnancy. Williams et al performed one of the few functional, in-vivo studies to identify differences in vascular reactivity in pregnant and non-pregnant women. [75] Using venous occlusion plethysmography, hand blood flow was assessed in three groups of women: non-pregnant, first trimester pregnancy and third trimester pregnancy. Basal blood flow was significantly increased in late pregnancy as compared with early and non-pregnant subjects ($p=0.007$) and brachial artery infusion of the NOS inhibitor L-NMMA (**Figure 1**) produced a greater reduction in hand blood flow in both pregnant groups compared with the non-pregnant women. ($p=0.0003$) Also vasoconstriction, that is reduced hand blood flow in response to administration of norepinephrine, was less in non-pregnant and early pregnant women as compared with those in late pregnancy. ($p=0.0029$) These observations were confirmed by similar work performed by

Anumba et al. [76] Again, the above evidence suggests that increased generation of endothelial cell derived nitric oxide may be the responsible mechanism for the fall in peripheral vascular resistance seen in normal human pregnancy.

Maternal Metabolism

Plasma cholesterol and triglyceride concentrations increase by 25-50% and 200-400% respectively throughout healthy pregnancy. In the latter stages of the second trimester of pregnancy an increased concentration of free fatty acids (FFA) is observed. This response may be produced as a result of stimulation of hormone sensitive lipases (HSL) by human placental lactogen (HPL) and/or the development of a relative resistance to insulin with a loss of the usual suppressive effect of insulin upon FFA release from adipose tissue. (reviewed in [77]) **(Figure 6)** Therefore an increased delivery of FFAs to the liver results in increased synthesis of triglyceride and subsequent hepatic assembly of VLDL particles. [78] **(Figure 6)** Also pregnancy results in a significant reduction in lipoprotein lipase activity, with consequent reduction in maternal triglyceride catabolism. [79]

Driven by elevated oestrogen concentrations which stimulate increased Apo A1 production, high-density lipoprotein (HDL) also increases in pregnancy, achieving a peak around 28 weeks gestation. Low-density lipoprotein (LDL) achieves a less significant elevation, although as triglyceride concentrations rise, a

threshold is reached after which proportionally more hepatic production of the subfraction LDL-III takes place. In the oxidised form, this molecule is believed to be highly atherogenic, promoting foam cell production and initiating endothelial dysfunction. [78] **(Figure 6)**

Therefore, as discussed above, excess peripheral lipolysis of fat stores provides increased non-esterified fatty acid (NEFA) concentrations. NEFAs, as already discussed, contribute to the dyslipidaemia observed in pregnancy but in addition impair peripheral glucose uptake and enhance glucose production as well as possibly inhibiting portal insulin extraction, resulting in increased insulin resistance and insulin concentrations. [80] A progressive increase in nutrient stimulated insulin responses consistent with insulin resistance has been described in normal healthy pregnancy. [81] Hyperinsulinaemic euglycaemic clamp techniques have demonstrated insulin efficacy to be as much as 50-70% less in late pregnancy as compared with non-pregnant women. To meet the increasing demands of the developing fetus and placenta, basal endogenous hepatic glucose production is thought to increase by 16-30% with an increased contribution of carbohydrate to oxidative metabolism in late pregnancy. (reviewed in [82])

Cardiovascular and metabolic complications of Pregnancy

1) Pre-eclampsia

Pre-eclampsia is a pregnancy-specific multi-system maternal syndrome, unique to humans, encompassing three main clinical aspects: elevated blood pressure, proteinuria and oedema. Historically, as the nomenclature suggests, it was the complications of this condition that were originally recognised and described, with the term "eclampsia" describing maternal seizures. However, pre-eclampsia is notoriously heterogeneous with a variety of clinical presentations and associated signs and symptoms. The condition is defined as developing after 20 weeks gestation, most commonly in the third trimester of pregnancy. The common factor in all cases appears to be the presence of the placenta [83]. In cases of excessive trophoblastic development such as a molar pregnancy, presentation has been described in the first trimester.

The complications of pre-eclampsia can detrimentally affect maternal and fetal well being. Cerebral haemorrhage and adult respiratory distress syndrome (ARDS) have persistently featured as among the most common causes of maternal mortality in the confidential enquiry into maternal deaths in the UK. [84] Perinatal mortality associated with this condition is around five times greater than that associated with normal pregnancy and the offspring of pre-eclamptic pregnancies account for 40% of iatrogenic premature deliveries. [85] Despite these

dramatic statistics, delivery remains the only effective strategy for management of this condition

As already described, pre-eclampsia is a multisystem disorder with potential to develop complications affecting almost all the major systems. These include cerebral vasospasm and haemorrhage, resulting in seizures, hepatic and adrenal haemorrhage and necrosis, glomerular endotheliosis and acute renal failure, subendocardial necrosis, consumptive coagulopathy and microangiopathic haemolysis. Some associated syndromes have been described as potential variations of pre-eclampsia. The liver and coagulation pathways are often involved with clinical presentation ranging from a mild derangement of liver enzymes to gross hepatic impairment and intravascular haemolysis as seen in the HELLP syndrome (haemolysis, elevated liver enzymes and low platelets). (Reviewed in [86])

Pre-eclampsia is also thought to be associated with an increased risk of cardiovascular disease in later life. Of interest, other poor outcomes of pregnancy, such as pre-term labour, a small baby and gestational diabetes have also been described to be associated with an increased risk of cardiovascular disease in the mothers in later life. (Reviewed in [87]) **(Table 1)** Hannaford et al studied general practitioners' records of 23000 women from UK in order to determine the rate of cardiovascular disease in parous women. Compared with parous women with no

history of pre-eclampsia, those previously affected by pre-eclampsia showed an increased risk of hypertensive disease (RR 2.35), acute myocardial infarction (RR 2.24), chronic ischaemic heart disease (RR 1.74), and angina (RR 1.53). [14] More recently these findings were confirmed in a local population. Smith et al examined data from 130,000 deliveries in Scotland between 1981 and 1985 inclusive. [88] This was linked with data on all hospital admissions and the General Registrars office, providing follow up information for 15-19 years. Again a two-fold increased risk of IHD in women with a past history of pre-eclampsia was demonstrated, but of interest, a cumulative risk associated with other complications of pregnancy was observed. Pregnancies affected by pre-eclampsia plus pre-term delivery were associated with a six-fold increase in risk of death from IHD compared to women with uncomplicated pregnancy. Pre-eclampsia and a baby in the lowest quintile for birthweight was associated with a four-fold increase in risk and a combination of all three poor outcomes a sixteen-fold increase in risk, some eight times the risk from pre-eclampsia alone. This group hyothesised that their findings may be explained by common genetic risk factors implicated in both the pathophysiology of poor pregnancy outcome and cardiovascular disease. An increased risk of insulin resistance has also been observed in a seventeen-year follow up of women affected by pre-eclampsia. [89]

Association of adverse pregnancy outcomes with risk of diabetes or risk factors for coronary heart disease and vascular disease

Pregnancy outcome	Incidence in pregnancy (%)	Risk factors shown to perturbed after pregnancy	Association or risk ratio (95% CI)
Gestational diabetes	1.9-5.0*	Lipids[2]	Increased risk of type 2 diabetes, especially if recurrence of gestational diabetes in a subsequent pregnancy. No data on coronary heart disease risk
		Blood pressure[2]	
		Large vessel function[3]	
		Small vessel function[3]	
Pre-eclampsia	2-4	Lipids[4]	1.9 (1.0 to 3.5) *v* pregnancy induced hypertension alone[7]
		Clotting[4]	1.7 (1.3 to 2.2) *v* no pre-eclampsia[8]
		Fasting insulin[5]	2.0 (1.5 to 2.5) *v* no pre-eclampsia[9]
		Large vessel function[6]	
Low birth weight (<2500 g)	5	Not studied	11.3 (3.5 to 36.1) *v* > 3500 g[9]
			7.1 (2.6 to 18.7) *v* > 3500 g[10]
Preterm delivery (<37 weeks)	5-6	Not studied	1.8 (1.3 to 2.5) *v* term delivery[9]
			2.1 (1.2 to 3.5) *v* term delivery[11]

*Dependent on population studied, ethnic group, and diagnostic criteria.

Table 1. Complications of pregnancy and associated relative risk of maternal cardiovascular disease in later life (From [87])

Pathophysiology

The pathophysiology of pre-eclampsia remains unclear, although there is increasing evidence that abnormal endothelial function may be implicated. Of significance, risk factors associated with the development of cardiovascular disease in later life, such as dyslipidaemia, insulin resistance and obesity, are also strongly associated with pre-eclampsia. The obese individual often demonstrates many of these risk factors with a resultant increase in the risk of developing hypertensive complications of pregnancy. Sattar et al demonstrated a nine-fold increase in the risk for development of pre-eclampsia in primigravid women with

a body mass index (BMI) over 25kg/m2 (OR 9.3, 95%CI 2-48). [90] In another study of 5800 UK females, the elevated risk of pre-eclampsia associated with a BMI greater than 28.6kg/m2 was three-fold. (OR 3.02, p<0.0001) [91]

Insulin dependent diabetes and gestational diabetes are also believed to be strongly associated with development of hypertensive complications of pregnancy. In a large study of 4589 healthy nulliparous women in America, even within the normal range, the level of plasma glucose after a 50g oral glucose challenge was positively correlated with likelihood of pre-eclampsia. Among women with gestational diabetes the relative risk of all hypertensive disorders of pregnancy were significantly increased (relative risk 1.54, 95% CI 1.28-2.11) [92]. In another smaller study from Europe, pre-eclamptic women were found to have 37% lower insulin sensitivity than normal pregnant controls (p=0.009) with 70% higher free fatty acid concentrations (p=0.004). Also of interest, insulin sensitivity in pre-eclamptic women was still 26% lower than controls some three months post-partum (p=0.04) [93] and persistent insulin resistance was demonstrated in a seventeen-year follow up of women with pregnancy complicated by pre-eclampsia. [89]

Pre-eclampsia has been postulated to be an "inflammatory disorder" with endothelial cells in maternal systemic vessels becoming activated and damaged by circulating factors released from a poorly perfused and ischaemic placenta [83 94 95]. Therefore, it would seem likely that the classical clinical picture of the

hypertensive, proteinuric patient is an illustration of the endpoint rather than the cause of this complicated condition. In fact, many of the pathophysiological changes associated with pre-eclampsia are demonstrable long before the full clinical syndrome becomes evident.

Increased sensitivity of the maternal vasculature to pressor agents such as angiotensin II can be demonstrated before hypertension develops [61], although in recent studies this has not been confirmed [96]. Markers of activation of the coagulation cascade, such as reduced antithrombin [97], increased thrombin-antithrombin complexes (TATs) [98] and increased fibrin degradation products (FDPs) [99] can be demonstrated weeks to months before the development of the clinical disorder. Vasoconstriction can also be demonstrated before any clinical changes in systemic vascular resistance. Women destined to develop pre-eclampsia have reduced plasma volume but despite this finding, demonstrate increased plasma concentrations of atrial naturetic peptide (ANP) [100] and reduced rennin concentrations [101] weeks before pre-eclampsia is clinically evident.

Fasting triglyceride concentrations are doubled compared with normal pregnancy and a three-fold increase in VLDL and LDL-III concentrations are also observed well in advance of the clinical manifestations of the disease. [102] As described above, these changes predispose to endothelial damage and insulin resistance. It is also notable that the specific vascular lesion of pre-eclampsia 'acute atherosis

with lipid laden foam cells' as observed in the placental bed, is similar to that seen in arteriosclerosis in the non-pregnant [77] **(Figure 6).**

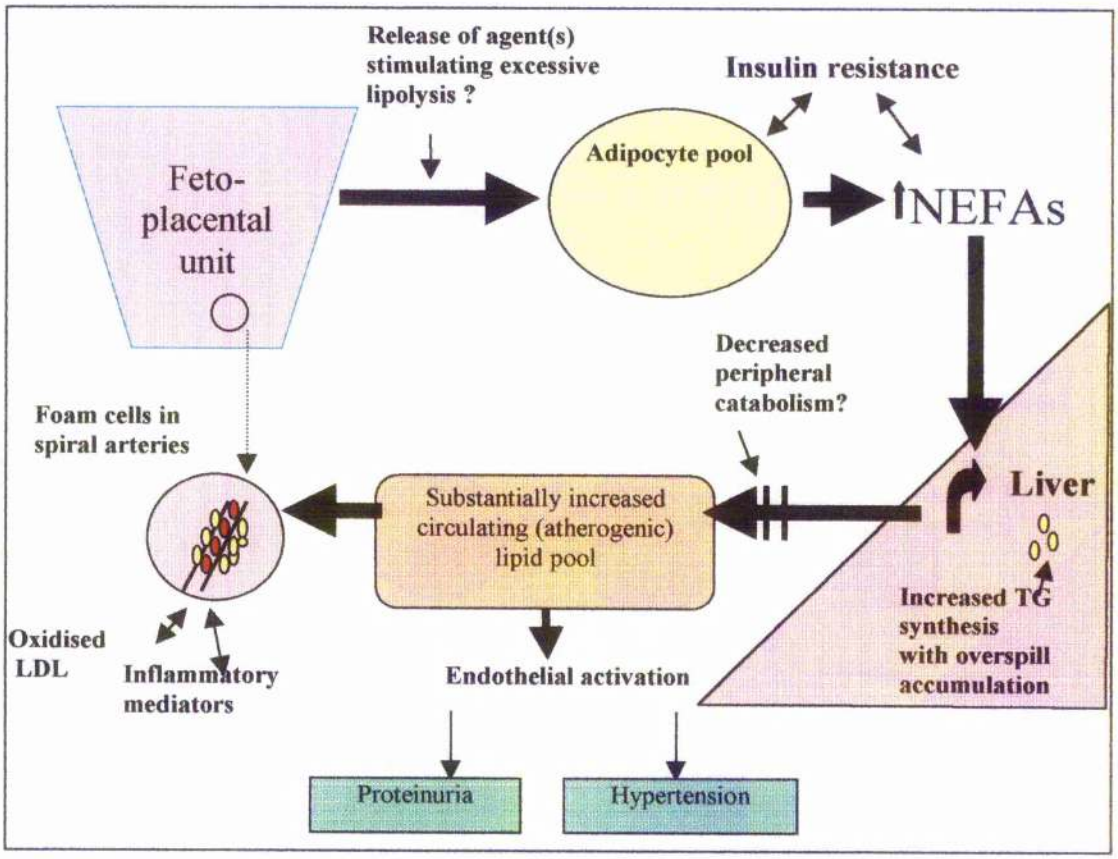


Figure 6: The potential role of a disturbance in lipid metabolism in the pathogenesis of pre-eclampsia. Placenta factor(s) enhances peripheral lipolysis which is already stimulated in normal pregnancy by HPL. This results in an increased flux of non-esterified fatty acids (NEFAs) to the liver. These are channelled predominantly into hepatic triglyceride synthesis with increased secretion (over and above normal pregnancy) of triglyceride-rich lipoproteins ($VLDL_1$). Accumulation of triglyceride occurs in the hepatocyte when this pathway is saturated. Increased concentrations of $VLDL_1$ in the circulation drives the production of an atherogenic lipoprotein profile by stimulating excessive synthesis of small, dense LDL (LDL-III) and by lowering HDL-cholesterol. This lipid profile may contribute to endothelial dysfunction and therefore the expression of pre-eclampsia in the mother. Finally, this pathway plays a part in the formation of lipid-laden macrophages (foam cells) in the spiral arteries of the decidua basalis, and as a result, may be involved in the enhanced placental production of pro-inflammatory mediators in PE. [77 102]

As discussed above, pre-eclampsia is believed to be a disease of the maternal inflammatory system and there is now substantial published evidence in this field. Granulocytes and monocytes are activated [103] [104] [105] [106], with increased release of pro-inflammatory cytokines such as TNFα, IL-6 and soluble phospholipase A2. [94] [107] An increase in serum concentrations of the cell adhesion molecules, VCAM-1 and ICAM-1, has been demonstrated in women with pre-eclampsia [108] [109] also suggesting a state of inflammation and endothelial activation. Further evidence of the link between inflammation and endothelial activation is provided by Greer et al. [110]. This group have shown that a significant correlation exists in patients with pre-eclampsia, between serum levels of IL-6 and VCAM-1 ($r=0.539$, $p<0.005$) suggesting, as is seen in cardiovascular disease, that the pro-inflammatory cytokine IL-6 may be pivotal in the pathophysiology of endothelial dysfunction and the development of pre-eclampsia.

As discussed earlier, more direct evidence of impaired endothelial function is difficult to obtain due to ethical issues of invasive testing in pregnancy. However, several ex-vivo techniques have supplied evidence of altered vascular reactivity in pre-eclampsia. Many groups, using various myographic techniques, have demonstrated reduced endothelial dependent vasodilatation in isolated subcutaneous resistance vessels from women with pre-eclampsia [73] [111] [74]. This effect was demonstrated in response to endothelial dependent vasodilators, acetylcholine and bradykinin or to shear force, also believed to be a stimulant of endothelial cell function. Hayman et al related lipid concentrations, specifically

lower apo-AI levels (the major lipoprotein in HDL particles), to impaired endothelial function in myometrial vessels bathed in plasma from pre-eclamptic pregnancies [112] again providing potential evidence of the detrimental effect of a circulating factor upon endothelial function. This effect was also demonstrated when isolated myometrial vessels from healthy pregnant women showed reduced endothelial dependent vasodilatation when incubated with plasma from pre-eclamptic women. [113]

Venous occlusion plethysmography provides a direct technique for vascular function assessment but is an invasive technique that is usually combined with intra-arterial infusion of potent vasoconstrictors. Anumba et al infused incremental doses of the nitric oxide synthase inhibitor, n-monomethyl-L-arginine (L-NMMA) and recorded vasoconstrictor responses in non-pregnant, pregnant and pre-eclamptic women. (Figure 1) Also, the same investigators employed an infusion of the endothelial dependent vasodilator, serotonin, to study these groups of women. These authors failed to demonstrate that reduced nitric oxide activity contributes to the increased vascular resistance in pre-eclampsia. [76]

2) Intra-Uterine Growth Restriction (IUGR)

Growth restriction in the neonate is unfortunately, more commonly identified after delivery than antenatally. This failure of antenatal detection is secondary to

inadequate effective screening tests. "Routine" ultrasound in the third trimester is usually arranged as a result of clinical concern or as a result of identifying a "high risk" pregnancy, that is an individual with a previous poor obstetric outcome or a complicated medical history.

Small for gestational age infants are defined as those with birth weights less than the 10th centile or two standard deviations below the mean for gestational age. However, the majority of this group are in fact normal healthy infants and do not have any particular increased perinatal morbidity or mortality. Also, growth charts for different ethnic groups must be considered, as the children of Asian parents cannot be assessed by western parameters. The classical, at-risk, growth restricted fetus or infant has a birth-weight under the fifth or even the third centile for gestational age, plus or minus abnormal umbilical artery Doppler flow velocity waveforms, including absent or reversed end-diastolic flow [114]. These babies have a perinatal morbidity rate in the range of about 40% [115] and may require premature delivery in order to pre-empt intra-uterine death [116].

The causes of growth restriction are varied and in general can be divided into two separate groups: intrinsic or symmetrical growth restriction and extrinsic or asymmetrical growth restriction. The symmetry in question is that between the growth of the fetal head and abdomen. Asymmetrical growth patterns involve "head sparing"; preferential shunting of blood to cerebral vasculature, and

impaired hepatic glycogen storage and this effect is usually secondary to "extrinsic" causes. Intrinsic causes of poor growth are usually fetal in origin, for example chromosomal abnormalities and congenital infections such as toxoplasmosis, rubella and cytomegalovirus. The outcome for these children is extremely poor in general. Extrinsic causes of poor fetal growth usually result from impaired placentation, either as a primary phenomenon or secondary to maternal medical conditions such as hypertension or social factors such as smoking [86].

The normal development of the placental vasculature relies on two distinct waves of trophoblastic invasion into the uterine spiral arterioles in the first and second trimesters of pregnancy. Failure of the secondary wave of trophoblast invasion into the myometrial segments of the spiral arterioles is observed in the placentae of growth restricted fetuses and in pregnancies complicated by pre-eclampsia [117] **(Figure7).**

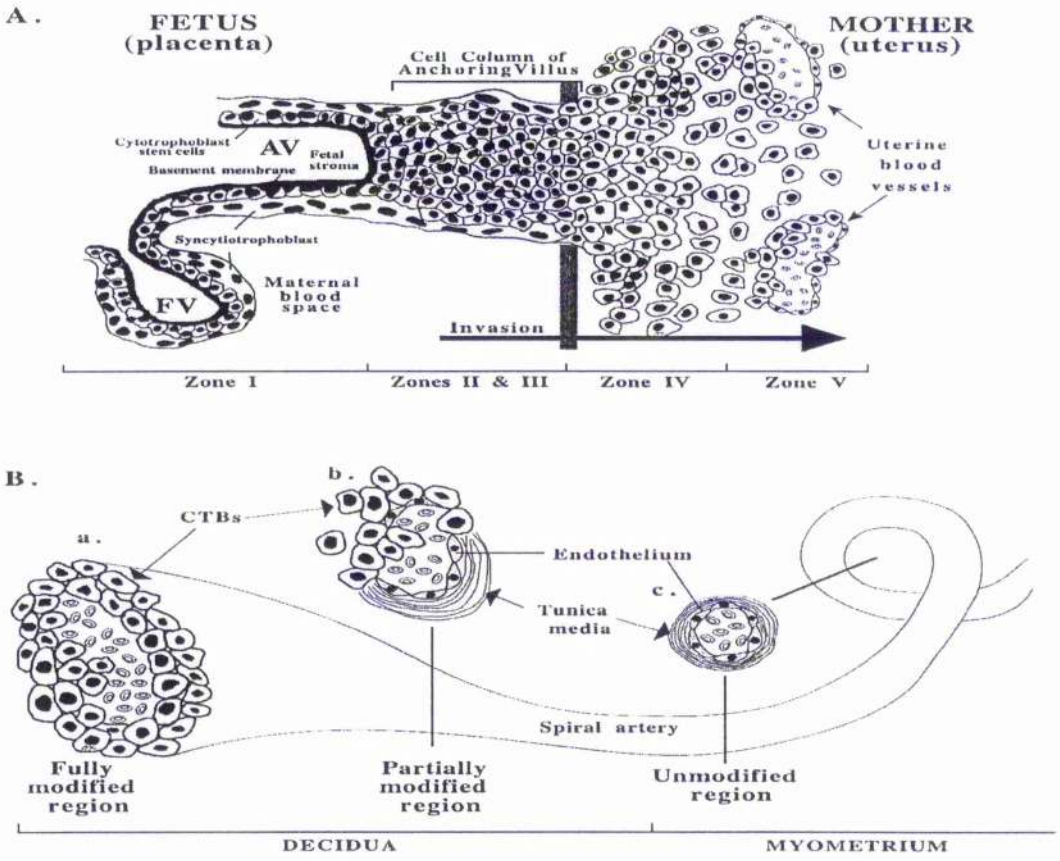


Figure 7. (A) Diagram of a longitudinal section of an anchoring chorionic villus (AV) at the fetal-maternal interface at (approximately) 10 wk gestational age. The anchoring villus (AV) functions as a bridge between fetal and maternal compartments, whereas floating villi (FV) are suspended in intervillus space and are bathed by maternal blood. CTB in AV (Zone I) form cell columns (Zones II & III). CTB then invade uterine interstitium (decidua and first third of the myometrium, (Zone IV) and maternal vasculature (Zone V), thereby anchoring the fetus to the mother and accessing maternal circulation. (B) Diagram of spiral artery in which endovascular invasion is in progress (10-18 wk gestation). Endometrial and then myometrial segments of spiral arteries are modified progressively. In fully modified regions (a) the vessel diameter is large. CTB are present in lumen and occupy entire surface of vessel wall. A discrete muscular layer (tunica media) is not evident. (b) Partially modified vessel segments. CTB and maternal endothelium occupy discrete regions of vessel wall. In areas of intersection, CTB appear to lie deep to endothelium and in contact with vessel wall. (c) Unmodified vessel segments in myometrium. Vessel segments in superficial third of myometrium will become modified when endovascular invasion reaches its fullest extent (by 22 wk), while deeper segments of same artery will retain their normal structure. (From [118])

This abnormal implantation can be detected by Doppler ultrasound assessment of the uterine artery blood flow, when a high resistance circulation and a "notched" appearance to the waveform would be detected. [119] This abnormal utero-placental circulation is believed to result in hypoxia and ischaemic damage of the placenta. However, what is poorly understood is why, with a similar placental abnormality, should some women be spared the systemic effects of widespread vascular damage and dysfunction manifest as hypertension and proteinuria. Little is known about maternal metabolism in association with pregnancy affected by intrauterine growth restriction. Maternal risk factors are similar to those associated with hypertensive disease in pregnancy, such as pre-existing hypertension, renal disease, and diabetes, that is maternal vascular disease. In fact, pre-eclampsia and IUGR often co-exist although IUGR can also occur in isolation.

Maternal metabolism associated with IUGR.

In 1985, Knopp et al demonstrated maternal ApoA-I (the principle apoprotein associated with HDL_2 subfraction) to correlate positively with birth weight while ApoA-II (associated with the smaller HDL_3 subfraction) correlated negatively with birth weight. [120] More recently, Sattar et al demonstrated that in women with IUGR there is a failure of the physiological hyperlipidaemia of pregnancy, in stark contrast to pre-eclampsia where, as discussed above, an exaggerated rise in lipids is observed. [121] In this study, no significant differences were found between triglyceride, HDL-C and $VLDL_1$-C between pregnant women with IUGR and

healthy controls. However women with pregnancies complicated by IUGR had significantly lower cholesterol LDL-C, VLDL-C and IDL-C concentrations ($p<0.01$). HDL-2 sub fractions stimulate and may affect fetal growth by controlling the provision of cholesterol via the placenta. [122] Another unifying hypothesis involves insulin resistance as this relates inversely to HDL metabolism. Support for this theory is demonstrated in a study by Breschi et al when in women with a BMI less than 25 kg/m^2, plasma insulin and insulin response to an oral glucose tolerance test were inversely related to birth weight ($p<0.02$). [123] Therefore as before, the metabolic and consequent vascular characteristics of the mother may influence fetal growth either indirectly through the initial development of the placental vascular architecture or directly by influencing the provision of fetal "building blocks". **(Table 2)**

Direct evidence

- Maternal obesity linked to increased CHD in adult offspring independently of birth weight [124]
- Maternal obesity increases risk for 2 diabetes in adult offspring [125]
- Maternal (but not paternal) obesity accounts for the association between birth weight and adult obesity [126]

Indirect evidence

Maternal obesity:

- Influences fatty acid compositions in maternal blood [127], and thus transfer to fetus. Ultimately, may influence insulin sensitivity in neonate
- A high saturated fat diet leads to endothelial dysfunction in rat offspring [128]
- Increases circulating total FFA concentrations, which correlate with diastolic blood pressure of adolescent offspring [129]
- Linked to increased inflammatory cytokine levels, which may adversely programme metabolic and neuroendocrine pathways in fetus [130]
- Linked to reduced fertility and increased miscarriage rates [131 132 133]

Maternal insulin resistance:

- Influences fetal body composition [134 135]
- Correlates with maternal blood pressure which in turn has been correlated with blood pressure in offspring [136 137]
- Could explain relationship between low birth weight and increased CHD risk in mothers [88 138], since the metabolic syndrome is a key factor governing CHD in women
- Is enhanced by smoking, which is linked to low birth weights
- Reduces fertility and increases miscarriage rates [133]

Exercise

- Improves fertility and reduces miscarriage rates [133]
- Increased birth weight in trial of exercise in pregnant women [139]
- Sedentary lifestyle associated with increased risk for low birth weight babies relative to women who exercise at moderate levels [140]
- Reduces rates of gestational diabetes [141]

Table 2. Direct and indirect evidence supporting a pivotal role for maternal obesity and insulin resistance in fetal programming of vascular and metabolic disease

AIMS

As discussed above, there are many common risk factors for atherosclerotic coronary heart disease and pre-eclampsia. These factors are believed to exert their effect via various mechanisms resulting in the ultimate end-point of endothelial activation and dysfunction. Our aim is to take advantage of recent advances in non-invasive micro-vascular assessment, using laser Doppler technology in order to attempt to demonstrate vascular dysfunction within these risk groups and potentially, to develop our understanding of the pathophysiological mechanism linking risk factors and vascular complications of pregnancy. Therefore we will measure basal and stimulated endothelial function in vivo:

- in healthy lean and obese pregnant individuals
- in women with vascular complications of pregnancy such as pre-eclampsia and intra-uterine growth restriction.
- in pregnant women with a pre-existing medical disorder (type 1 diabetes).
- We also will endeavour to assess the effects of pregnancy itself on endothelial function both in the short term, i.e. 6-12 months post-partum and in the long term, i.e. greater than 15 years post partum.

Forearm skin perfusion is assessed under basal conditions and following multiple doses of iontophoretically applied 1% acetylcholine (Ach, endothelium-

dependent) and 1% sodium nitroprusside (SNP, endothelium-independent) using the laser Doppler imager (LDI). As discussed, most studies concerning endothelial function in normal and complicated pregnancy have utilised indirect or ex-vivo methods, and pre-existing in-vivo techniques are invasive and have produced conflicting results. Therefore, the development of this technique will represent a significant advance in this area. Additionally, we plan to consider endothelial function in relation to circulating parameters such as cytokines and lipoproteins which have been shown to be perturbed in pre-eclampsia and which may contribute to endothelial damage.

The important feature of this proposal is the application of a novel in-vivo technique to a new area. If this technique allows detection of quantifiable differences in endothelial function in pre-eclamptic women, then its non-invasive and direct nature offer the potential to measure vascular function at differing gestations. This flexibility may facilitate better identification of factors associated with or responsible for endothelial dysfunction, and as a result lead to a clearer understanding of the pathogenesis of pre-eclampsia. It may also present, in the longer term, a suitable means of early identification of women at a high risk of developing pre-eclampsia, thereby further enabling better research into potential therapeutic options.

Chapter Two

METHODS

Laser Doppler perfusion imaging in conjunction with iontophoresis of vasoactive solutions for the assessment of microvascular function in-vivo.

Factors critical to iontophoretic assessment of vascular reactivity: Variability in drug delivery.

Introduction

Increasing evidence relates impaired endothelial vasomotor function to coronary heart disease [7]. Endothelial dysfunction is not limited to the coronary circulation but is also detected in the peripheral circulation proportionate to the degree of endothelial dysfunction occurring in the coronary arteries [142]. Furthermore, assessment of endothelial vasomotion in these peripheral arteries has been shown to correlate with and relate directly to coronary dysfunction [143]. Thus, there is considerable interest in non-invasive methods for assessing peripheral vascular function. This is particularly relevant in the pregnant woman, with regards to the investigation of vascular reactivity in pre-eclampsia, a condition also suggested to arise secondary to microvascular dysfunction. Iontophoresis for transdermal delivery of the vasodilator agents acetylcholine (ACh) and sodium nitroprusside (SNP) can be used for this purpose. The technique is based upon the fact that a charged molecule migrates across the skin under the influence of an applied electrical field and ionised drug delivery is dependent on the magnitude of the applied current and its duration (current × time = charge, in Coulombs). In the past iontophoresis has been used in conjunction with laser Doppler flowmetry (LDF), a non-invasive method for assessing microvascular perfusion at a single point [144]. More recently, iontophoresis has been combined with laser Doppler imaging (LDI) which reduces measurement variability [145 146] because, unlike LDF, LDI measures perfusion across many points [147] and thus an average measure of perfusion can be computed for any chosen area. Iontophoresis of ACh tests endothelial function since binding to muscarinic receptors with subsequent

generation of NO requires intact endothelial cells and is therefore said to be "endothelium-dependent". Vasodilatation is ultimately mediated by action of NO on vascular smooth muscle (via the cGMP pathway) and so iontophoresis of SNP, an NO donor, is used as an "endothelium-independent" control. This methodology has been widely used to investigate microvascular function in a variety of disease states, most commonly diabetes mellitus where endothelial dysfunction has been implied by a decreased response to ACh iontophoresis [36 148 149].

Iontophoresis has a number of attractions. It provides a direct assessment of microvascular function, is simple to use, and most importantly for clinical application, it is non-invasive. However, there are some important factors which can influence iontophoretic drug responses and its interpretation which in the past have not been addressed. One of these is the assumption that drug delivery is solely influenced by the magnitude of current applied and its duration (charge). This ignores the fact that the electrical properties of skin differ between subjects and this could impact on effective drug delivery. By monitoring the voltage across the iontophoresis chambers we investigated for the first time whether variation of the electrical properties of skin between normal subjects influences the magnitude of the responses to ACh and SNP. The results of these investigations have critical implications for future clinical studies of endothelial function that employ iontophoresis of vasoactive drugs.

Methods

Experiments were performed in twenty healthy subjects aged 22-50 years of both sexes with no history of peripheral vascular abnormalities such as Raynauds syndrome, dermatological diseases or systemic disease processes such as diabetes mellitus and all were non-smokers. All subjects were fasted overnight and asked to refrain from drinking any fluids except water prior to measurements. These were undertaken in a temperature controlled room (23 ± 1°C) and all subjects were allowed to acclimatize for 30 minutes prior to measurement. The study was performed according to the Declaration of Helsinki, the institutional ethics committee approved procedures and informed consent was obtained.

Iontophoresis

Drug delivery was achieved using a battery-powered constant current iontophoresis controller (MIC-1e; Moor Instruments Ltd, Axminster, UK). The chambers used for iontophoresis (ION 6; Moor Instruments Ltd) were constructed of Perspex (internal diameter 22mm; area 3.8cm^2) with an internal platinum wire electrode. Two chambers were attached to the skin of the volar aspect of the forearm by means of doubled-sided adhesive disks avoiding hair, broken skin and superficial veins. The chambers were connected to the anode and cathode connections on the iontophoresis controller. A digital multimeter was connected in parallel to monitor voltage across the chambers (**Fig 1 and 2**). As a constant current source was used, resistance values were calculated from the recorded voltages using Ohm's Law.

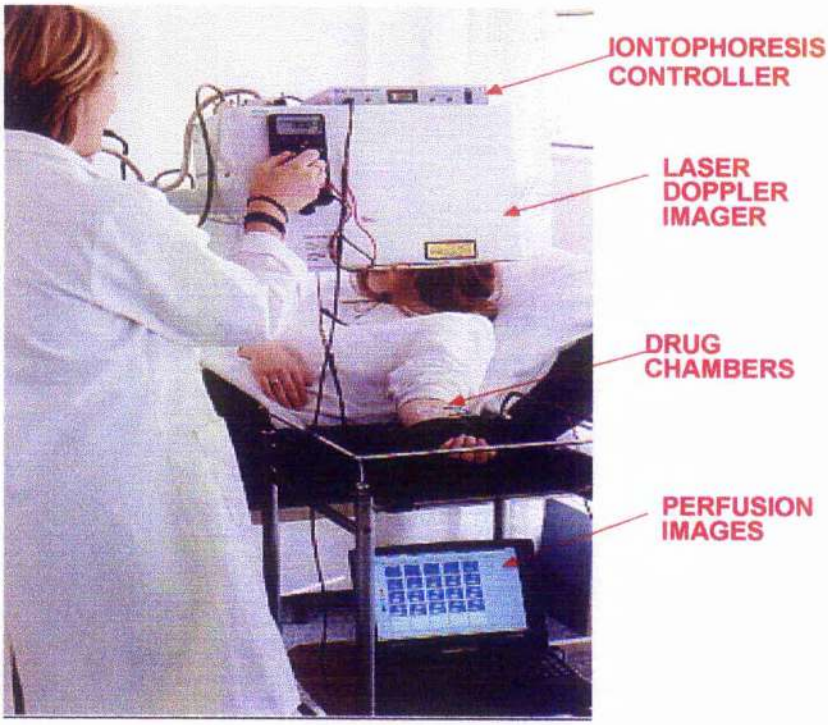


Figure 1: Laser Doppler Imager and iontophoresis set-up.

Control of current delivery was programmed into the software for the laser Doppler imager such that current was switched on at the beginning of a scan and remained on throughout the scan until the start of the following scan. The current was then either left on for the next scan or was switched off once the total charge had been delivered. Current duration was determined by the time taken to complete each scan (50 seconds) multiplied by the total number of scans programmed. To limit the iontophoresis dose, resulting from relatively long scan times, low currents were used: the protocol involved incremental current delivery with four scans at 5μA, four at 10μA, four at 15μA and two at 20μA giving a total charge of 8mC (**fig 3A**). Each frame is associated with the current delivery during

that scan, although the resulting vascular response could be delayed due to the time required for chemical factors to initiate it.

2.5 ml of 1% acetylcholine chloride (Sigma) was introduced into the anodal chamber whilst 2.5ml of 1% sodium nitroprusside (Sigma) was placed in the cathodal chamber. Thus both agents were delivered simultaneously during each period of current administration. The vehicle for these drugs was 0.5% NaCl. Fluid was prevented from escaping by placing circular 32mm coverslips over the chambers.

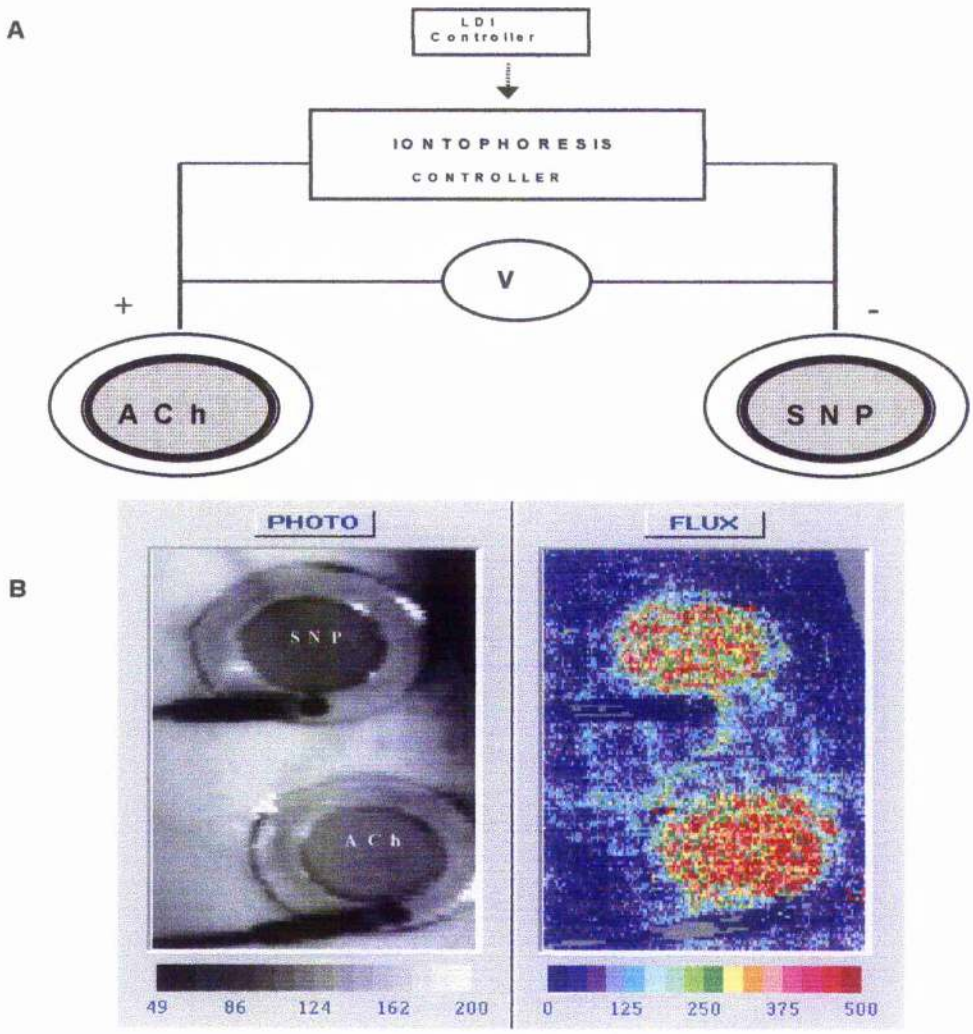


Fig 2. A: Diagram of the experimental arrangement. Acetylcholine (ACh) was placed in the anodal chamber and sodium nitroprusside (SNP) in the cathodal chamber. A digital voltmeter (V) was connected between these.

B: The backscattered light intensity provides the Photo image with the Doppler shifted component providing the Flux image. This scan was taken at the conclusion of the administration of current. Flux is colour-coded with lowest perfusion dark blue (0 PU) and highest in dark red (500 PU).

Perfusion measurements

Non-invasive measurement of skin perfusion was performed by means of a laser Doppler imager (Moor Instruments Ltd, Axminster, UK) equipped with a red laser (wavelength 633nm; power 1mW; beam diameter 1mm). The technique is based upon the Doppler shift imparted by moving blood cells in the underlying tissue to the backscattered light. The laser is scanned in a raster fashion over both chambers and through the coverslips. The backscattered light is collected by photodetectors and converted into a signal proportional to perfusion in arbitrary perfusion (flux) units (PU) that is displayed as a colour-coded image on a monitor (**fig 2B**). Perfusion measurements were obtained using the imager manufacturer's image analysis software by outlining a region of interest (ROI) around the internal circumference of the chamber. Statistical analysis of the ROI was subsequently performed offline to yield the median flux value across approximately 700 measurement points. Twenty repetitive scans were taken, the first being a control (pre current administration) followed by the incremental current protocol described above (14 scans) followed by five further scans with no current administration. The biological zero was measured by taking a single scan after occluding the arterial blood supply with a sphyngomanometer cuff. This was found to be consistent between and within subjects with a mean (± SEM) of 23.9 ± 0.72 PU.

To determine whether the combination of fluid in the iontophoresis chambers and the coverslip affect the flux signal, scans were obtained comparing basal

perfusion in the anodal chamber (containing ACh) and in the cathodal chamber (containing SNP) to that occurring in a surrounding area of skin. The latter showed significantly ($P = 0.011$, 1-way ANOVA; $n = 14$) higher basal perfusion (71.6 ± 6.4 PU; mean ± SEM) compared to the values at the ACh-containing chamber (54.4 ± 2.7 PU) and the SNP-containing chamber (53.9 ± 3.7 PU), indicating that the chambers produce some attenuation of the LDI signal, but there was no difference between chambers. Although some signal attenuation occurs, perfusion changes in response to intervention are unaffected. Following occlusion of the blood supply to the arm, the ratio of the increase in perfusion from biological zero showed no significant differences between the anodal and cathodal chambers and adjacent skin (1.93 ± 0.09, 2.23 ± 0.15 and 2.18 ± 0.16 respectively; $P = 0.26$).

Drugs

In separate experiments, seven subjects were administered 600mg aspirin orally half an hour prior to iontophoresis in order to inhibit prostaglandin synthesis. This has been shown to be an adequate period of time to produce maximal inhibition of endothelium-derived prostacyclin [150]. Aspirin was dissolved in orange juice to disguise its taste, which allowed use of plain orange juice as a placebo. Measurements with placebo or aspirin were performed on one occasion each, separated by a minimum of 14 days. The investigators were aware of the status of the subjects, ie not blinded.

A

B

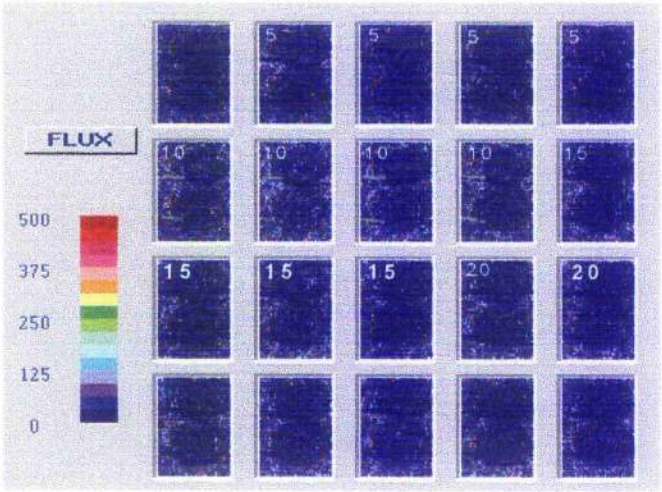


Fig 3.A: Scans taken with SNP in the upper chamber and ACh in the lower chamber. The differences in the time courses of the drug-induced vasodilator responses are clear. The numbers in each frame refer to the applied current (in μA).

B: Repetitive scans in a single subject with 0.5% NaCl vehicle. Each scan duration was 50s with no delay between scans.

Statistical analyses

Measurement of responses was performed using raw values but in some cases an assessment of the overall response to drugs was obtained by taking the area under the perfusion.time curve. For resistance data, resistance.time integrals were computed. Comparisons were by ANOVA or Student t-tests, paired or unpaired as appropriate. All tests were two-tailed and data are expressed as means ± SEM or ± SD. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, $\log_{10}$ transformation of the data was performed to equalize the variances and thereby permit parametric data analysis. The variance ratio, comparing maximal drug responses to the control (pre current application) scan, was found to vary between 9-60 for the raw perfusion data but after log transformation the same variance ratio range was reduced to 0.7-1.2.

Results

Responses to ACh and SNP

Iontophoresis of ACh and SNP resulted in progressive increase in perfusion, although with differing timecourses (**fig 3A**). ACh showed a more rapid onset than SNP but also a rapid decline once current was terminated. This rapid fall in the ACh response may be due to the presence of acetylcholinesterase in human blood vessels [151]. Current administration with only vehicle present failed to elicit vascular responses (**fig 3B**). The time course of the responses to both drugs for seven subjects is shown **fig 4A**. The drug responses differed significantly

($P<0.001$; 2-way ANOVA) but it is clear that the current protocol used did not elicit hyperaemic responses with only the vehicle present. With responses expressed relative to cumulative charge, both ACh and SNP reached a plateau by the time about half the charge was delivered (**fig 4B**).

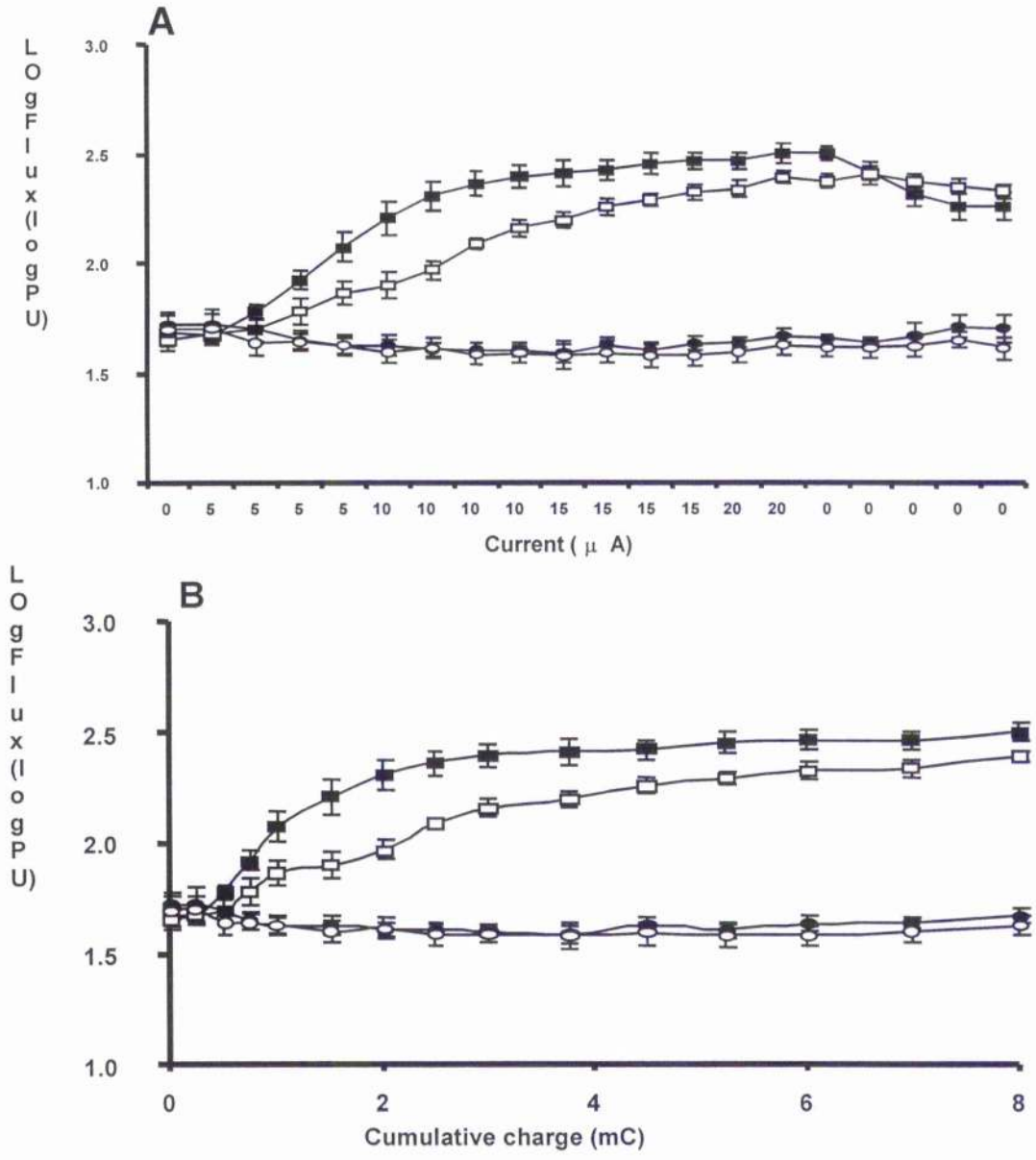


Fig 4.A: Time course of the response to iontophoretic administration of ACh (v) and SNP (□) dissolved in 0.5% NaCl as well as the response to this vehicle at the anode (v) and cathode (□). Dose dependent increase in perfusion is significant ($P<0.0001$, 1-way ANOVA) for drugs but not for vehicle. Mean ± SEM; n = 7 subjects.

Fig 4 B: Same perfusion data as in A, but plotted against cumulative charge.

A striking observation is that drug delivery is not only influenced by the applied charge but also by the electrical resistance of the skin which varied in different subjects **(fig 5).**

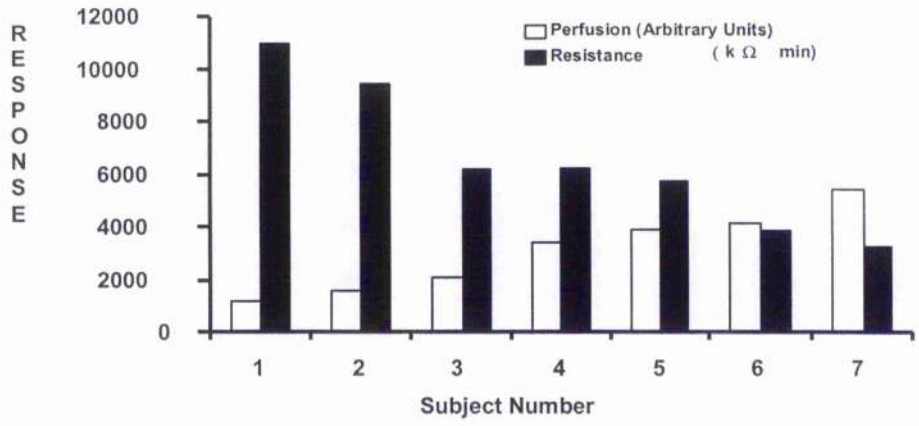


Fig 5. Relationship between perfusion.time integrals and the resistance.time integrals for the response to ACh in a series of subjects. The inverse nature of the relationship is clear.

There is an inverse linear relationship between the perfusion integral and the resistance integral for both ACh (**fig 6A**; $r = -0.86$) and SNP (**fig 6B**; $r = -0.96$) i.e. higher resistance is associated with smaller vasodilator responses for both drugs and *vice versa*. These relationships were significant, $P < 0.0001$ in both cases. Correcting for this variable, by dividing by the integral of conductance (the reciprocal of resistance) over time or more simply by multiplying individual perfusion values by the integral of resistance over time, normalises responses.

Correlating these corrected perfusion integrals to their respective resistance integrals now yields non-significant r^2 values of 0.011 for ACh and 0.025 for SNP (**fig 6C, D**), indicating the effectiveness of this correction and lowering apparent inter-subject variability. The coefficient of variation for the log perfusion integral of ACh was 3.36% ($n = 7$) prior to correction for resistance and 1.86% thereafter. The respective coefficients for SNP were 2.18% and 1.56% respectively. These corrections reduce variability between subjects by about a half and a third respectively. The between-day coefficient of variation of the ACh response for the same four subjects described in methods after correction for resistance was 3.04 ± 0.67% (mean ± SD), which was about half of the uncorrected value.

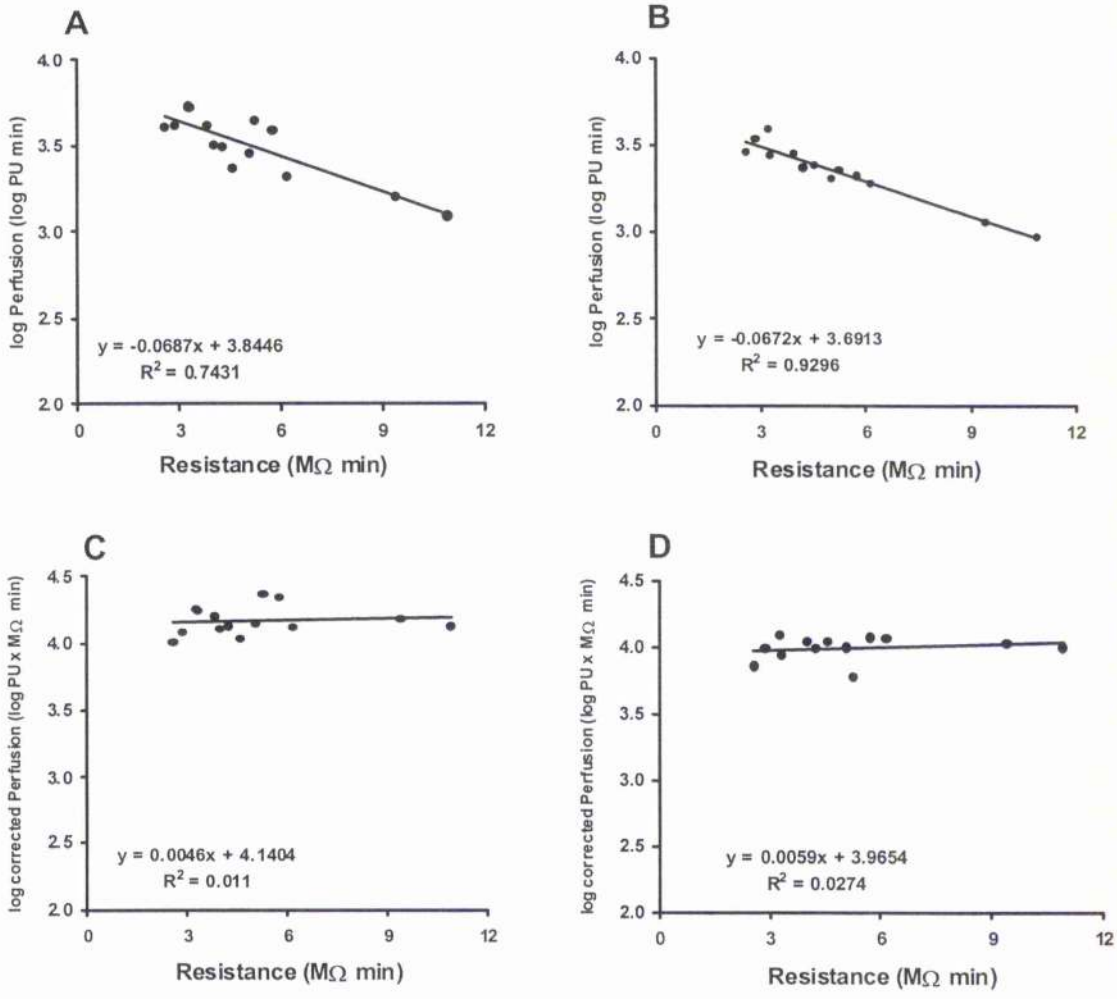


Fig 6 **A:** Relationship between the log transformed perfusion.time integral and the resistance.time integral for ACh.

B: Relationship between the log transformed perfusion.time integral and the resistance.time integral for SNP. $n = 14$ and $P<0.0001$ for both A and B.

C: Correction of log transformed perfusion.time integral (multiplying flux by the resistance.time integral) for ACh and plotting against the resistance.time integral no longer yields a significant correlation.

D: Similarly, the corrected data for SNP no longer shows a correlation.

Influence of prostaglandins on cutaneous vascular responses

The vascular responses to both ACh and SNP appeared to be significantly depressed after administration of aspirin (P<0.0001; 2-way ANOVA; **fig 7 A & B**). However, it was found that resistance during ACh and SNP administration was significantly greater ($P = 0.03$; Student paired t test, $n = 7$; means ± SD) after aspirin (6.25 ± 2.08 MΩ min) than with placebo administration (4.09 ± 1.04 MΩ min). After correcting for resistance, there was no longer any significant difference between the responses for either ACh or SNP ($P = 0.64$ and $P = 0.75$ respectively, 2-way ANOVA; **fig 7 C & D**). Measurement of drug responses in four subjects on two separate days but without aspirin administration showed no significant differences in resistance between days (4.32 ± 0.94 and 4.74 ± 0.6 MΩ min).

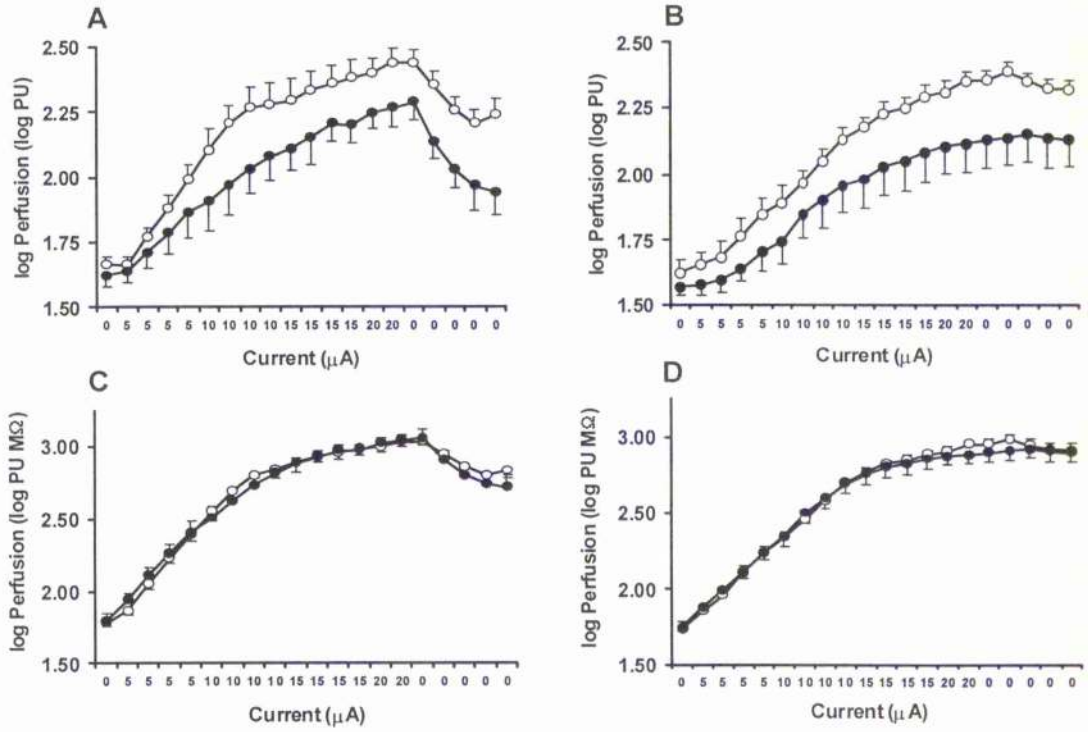


Fig 7. Vascular responses to administration of ACh (**A**) and SNP (**B**) in 0.5% NaCl vehicle before (open symbols) and following (closed symbols) administration of aspirin. For both ACh and SNP the responses before and after administration of aspirin differ significantly ($P<0.0001$, 2-way ANOVA). Vascular response to administration of ACh (**C**) and SNP (**D**) before (open symbols) and following (closed symbols) administration of aspirin after correcting flux values for resistance in each subject. Mean ± SEM; $n = 7$ subjects.

Discussion

There is mounting interest in the measurement of vascular function in cardiovascular research since endothelial dysfunction may be an early feature of the atherogenic process and is amenable to therapeutic intervention. It can be argued that endothelial function integrates the stress of other risk factors. These observations may be extrapolated to cardiovascular complications of pregnancy

such as pre-eclampsia. Laser Doppler imaging with iontophoresis is the most recent technique used for assessment of endothelial function and is particularly attractive because of its non-invasive nature. Although iontophoresis only assesses the cutaneous microcirculation, this is effectively a robust surrogate marker of vascular function in other vascular beds. Reduced responsiveness to iontophoretic administration of ACh has been observed in diabetes [36 148 149] and hypercholesterolaemia [152], and in both these conditions there is a parallel reduction of the ACh response in the forearm circulation (predominantly a skeletal muscle vascular bed) assessed by venous occlusion plethysmography [153 154]. Moreover, attenuated response in the skin of heart transplant patients to ACh iontophoresis [155] is paralleled by reduced responsiveness of coronary blood vessels to ACh in this group [156]. In addition, ACh-induced vasodilatation is reduced in both the forearm musculature [40] and skin [157] in patients with essential hypertension. Thus, many conditions affecting the cardiovascular system appear to result in global endothelial dysfunction and therefore assessment of the cutaneous microcirculation yields valuable insights into peripheral vascular function.

The current protocol used in the present study resulted in a overall lower charge (8mC) than that used in previous studies [145 146] and this, combined with a large surface area for iontophoresis and the use of a weak saline vehicle prevented the development of hyperaemic artefacts at the cathode. This obviated the need to use a topical anaesthetic such as EMLA® cream [145 146] which produces cutaneous

vasoconstriction [158], presenting a problem as any vasodilator responses would then be superimposed upon a basal vasoconstrictor tone. In preliminary experiments we have observed that iontophoresis of ACh and SNP to EMLA-treated skin elicited substantially reduced vasodilator responses (by 30-50%, unpublished observations) using the protocol employed in the present investigation.

Past work aimed at establishing the role of prostaglandins in mediating the response to ACh has produced contradictory results. It was previously observed that the vasodilator response to iontophoresis of ACh was reduced by administration of oral [159] or intravenous aspirin [160]. However, Morris & Shore [145] found no difference in the ACh response after oral aspirin. Our finding of an apparent difference in the vascular responses to both ACh and SNP after oral aspirin suggests a possible explanation for discrepancies between these earlier studies. After correction for resistance, there was no longer any difference in the ACh and SNP responses and this may indicate that variations in resistance across the iontophoresis circuit during drug administration could have been a confounding factor in previous studies. Aspirin itself could have changed skin resistance, and the time control experiment supports this as no change in resistance was found when measured on separate days but without aspirin administration. The change in resistance could not be ascribed to variations in room temperature, as all measurements were undertaken in a temperature-controlled room and subjects allowed to acclimatize. It is therefore possible that

in previous studies, variations in skin resistance might have influenced effective drug delivery leading to differing results. This emphasizes the importance of correcting for resistance to avoid potentially spurious results, particularly if measurements are taken at different sites or on different days.

The variation in calculated resistance between subjects suggests that there may be variable numbers of resistance pathways in the skin of different subjects. As the equipment and the composition of the solutions used was the same between subjects, the site of the resistance must be related to the skin. It might be the case that lower resistance is associated with a greater number of low resistance pathways, such as sweat ducts or hair follicles, being available and these pathways are close to blood vessels. This is quite likely given that sweat glands and their associated ducts, as well as hair follicles, are known to be richly vascularized [161]. Higher resistance may be associated with high resistance pathways for ion flow, mostly likely through the stratum corneum, and these are more remote from blood vessels. Thus, *effective* drug delivery could differ even though the charge remains constant. In effect, although the same total amount of drug may be delivered, the vascular response could differ depending on the relative number of low resistance pathways available. The inverse relationship between drug response and resistance, with calculated resistance integrals showing on average over twofold variation between subjects, indicates that skin resistance may contribute to inter-subject variability of drug responses. This has important implications for interpreting responses to drug administration in clinical

studies. This factor has not been taken into account in any previous study using iontophoresis and variations in observed responses between subject groups could have been influenced by a systematic variation in resistance rather than a true difference in vascular reactivity. This said, it remains likely that diabetic patients do have vascular dysfunction as most studies using venous occlusion plethysmography and intra-arterial drug administration show a reduced response to ACh compared to control subjects [153 162 163]. However, observed differences to iontophoresis of drugs at different sites could be explained by variations in skin resistance. Reduced vascular responses have been observed in the skin over the dorsum of the foot compared to the forearm for both normal [149 164] and diabetic subjects [165]. Similarly, marked differences in response to iontophoretic administration of ACh occurs at various sites on the hand and forearm in normal subjects [166], assessed by using laser Doppler flowmetry. Even when LDI is used, between-site variation in the magnitude of responses occur, although variability can be reduced by ensuring that consecutive measurements are taken from the same site [146]. This reinforces the need to monitor voltage during iontophoresis so that resistance can be estimated. Only by doing this is it possible to accurately assess the extent to which effective drug delivery is affected by skin resistance and to correct for this variable.

In conclusion therefore, these investigations have shown for the first time that resistance is an important but previously unrecognised variable influencing iontophoresis. The inverse relationship between skin resistance and blood flow

responses to both ACh and SNP indicates that resistance influences effective drug delivery. We anticipate that correction for resistance, coupled with use of appropriate vehicles, chambers and iontophoresis protocols, will lead to significant improvement of the iontophoresis technique and permit its additional development, thereby further increasing its robust nature as a non-invasive tool for assessment of endothelial function in clinical studies.

Chapter Three

METHODS

Laser Doppler perfusion imaging in conjunction with iontophoresis of vasoactive solutions for the assessment of microvascular function in-vivo.

Factors critical to iontophoretic assessment of vascular reactivity: Elimination of electrically-induced artefacts.

Introduction

The technique of iontophoresis is based upon the fact that a charged molecule migrates across the skin under the influence of an applied electrical field and ionised drug delivery is dependent on the magnitude of the applied current and its duration (current × time = charge, in Coulombs). Iontophoresis of acetylcholine (ACh) takes place at the anode as this compound is positively charged in solution. Therefore the "like" charge repels the ions and enables their absorption into the skin. Likewise for sodium nitroprusside (SNP), this compound is delivered at the cathode and negatively charged in solution. However, application of electrical charge at the cathode with only the vehicle for SNP present (distilled H_2O) can produce an electrically induced hyperaemic response [167] [168] sometimes referred to as a "galvanic" response. This artefact was also observed by Grossmann *et al* [169] at both the anode and cathode in response to iontophoresis of a variety of cations and anions respectively, although the control experiment of applying the same current protocol with only the vehicle (propylene glycol) present was not performed.

Mechanisms underlying electrically-induced hyperaemia are still poorly understood. Prostaglandins have been implicated, as aspirin, a prostaglandin synthase inhibitor, can produce inhibition although only at the cathode [170]. Whatever the mediators, there are a number of factors which may influence the development of this artefact such as pH changes at the electrodes, skin resistance, the nature of the solution in the chamber and the chamber size itself. The aim of

this investigation was to examine conditions under which electrically-induced hyperaemia is generated and whether it can be eliminated. We hypothesized that the electrical properties of the solution used for delivery of the drugs might influence the development of such hyperaemia, which could be minimised by changing the composition of the drug vehicle. Sub-hypotheses included whether factors such as chamber size, pH changes at the electrodes and skin thickness might influence the development of these artefacts. The contribution of prostaglandins to these responses was also investigated. The results of this investigation have implications for future clinical studies of endothelial function that employ iontophoresis of vasoactive drugs.

Materials and Methods

Experiments were performed in twenty healthy subjects aged 22-50 years of both sexes with no history of peripheral vascular abnormalities such as Raynauds syndrome, dermatological diseases or systemic disease processes such as diabetes mellitus. The institutional ethics committee approved all procedures and informed consent was obtained. Subjects were fasted prior to measurement and were either life-long non-smokers or had no recent history (within 1 year) of smoking. Measurements were performed with the subjects supine in a quiet room with an ambient temperature of $22 \pm 1^{\circ}C$.

Iontophoresis protocols

Drug delivery was achieved as described in chapter one, using a battery-powered constant current iontophoresis controller (MIC-1e; Moor Instruments Ltd, Axminster, UK). To assess the effect on hyperaemic responses of chamber size, two chambers with internal diameters of 22mm, area 3.8cm^2 and 10mm, area 0.78cm^2 were used for iontophoresis. Chambers were applied as previously described in chapter one. Skin temperature close to the iontophoresis chambers was monitored continuously throughout.

2.5 ml of 1% acetylcholine chloride (Sigma) was introduced into the anodal chamber whilst 2.5ml of 1% sodium nitroprusside (Sigma) was placed in the cathodal chamber. Thus both agents were delivered simultaneously during each period of current administration. The vehicle for these drugs was either distilled H_2O or saline solutions. These solutions were prevented from escaping from the chambers by placing circular 32mm coverslips over the chambers. In four subjects the electrical resistance of the skin within the chamber was increased by applying Vaseline thinly over the skin surface and then current was applied with 0.5% NaCl alone in the chambers.

Current delivery was achieved as described previously. Two protocols were used in the present experiments:

(i) An incremental protocol involving stepped current delivery with four scans at 5μA, four at 10μA, four at 15μA and two at 20μA giving a total charge of 8mC over 11.7min. This was intended to minimise electrically-induced hyperaemic artefacts with the relatively large surface area and low currents yielding a cumulative charge density of only 2.1mC/cm^2.

(ii) A 'galvanic' protocol, consisting of three consecutive periods of current at 100μA giving a total charge of 15mC over 2.5min, was employed with the specific purpose of eliciting electrically-induced cutaneous hyperaemia as this gives a cumulative charge density of 3.95mC/cm^2, almost double that in the incremental protocol.

Vehicles

The drug vehicles used were either distilled water, or 0.5%, 1% or 5.8% saline solutions (5.8% = 1M NaCl). The numbers of subjects in whom these were used are given in the text and figure legends.

Perfusion measurements

Non-invasive measurement of skin perfusion was performed as described in chapter one. Twenty repetitive scans were taken, the first being a control (pre current administration) followed by the incremental current protocol described above (14 scans) followed by five further scans with no current administration. For the 'galvanic' protocol, after a control scan three scans were administered

during which the 100μA current was passed followed by a further 16 scans without current.

Drugs

In separate experiments, seven subjects were administered 600mg aspirin orally half an hour prior to iontophoresis in order to inhibit prostaglandin synthesis. This has been shown to be an adequate period of time to produce maximal inhibition of endothelium-derived prostacyclin [150]. Aspirin was dissolved in orange juice to disguise its taste, which allowed use of plain (aspirin-free) orange juice as a placebo. Using the 'galvanic' protocol, measurements were performed sequentially, one on each arm, before and after aspirin in a single blind fashion, with the two measurements being separated by less than one hour. The investigators were aware of the status of the subjects, ie not blinded.

pH measurements

As it has been shown that pH changes can occur at sites of iontophoretic current administration [171], in five subjects the fluid from each chamber was removed after the incremental iontophoresis protocol and its pH measured. This was preceded by measurement of the pH of fluid left in the chambers for the same duration as the iontophoresis protocol, but without applying any current.

Skin thickness measurement

It is well known that skin thickness is quite variable between subjects, but whether this influences iontophoresis of drugs or the apparent electrical properties of skin is unknown. We used callipers (Kroeplin Oditest OD 1020T10, Germany; resolution 0.01mm) to measure skin thickness at the anodal site in a series of subjects prior to iontophoresis.

Statistical analyses

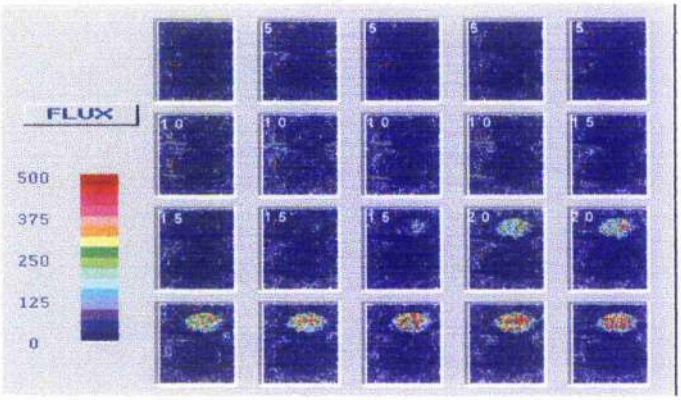
Statistical analyses, variance ratios and coefficients of variation are described in chapter one

Results

Electrically-induced hyperaemia

Using the distilled H_2O vehicle alone, it was apparent that in about 75% of subjects a hyperaemic response developed at the cathode during incremental current administration and gradually increased (**fig 1A**). This response clearly overlaps with the response to iontophoresis of SNP that typically tends to plateau towards the end of the scan period (**fig 1B**). In general, the electrically-induced artefact was less of a problem with respect to the ACh response as this response was only observed at the anode in one out of twenty subjects using this current protocol. No significant temperature change occurred between the beginning and end of perfusion measurements, indicating stable conditions.

A



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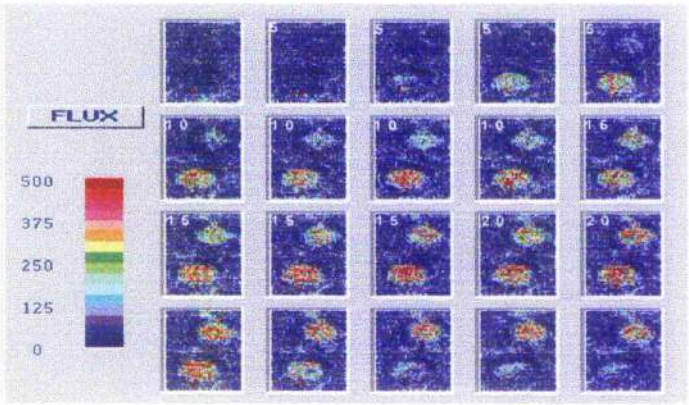

Fig 1.A: Repetitive scans in a single subject taken using the incremental current protocol (see Methods) with distilled H_2O vehicle alone. Scan duration was 50s with no delay between scans. Electrically-induced hyperaemia develops at the cathode towards the end of the period of current administration.

Fig 1.B: Scans taken from the same subject using the incremental current protocol with SNP in the upper chamber and ACh in the lower chamber. The differences in the time courses of the vasodilator responses to ACh and SNP are clear. The numbers in each frame refer to the applied current (in μA).

0.5% saline vehicle

Use of distilled water as the vehicle resulted in higher voltages than when using the 0.5% saline vehicle (**fig 2**). However, comparison of the perfusion integrals indicated that there was no significant difference for either the ACh or SNP responses irrespective of whether these drugs were dissolved in water or 0.5% NaCl vehicles (**fig 3**). Furthermore, the time course and magnitude of the responses to ACh and SNP were the same irrespective of the vehicle used (**fig 4A cf 4B**) but the development of electrically-induced hyperaemia is obvious when current was passed using the water vehicle alone (**fig 4A**) compared to the 0.5% saline vehicle (**fig 4B**).

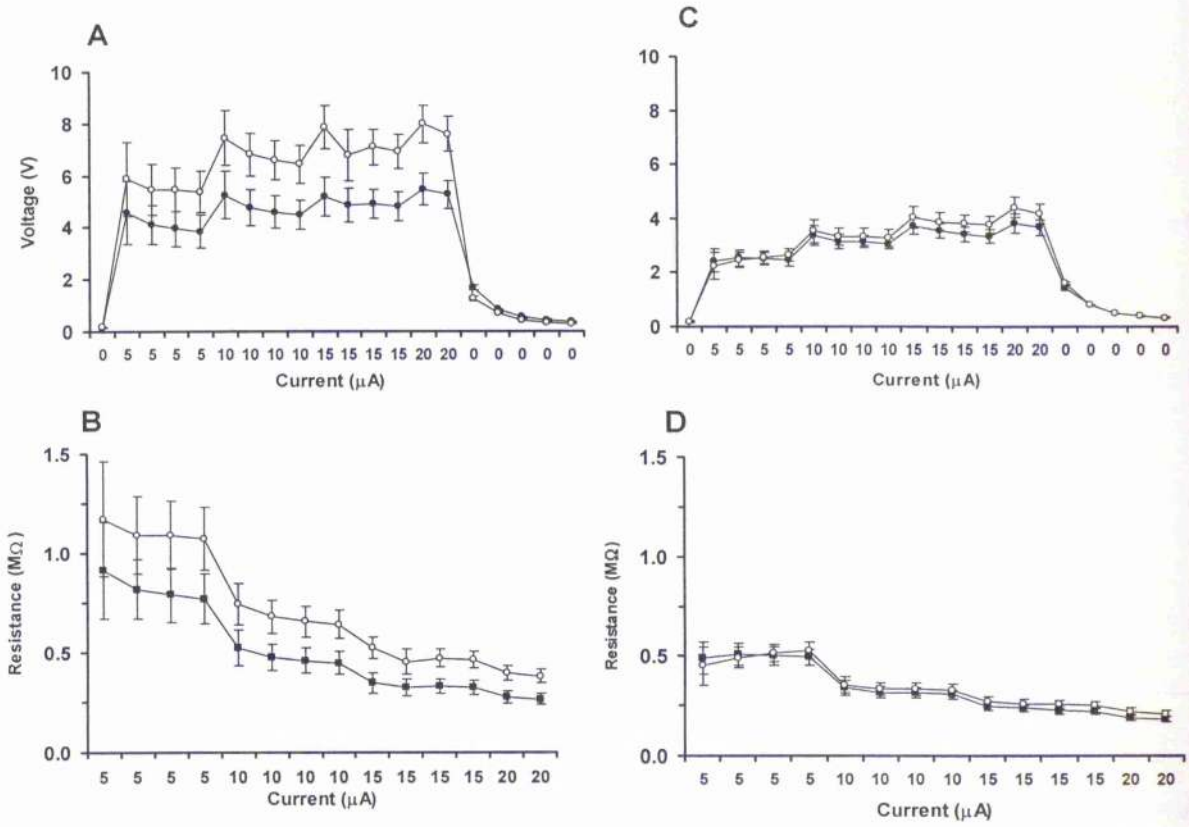

Fig 2. **A:** Voltage recorded across iontophoresis chambers during current delivery with H_2O vehicle (O) and drugs dissolved in vehicle (●). These differ significantly ($P<0.001$, 2-way ANOVA) and current-induced increase in voltage is significant in both cases ($P<0.0001$, repeated measures 1-way ANOVA).

B: Resistance calculated from voltages recorded in A for H_2O vehicle (O) and drugs dissolved in vehicle (■). These differ significantly ($P<0.001$, 2-way ANOVA) and progressive fall in resistance with increasing current is significant in both cases ($P<0.0001$, repeated measures 1-way ANOVA).

C: Voltage recorded across the iontophoresis chambers during current delivery with 0.5% NaCl vehicle (O) and drugs dissolved in this vehicle (●).

D: Resistance calculated from voltages recorded in C for 0.5% NaCl vehicle (O) and drugs dissolved in vehicle (■). Mean ± SEM; data from seven subjects for all groups. No significant differences between 0.5% NaCl vehicle and drugs dissolved in this vehicle for either C or D.

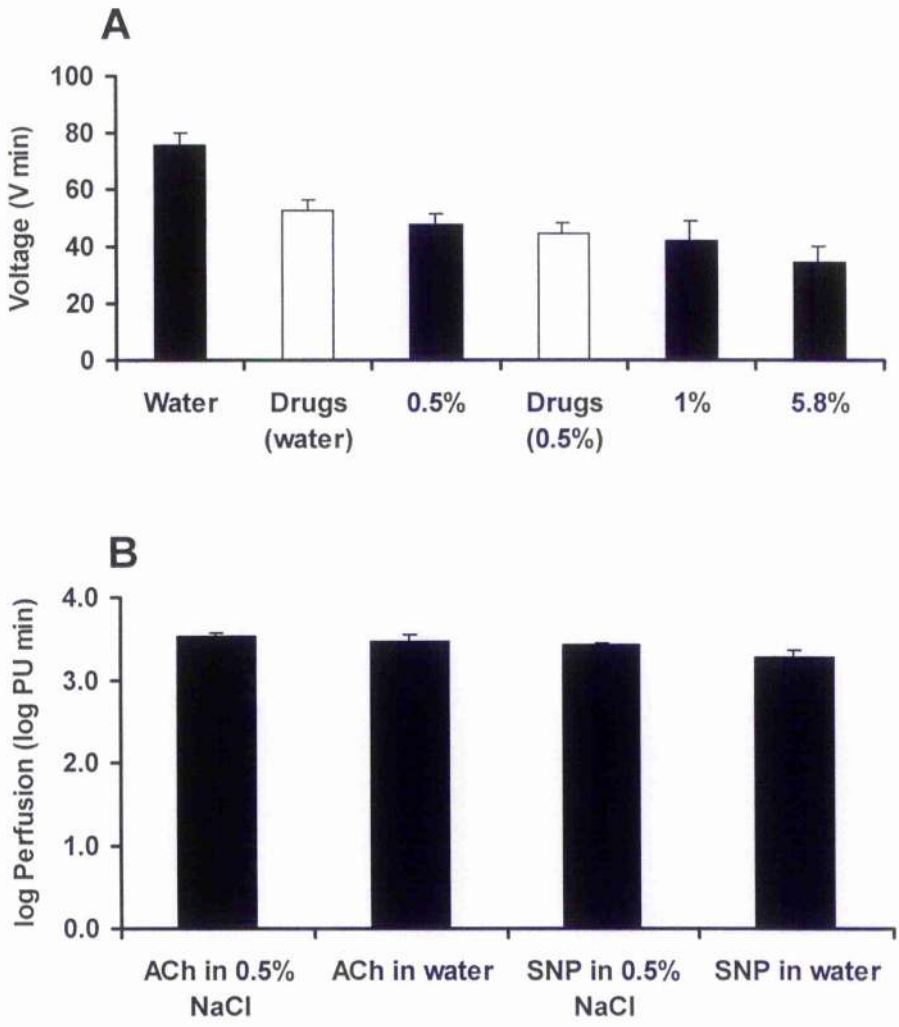

Fig 3. **A:** Comparison of voltage.time integrals for H_2O and the different concentrations of saline vehicles. Open columns represent values for drugs dissolved in H_2O and 0.5% saline. n = 5-14 subjects. Water vehicle differs significantly from all saline vehicles ($P<0.05$; Scheffe F-test), but saline vehicles do not differ from each other.

B: Log perfusion voltage.time integrals showing no difference in the magnitude of the responses of ACh and SNP in H_2O and 0.5% NaCl vehicles. Mean ± SEM; n = 7 subjects for each group in both A and B.

The relevance of monitoring voltage becomes apparent when examining the development of the electrically-induced hyperaemic response. For individual subjects, taking the integral of voltage over time for different vehicles and plotting these against the perfusion values at the cathode for scan 20 reveals that there is a threshold voltage integral beyond which hyperaemic responses are triggered (**fig 5**). It is noticeable that none of the saline vehicles (0.5%, 1% and 5.8% NaCl) was associated with an electrically-induced hyperaemic response (defined as a flux reading ≥ 100 PU) whereas the water vehicle is associated with these hyperaemic responses in all but two cases and in both of the latter the voltage integrals were the lowest for this group. However, when skin resistance was increased by Vaseline, even with the 0.5% saline vehicle higher voltages occurred and hyperaemic responses were generated. Also noticeable was the absence of a consistent relationship between the voltage integral and the magnitude of the hyperaemic response, perhaps indicative of varying magnitudes of responsiveness in different subjects.

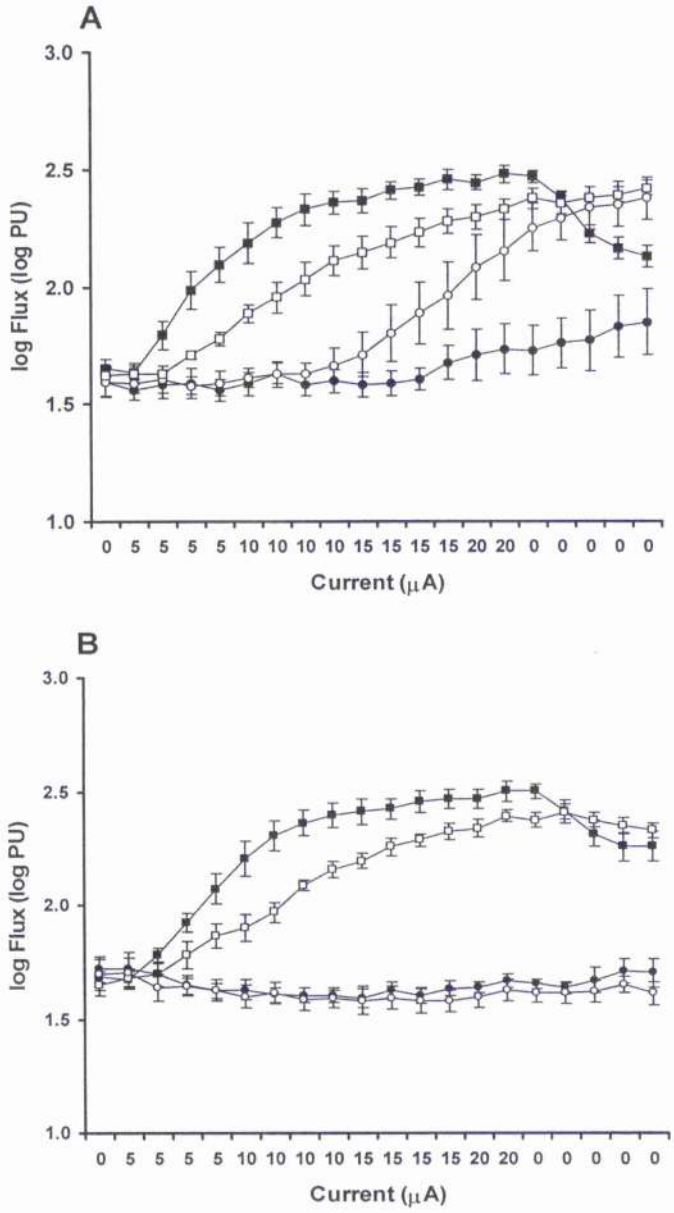

Fig 4. **A:** Time course of the response to iontophoretic administration of ACh (■) and SNP (□) dissolved in H_2O as well as the response to this vehicle at the anode (●) and cathode (○). Dose dependent increase in perfusion is significant ($P<0.0001$, 1-way ANOVA) for all except for water vehicle in the anodal chamber.

B: Time course of the response to iontophoretic administration of ACh (■) and SNP (□) dissolved in 0.5% NaCl as well as the response to this vehicle at the anode (●) and cathode (○). Dose dependent increase in perfusion is significant ($P<0.0001$, 1-way ANOVA) for drugs but not for vehicle. Mean ± SEM; $n = 7$ subjects for both A and B.

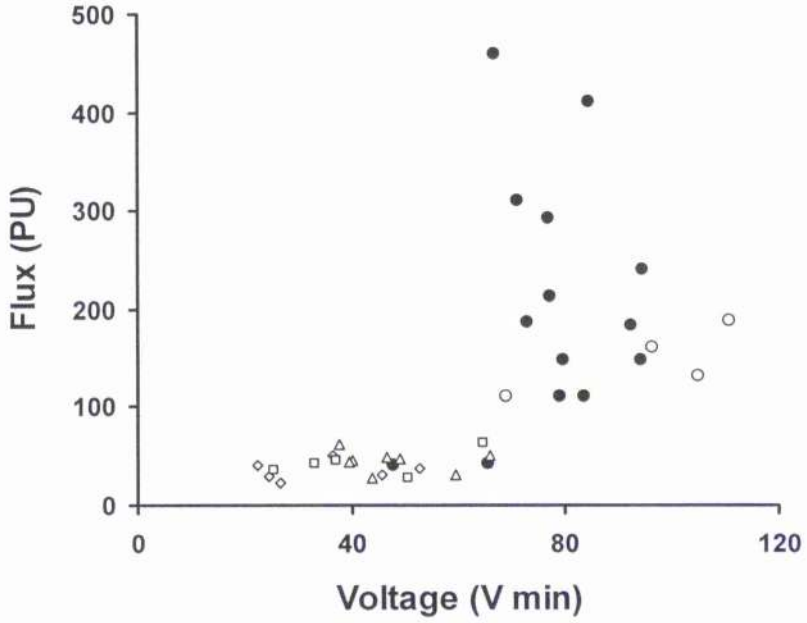

Fig 5. Analysis of flux responses (at scan 20) occurring during incremental current administration using water (●; $n = 14$), 0.5% NaCl (Δ; $n = 8$), 1% NaCl (□; $n = 5$) and 5.8% NaCl (◊; $n = 6$) NaCl vehicles plotted against voltage.time integral (area under the curve - AUC) for each vehicle. Also plotted are values for the 0.5% NaCl vehicle in chambers where Vaseline was applied to the skin (○; $n = 4$).

pH measurements

Distilled H_2O (pH 7) instilled in both chambers and left in contact with skin for the duration of the incremental current protocol (16.7 min), but without application of current, resulted in a significant ($P<0.01$; $n = 5$) reduction in pH at both the anodal (5.9 ± 0.33) and cathodal (5.96 ± 0.33) chambers (mean ± SEM). However, following current application, chamber fluid pH was not found to have changed significantly at either the anodal (5.72 ± 0.37) or cathodal (6.02 ± 0.26)

chambers. Comparison of the anodal and cathodal chambers showed no significant difference either prior to (P=0.9) or after (P=0.52) current administration. Unsurprisingly, there was no pH difference between anodal and cathodal chambers with ACh and SNP in either water or 0.5% NaCl vehicles.

Chamber size

Another factor which could influence the electrically-induced hyperaemic response is the size of the iontophoresis chamber. Using the incremental protocol, and water as the vehicle, employing a smaller chamber (as described in methods) resulted in higher voltage integrals and almost tripled the perfusion integral of the electrically-induced hyperaemic response at the cathode (3546 ± 1277 PU min) compared to that obtained using the large chamber (1360 ± 823 PU min), this difference being significant ($P = 0.005$; $n = 4$; paired t-test; means ± SD). This difference is likely to arise due to the difference in charge density (2.1mC/cm^2 and 10.2mC/cm^2 respectively). The smaller chamber also showed an earlier response onset and hyperaemia spreading well beyond the chamber. Additionally, use of the smaller chamber resulted in hyperaemic responses occurring at the anode in three out of four subjects.

Effect of skin thickness on resistance and vascular responses

It was noticeable that during current delivery using the incremental protocol, the calculated resistance values varied between subjects. To establish whether this

was a consequence of variations in skin thickness, skin fold thickness at the anodal site was measured using callipers in each subject prior to current administration. No significant correlation between calculated resistance and skin fold thickness was found ($r^2 = 0.0002$; $P = 0.96$; $n = 9$), indicating that subcutaneous fat does not affect resistance. In addition, no significant correlation was observed between skin fold thickness and the ACh perfusion.time integral ($r^2 = 0.13$; $P = 0.38$; $n = 8$).

Prostaglandin dependence of electrically-induced hyperaemia

To establish whether prostaglandins mediate electrically-induced hyperaemia, the effect of oral aspirin administration on the electrically-induced response was investigated. The 'galvanic' protocol elicited hyperaemic responses at the cathode in all subjects tested ($n = 7$) and at the anode in some ($n = 3$) and these were both strongly inhibited by aspirin ($P < 0.001$ in both cases; 2-way ANOVA; $n = 7$; **fig 6**). Resistance integrals before (0.38 ± 0.05 MΩ min) and after (0.39 ± 0.05 MΩ min) aspirin did not differ significantly ($P = 0.76$, Students paired *t* test; means ± SD).

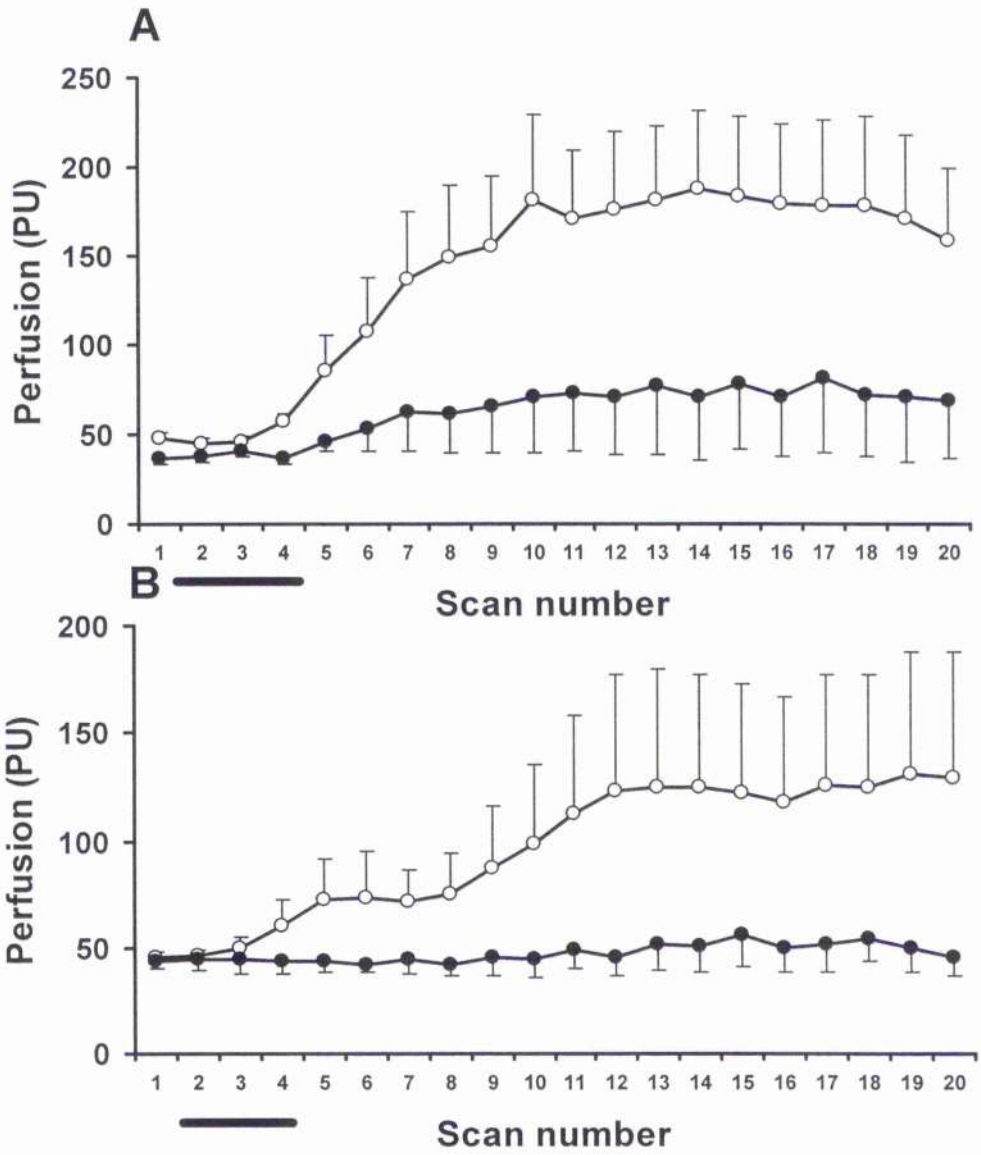

Fig 6. A: Response at the cathode to application of three 50s periods of current (black bar) at 100μA using the galvanic protocol with the distilled water vehicle before (O) and following (●) administration of aspirin.

B: Response to the same current protocol and water vehicle at the anode before (O) and following (●) administration of 600mg aspirin. Mean ± SEM; $n = 7$ subjects. Responses before and after aspirin differ significantly ($P<0.001$, 2-way ANOVA) in both cases.

Discussion

The aim of this investigation was to monitor voltage changes associated with the application of constant current during iontophoresis, in order to understand better the generation of hyperaemic artefacts associated with this procedure. Another aim was to establish whether such hyperaemic responses could be attenuated using a 0.5% NaCl vehicle rather than distilled water.

Administration of incremental current in the presence of the water vehicle resulted in the development of a hyperaemic response, most obvious at the cathode, which is in agreement with previous investigations [167 168]. The finding that a threshold voltage.time integral had to be exceeded before such a hyperaemic response was triggered (fig 5) clearly indicates that it is not simply the total charge that determines this response. The same total charge (8mC) failed to elicit cathodal hyperaemia using the 5.8%, 1% and 0.5% saline vehicles but did so with the water vehicle, the only apparent difference being the voltage integrals, implying this may be an important parameter in the generation of such artefacts. The finding that increasing epidermal resistance by topical application of Vaseline resulted in higher voltage integrals and hyperaemic responses, even when employing the 0.5% NaCl vehicle, further supports this. There was no obvious relationship between the magnitude of the hyperaemic response and voltage.time integral of individual subjects. It is possible that there is a particular threshold beyond which hyperaemia is triggered, but the magnitude of this response need not be correlated with the voltage.time integral. This would be consistent with the

observation that the magnitude of 'axon reflex' flares to identical stimuli shows substantial variability between individual subjects [172]. The fact that the voltage profile for the water vehicle differed markedly from that occurring with the drug solutions questions the appropriateness of examining 'control' responses using only the drug vehicle whose electrical resistivity may be quite different compared to the solutions containing ACh and SNP.

Although it has been shown previously that water iontophoresis results in pH changes at both anode and cathode [171], the charge used with the incremental protocol in the present study was substantially lower and did not result in pH changes at the chambers even though hyperaemic responses still occurred. This indicates that cathodal hyperaemia induced by electrical stimulation is not mediated by hydroxyl (OH^-) ions. Again, the voltage.time integral is the only factor linking to electrically-induced hyperaemic responses. However, as pH changes were not measured in the skin itself, an effect due to changes in subdermal H^+ or OH^- concentrations cannot be excluded by this study.

Use of a saline vehicle virtually eliminated the electrically-induced hyperaemic artefact and was associated with voltage.time integrals being below the threshold for triggering this response. This is achieved by the presence of ions in solution resulting in a lowered resistance within the circuit. This concurs with previous work that showed electrically-induced hyperaemia to be greatly attenuated using a hyperosmolar (5M) solution of NaCl [168]. This work also showed a reduction in the

applied voltage with the hyperosmolar solution compared to water, but this was only measured for a single current pulse application and not across a range of currents as in the present investigation. The main problem associated with use of a hyperosmolar vehicle is that the response to iontophoresis of ACh using this vehicle was attenuated and both intra- and inter-individual variations were increased [168]. This may be related to competition between the drug and vehicle ions during iontophoresis, particularly if there is a major disparity in concentration between ionic species. Increasing the ACh concentration lowered variability but did not increase the response [168]. In the present investigation the magnitude of the ACh and SNP vascular responses were unaffected when the 0.5% NaCl vehicle was used compared to the water vehicle (fig 3B, fig 4 A,B). The fact that the vasodilator responses to ACh and SNP were unaffected by the presence of Na^+ and Cl^- ions respectively in the vehicle, and that the latter ions had no effect on their own (fig 4B), indicates they cannot contribute to the vasodilator responses of ACh and SNP. This implies that sufficient drug gained access to elicit vasodilatation, despite the presence of other ionic species. It must therefore be the case that there is sufficient charge to carry both drug ions and salt ions into the skin without significant competition, as long as the salt solution is relatively low in concentration. The present experiments were limited to the use of ACh and SNP as these are the most commonly used agents in studies employing iontophoresis, but it is likely that similar principles would apply if other ionised drugs were to be used. Also, the make of manufacture of the equipment should not introduce variability, as long as a constant current delivery

system is used, large diameter chambers employed and the voltage monitoring device has a high input impedance.

In the present investigation, a low concentration of NaCl (0.5%) was used in the vehicle and this successfully eliminated the electrically-induced hyperaemic response. However, this disagrees with a previous study where such responses occurred using 0.9% NaCl [160]. Our results suggest that the differences arise because of differences in the chamber sizes and the currents employed. We have shown that use of a smaller chamber ($0.78cm^2$), similar to that used in previous studies [168 160], is associated with higher voltages and greater electrically-induced hyperaemic responses compared to the responses with the larger ($3.8cm^2$) chambers. This is explained by greater current density occurring within the smaller chamber and, as all previous studies appeared to have used such chambers, this could account for the occurrence of electrically-induced hyperaemia even with 0.9% NaCl. Noon *et al* [160] previously proposed that use of a 2% methylcellulose vehicle eliminates the electrically-induced hyperaemic response, but we have been unable to confirm this finding. Hyperaemic responses still occurred when using 2% methylcellulose despite the total charge used in our protocol (8mC) being half that used by Noon *et al* [160].

The mechanism underlying the electrically-induced hyperaemic response in skin remains poorly understood. One hypothesis suggests this response is achieved by generation of an "axon reflex" via activation of mechano-insensitive C-

nociceptors [173], as application of a topical anaesthetic (EMLA® cream) resulted in its elimination. (See chapter one) However, application of EMLA cream results in cutaneous vasoconstriction [158] and this presents a problem as any vasodilator responses are then superimposed upon an artificially-induced vasoconstrictor tone. An additional complication is that local anaesthetics can also significantly alter ionic fluxes across membranes of many cell types, potentially blunting endothelial responses to applied drugs. Whatever the actual mechanism, electrically-induced hyperaemia appears to be mediated by prostaglandins as aspirin strongly inhibited this response at both cathode and anode, implying that similar mechanisms are involved in the generation of this response at both sites. This observation is at variance with a previous investigation where aspirin substantially reduced cathodal hyperaemia but showed no significant effect on the anodal response [170]. This discrepancy may be related to differences in the experimental conditions between the studies.

In previous investigations there has been some disagreement over whether vasodilatation in response to iontophoretic administration of ACh is prostaglandin dependent as two studies found that aspirin administration reduced the magnitude of the ACh-induced response [160 159], but in another study aspirin was shown to have no effect [145]. The fact that electrically-induced hyperaemia can occur at the anode, the site of delivery of ACh, and that such artefacts are reduced by aspirin administration (fig 6) suggests that in some of these earlier investigations the ACh response might have been "contaminated" by this artefact. As different current

protocols were used in these studies and as the chamber (drug delivery) areas also differed (0.5 – 0.78cm^2) it is not possible to compare them directly. Monitoring of the voltage across the circuit could provide one means of comparison between studies if, as the present investigation indicates, electrically-induced artefacts are mostly influenced by the voltage.time integral as a consequence of circuit resistance. This resistance arises partly due to the ionic nature of the fluid within the chamber and partly due to the surface area for drug delivery. However, variations in calculated circuit resistance between subjects, using the same chamber size, vehicle and current protocol, suggests that there must also be variations in resistance pathways through the skin. Exactly through which pathways current flows is unknown at present, but could include hair follicles and sweat gland ducts and thus variations in densities of these could account for some variation in calculated resistance between subjects.

In conclusion, we have shown that resistance is an important but previously unrecognised variable influencing development of hyperaemic responses during iontophoresis. The use of vehicles with low ionic content results in high circuit resistance, leading to development of high voltages that in turn are likely to generate electrically-induced hyperaemia. This complicates the interpretation of SNP responses. We anticipate that use of appropriate vehicles, chambers and iontophoresis protocols will lead to significant improvement in this technique and permit its development, thereby increasing its robust qualities as a non-invasive tool for endothelial assessment in clinical studies.

Chapter Four

Maternal obesity is associated with dysregulation of metabolic, vascular and inflammatory pathways

Introduction

Obesity is a health problem of epidemic proportions in Western society with 10% of children and 20% of adults in the UK now classified as clinically obese (BMI>30). [174 175] In the USA, these figures are even more alarming [176]. World wide it is estimated that more than 300 million people are clinically obese. It is now well established that obesity and body fat distribution strongly correlate with deranged metabolic function and are associated with an elevated incidence of cardiovascular disease (CVD) [49 10 50].

Obesity in pregnancy has implications for morbidity and mortality in both mother and baby. The risk of maternal hypertensive complications such as pregnancy-induced hypertension or pre-eclampsia are significantly greater if the mother is overweight as assessed by body mass index (BMI) in early pregnancy, or centrally obese as assessed by waist circumference [90 177]. Maternal obesity also increases the risk of metabolic complications such as gestational diabetes. Furthermore, some data suggest that adiposity in the mother may critically influence the programming of metabolic pathways of her fetus and its risk for diabetes and cardiovascular disease in later life [124].

Although there is abundant evidence concerning the effects of obesity on metabolic pathways and vascular function in the non-pregnant individual, such information is currently sparse with respect to pregnancy. Therefore, the aim of this study was to examine classical (lipids, blood pressure) and novel

(inflammation, insulin resistance) cardiovascular risk factors in lean and obese women in the third trimester of pregnancy. To relate these risk factors to blood vessel function we took advantage of recent developments in non-invasive microvascular assessment to examine endothelial function, using a novel technique ideally suited to the study of pregnant women [178]. Our hypothesis was that all of the pathological associations of obesity in non-pregnant subjects would be sustained in pregnant women in the third trimester, and as such may underlie the elevated risk for pregnancy complications in obese women.

Subjects and methods

Forty-seven consecutive subjects in the third trimester of pregnancy were recruited from antenatal clinics. These women were divided into two groups of lean and obese around the median early pregnancy (10 – 12 weeks' gestation) body mass index of 27.7 kg/m^2. This median value is similar to criteria used in previous studies [39]. Our power calculations suggested that 20 women in each group would provide 90% power to detect differences in endothelial function. Women participating in the study were healthy and normotensive with no significant past medical history, such as peripheral vascular abnormalities, dermatological diseases or systemic disease processes such as diabetes mellitus and no relevant complications of pregnancy. In addition, no participant had ongoing infection or a recent history of infection or injury. The study was performed according to the Declaration of Helsinki and approval was granted by the institutional ethics committee. All women gave written informed consent.

Clinical and Laboratory measurements

Women attended for participation in the study after an overnight fast (>10 hours) and underwent testing between 0900 and 1100 hours. Blood pressure was recorded using a standard sphygmomanometer and appropriately sized cuff (Table 1). Fasting blood was withdrawn for lipid profile (via modification of the standard Lipid Research Clinics Protocol. The intra-assay and inter-assay coefficients of variation (CVs) for all lipid measures were less than 3%). Other measures included glycosylated haemoglobin (HbA1c) (High performance liquid chromatography, HA8121 analyser, Menarini Diagnostics, Berkshire, UK), insulin (a competitive radioimmunoassay, Coat-A-Count® I, DPC, Los Angeles, USA), interleukin-6 (IL-6) (Quantikine High Sensitivity Human Il-6 Immunoassay, R&D systems Inc., Oxon, UK), and C-reactive protein (CRP) (Double antibody sandwich ELISA with rabbit anti-human CRP and peroxidase conjugated rabbit anti-human CRP: DAKO A/S, DK-2600 Glostrup, Denmark). For the CRP assay, standard curves were linear up to 5mg/l and logarithmic thereafter. The inter-assay and intra-assay coefficients of variation were less than 10% across the range of measured results. Plasma leptin was measured by an 'in house' radioimmunoassay validated thoroughly against the commercially available Linco assay [179]. The intra- and inter - assay coefficients of variation were <7% and <10%, respectively, over the sample concentration range. The detection limit of the assay was 0.5ng/ml.

Perfusion measurements

Perfusion measurements were also performed after an overnight fast, therefore ensuring no caffeine containing drinks had been consumed prior to testing. Also no "over the counter" medications were taken by any of the participants for at least 48hours prior to testing. Before testing, a ten-minute rest period was spent acclimatising in a temperature-controlled room. The women lay in a semi-recumbent position with the flexor aspect of the forearm exposed on an armrest. Non-invasive measurement of skin perfusion was performed by means of a laser Doppler imager (LDI) (Moor Instruments Ltd, UK) as described in chapter two and three [178]. Iontophoretic application of 2.5 ml of 1% acetylcholine (ACH) and 2.5ml of 1% sodium nitroprusside (SNP) (both Sigma, Poole UK) was performed using an incremental current protocol also as previously described. ACH and SNP are proposed to examine endothelium-dependent and independent vasodilatation. The vehicle for these drugs was 0.5% NaCl in deionised water. Responses were also observed with the vehicle alone as a control experiment. Twenty repetitive scans were taken with assessment of the overall response defined as the area under the perfusion.time curve (AUC). Units of perfusion were derived from multiplying the perfusion.time integral by the resitance.time integral as calculated by continous voltage monitorring as described on page 63.

Statistical analyses

Dose response curves were expressed as means ± SEM and compared using 2-way ANOVA. Metabolic parameters were compared using the Mann Whitney U

test. Univariate analysis relationships were expressed as Pearson's correlation coefficients after log transformation of skewed variables. Linear regression was employed to examine independent associates of endothelial and metabolic measures.

Results

Demographic characteristics of each subject group are given in **Table 1.** The groups were not significantly different with regard to age, smoking history, parity and weeks of gestation. The lean group consisted of 24 women with median BMI of 22.15kg/m^2 (IQ range 20-24), and the obese group 23 women with a median BMI of 31kg/m^2 (IQ range 29-34). Although within normal ranges, diastolic blood pressure recorded in the first trimester and systolic blood pressure recorded in the third trimester, were significantly higher in obese compared with lean subjects. Three women in the lean group subsequently gave birth to babies with birthweights below the 5th centile and one women from each group went on to develop mild PET at term. There were no significant differences between gestation of delivery or mode of delivery between the groups although the birthcentile of offspring was significantly greater from the obese women. All babies were liveborn (**Table 1**).

	Lean n=24	Obese n=23	*P* value
1st trimester BMI (kg/m^2)	22.1 (20-24)	31 (29.1-34)	<0.0001
Age (years)	27 (21.5-32)	30 (25-34)	0.24
Smokers	4	7	0.26[+]
Parity (primigravidae)	17	15	0.68[+]
Gestation (weeks)	36 (35-37)	35 (34-38)	0.79
1st trim. Systolic (mmHg)	117 (110-125.7)	119 (110-130)	0.5
1st trim. Diastolic (mmHg)	65 (60-72.75)	75 (64-78)	0.035
3rd trim. Systolic (mmHg)	115 (110-120)	130 (120-130)	0.01
3rd trim. Diastolic (mmHg)	70 (62-80)	80 (70-80)	0.2
Gestation of delivery (wks)	40 (39-40)	39 (38-40)	0.44
Birth centile	40 (10-60)	60 (40-90)	0.03
Vaginal delivery	68%	91%	0.07

Table 1 Demographic characteristics of study subjects.

All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test, [+] Chi-squared test. BMI = Body Mass Index. Trim = trimester.

Plasma Analyses

Results of plasma lipid, HbA1c, IL-6, CRP, leptin and insulin concentrations are displayed in **Table 2.** Although total cholesterol and LDL-C concentrations were not significantly different, fasting plasma triglyceride and VLDL-C concentrations were significantly higher and HDL-C concentrations lower in obese women. Insulin and leptin concentrations were more than two-fold higher in obese women as compared with lean controls, although percentage of HbA1c

demonstrated no significant difference between the two groups. IL-6 and CRP concentrations were also both significantly higher in the obese group (all P <0.05). As the women were recruited consecutively and they covered a continuous range of BMI, we were able to examine cross-sectional relationships between BMI and other parameters. Simple linear regression analysis of the entire cohort revealed significant correlation of BMI with log triglyceride ($r = 0.326$, $P = 0.025$), log insulin ($r = 0.684$, $P < 0.005$), log leptin ($r = 0.729$, $P < 0.005$), log IL-6 ($r = 0.523$, $P < 0.005$) and log CRP ($r = 0.476$; $P < 0.005$).

	Lean	Obese	*P* value
	n=24	n=23	
Lipids			
Cholesterol (mmol/l)	6.35 (5.87-6.95)	6.25 (5.6-7)	0.43
LDL-C (mmol/l)	4.05 (3.75-4.79)	3.9 (3.1-4.7)	0.32
Triglyceride (mmol/l)	2.17 (1.9-2.6)	2.7 (2.3-3.2)	0.02
VLDL-C (mmol/l)	0.52 (0.31-0.64)	0.75 (0.6-1)	0.008
HDL-C (mmol/l)	1.77 (1.46-1.99)	1.55 (1.15-1.7)	0.02
Metabolic			
Insulin (mu/l)	6.15 (4.47-9.5)	14.2 (11.3-27)	<0.0001
HbA1c (%)	4.4 (4.2-4.7)	4.5 (4.3-4.7)	0.89
Leptin (ng/ml)	23.4 (12.4-30.9)	56.8 (46.2-65.2)	<0.0001
Inflammatory			
CRP (mg/ml)	2.13 (0.89-3.29)	4.45 (3.09-6.78)	0.0002
IL-6 (pcg/ml)	2.1 (1.7-2.8)	3.15 (2.36-3.59)	0.0016

Table 2 Lipid, other metabolic and inflammatory parameters in lean and obese groups

All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. HbA1c = Glycosylated Haemoglobin. IL= interleukin, CRP = C-reactive protein.

Endothelial dependent vasodilatation

Dose dependent perfusion response to ACH was significantly greater in the lean as compared with obese women (**Figure 1**, P =0.0003). Corrected area under the perfusion.time curve for ACH response was also calculated for each individual woman, and used in simple linear regression in order to consider the relationship of endothelial function to metabolic parameters. Log CRP and log fasting insulin concentrations inversely correlated with microvascular endothelial function (ACH

response). (r = - 0.289, $P = 0.049$, r = -0.339, $P = 0.02$). In addition, CRP correlated strongly with fasting insulin (r = 0.473, $P = 0.001$).

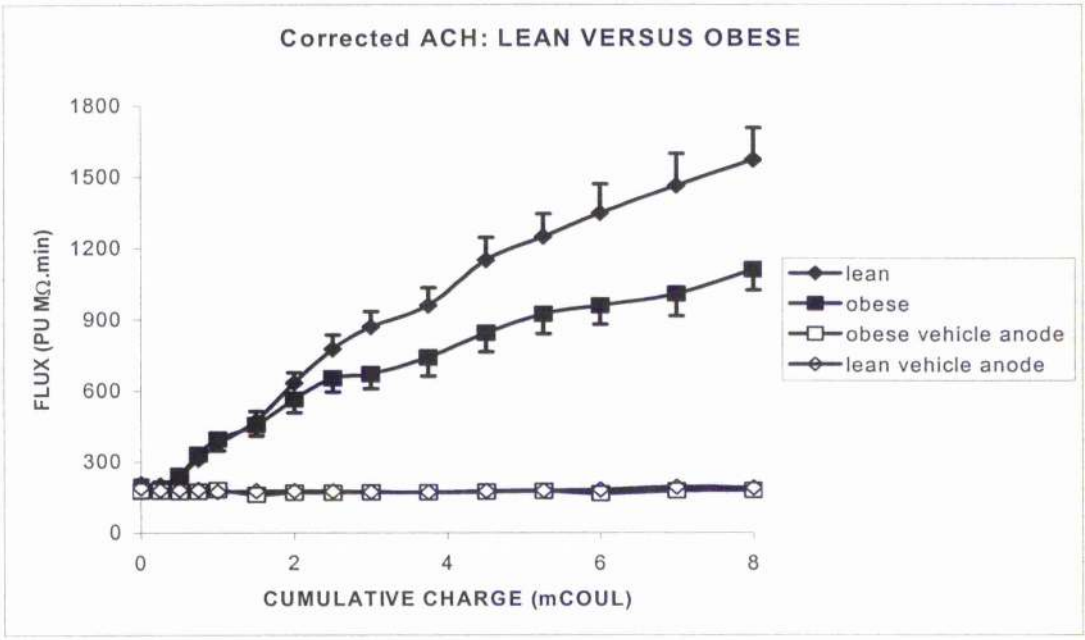

Figure 1: Endothelial dependent vasodilatation

Dose dependent perfusion response to Acetylcholine (ACH) in pregnant lean (n=24) versus obese women (n=23). Data are mean +/- standard error (SEM). P = 0.0003, ANOVA.

Endothelial independent vasodilatation

There was a small but significant difference in dose dependent perfusion responses to SNP between lean and obese groups (**Figure 2**, $P = 0.02$).

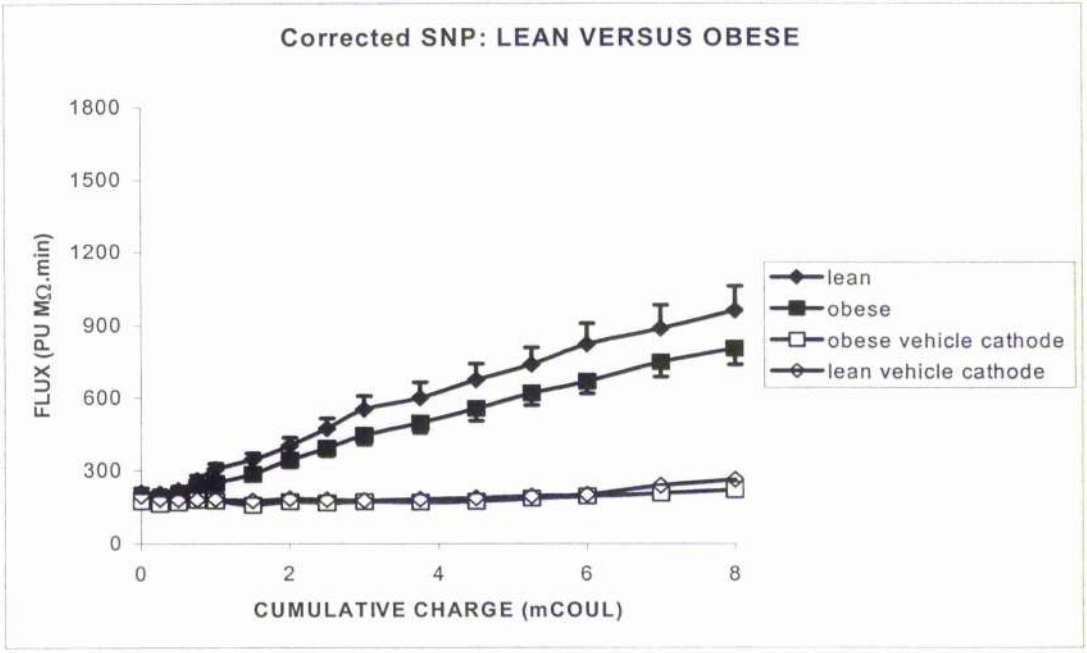

Figure 2: Endothelial independent vasodilatation

Dose dependent perfusion response to sodium nitroprusside (SNP) in pregnant lean (n=24) versus obese women (n=23). Data are mean +/- standard error (SEM). P = 0.02, ANOVA.

Predictors of CRP and fasting insulin

Given the recent evidence for the association of CRP with obesity and its independent prediction of diabetes in the non-pregnant individual [180 181], we were interested in the correlates of CRP and insulin in pregnancy. Potential correlates examined were based on biological plausibility and previous literature as above and included BMI, leptin, insulin, and IL-6. Log leptin was the strongest linear correlate to both CRP ($r = 0.532$, $P < 0.001$, **Figure 3**) and fasting insulin ($r = 0.738$, $P < 0.001$, **Figure 4**) but other parameters were also correlated (data not shown). We therefore examined the independent predictors of CRP and insulin using step-wise regression analysis. For CRP, leptin ($T=3.06$, $P=0.004$) and IL-6

(T=2.16, P =0.036) were retained in the final model and together explained 32.2 % of its variability. For insulin, the independent predictors were again leptin (T=3.62, P =0.001) and BMI (T=2.21, P =0.032) and together accounted for 57.1% of its variability.

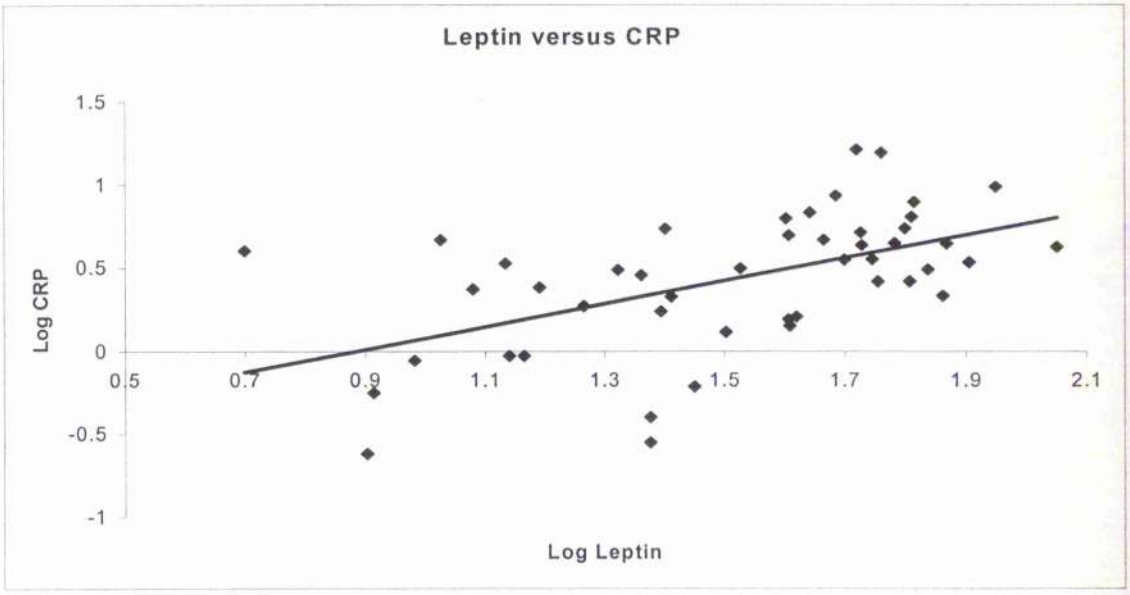

Figure 3: Positive correlation between $\log^{10}$ leptin and $\log^{10}$ C-reactive protein (CRP) concentrations (n=47) r=0.532, p<0.001.

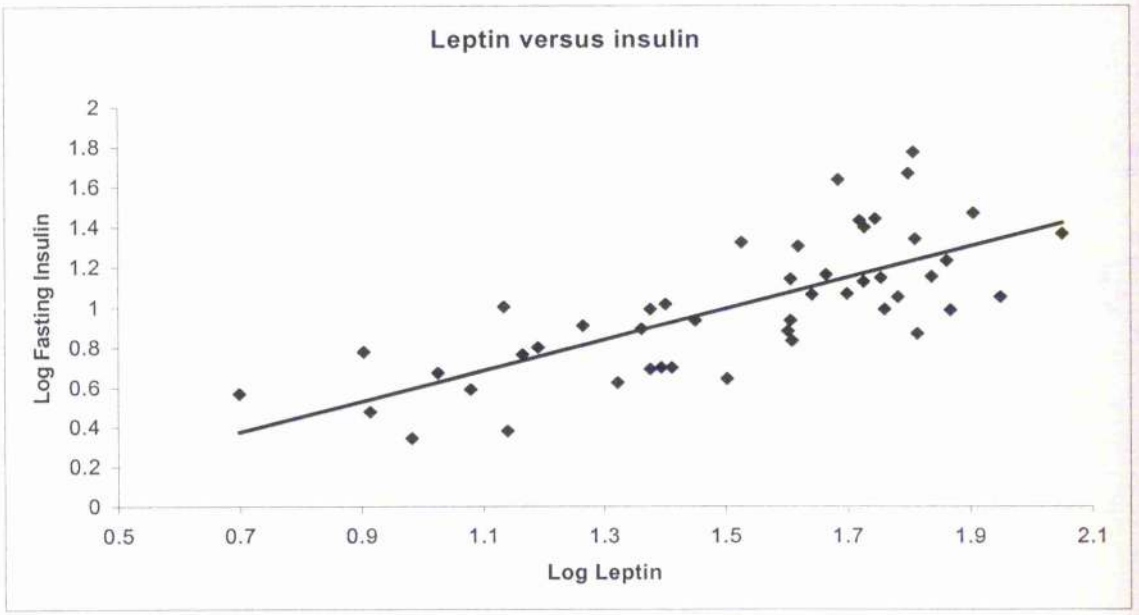

Figure 4: Positive correlation between $\log_{10}$ Leptin and $\log_{10}$ fasting Insulin concentrations (n=47) r=0.738, p<0.001.

Discussion

We demonstrate for the first time, that microvascular endothelial function assessed by a novel non-invasive technique is impaired in obese pregnant women as compared with lean counterparts. These obese women also show a perturbed metabolic state with dyslipidaemia, as characterised by higher triglyceride and lower HDL-cholesterol concentration, hyperinsulinaemia, elevated leptin concentrations and a low-grade inflammatory response. Interestingly, just as in the pre-diabetic state, this perturbation occurred in advance of any notable glucose dysregulation, since HBA1c concentrations were near identical. Thus, just as in the non-pregnant state, obesity in pregnancy results in a plethora of metabolic and vascular abnormalities that could collectively exacerbate the risk of maternal

complications. As the pattern of metabolic and inflammatory perturbances associated with obesity is also observed in our group of pregnant women, this indicates that the marked pregnancy-associated hormonal increments (e.g. oestrogen, progestogen, and hormone placental lactogen) do not over-ride these changes.

Microvascular function was investigated using a well-tolerated in-vivo method of assessment, ideal for the pregnant patient. LDI in combination with iontophoresis examines the cutaneous microcirculation, a robust surrogate marker of vascular function in other vascular beds. Reduced responsiveness to iontophoretic administration of ACH has been observed in diabetes [36 148 149] and hypercholesterolaemia [152] and in both these conditions there is a parallel reduction of the ACH response in the forearm circulation (a predominantly skeletal muscle vascular bed) assessed by venous occlusion plethysmography [153 154]. Furthermore, assessment of endothelial vasomotion in peripheral arteries has been shown to correlate with and relate directly to coronary dysfunction [143].

We noted not only a reduced response to ACH (endothelial dependent), but also reduced responses to administration of SNP (endothelial independent). Therefore impaired perfusion responses in obese subjects may not necessarily represent solely endothelial dysfunction, but vascular dysfunction further downstream at the level of the vascular smooth muscle. However, the smaller magnitude of the SNP response as compared with the ACH response may suggest endothelial dysfunction to be relatively more important. Other groups utilising laser Doppler

imaging technology to investigate microvascular function in diabetic subjects and healthy relatives of Type II diabetics, a condition strongly linked with obesity, also demonstrated impaired endothelial independent vascular responses. [149] [163]

These observations may have particular significance with regards to maternal vascular complications. Maternal obesity is established as a significant risk factor in the development of hypertensive complications of pregnancy, particularly pre-eclampsia. [90] [91] Deranged endothelial function is proposed as the pathophysiological mechanism of this condition and several ex-vivo techniques have supplied evidence of altered vascular reactivity in pre-eclampsia. [73] [111] [74] Thus the metabolic, inflammatory and functional abnormalities which we have described for the first time in obese pregnancy, may predispose to vascular compromise and may contribute to the mechanism by which maternal adiposity is associated with an elevated risk of pre-eclampsia.

This report, to the best of our knowledge, is also the first to comprehensively demonstrate that obesity in pregnancy is associated with hypertriglyceridaemia, and low HDL. This dyslipidaemic pattern is consistent with the metabolic syndrome [182] [183]. Interestingly, although LDL-cholesterol was not raised, smaller, dense, atherogenic LDL species are likely to be elevated, since circulating triglyceride-rich particles drive production of smaller particles from larger more buoyant species [78]. Evidence from the cardiovascular arena links such a pattern, directly and indirectly, to endothelial dysfunction and greater oxidative stress [7]. Indeed, we and others have shown the same pattern in pre-eclampsia [93] [184] [102].

Clearly, prospective studies are required to examine whether the magnitude of the lipid dysregulation in the third trimester, associated with maternal obesity, is more or less exaggerated than would be seen in the non-pregnant state.

Our novel findings of elevated concentrations of IL-6 and CRP in obese pregnant women agree with the recent literature implicating adiposity as a key factor in low-grade chronic inflammation [11 59 58]. We found that IL-6 was 50% higher and CRP was almost double in the obese pregnant women relative to the lean group. High CRP correlates with endothelial dysfunction [56 185] and impaired insulin sensitivity [186 187] in non-pregnant populations and predicts risk for type 2 diabetes in women [181]. In line with these observations, we noted that CRP correlated negatively with acetylcholine-mediated endothelial vasodilatation (P =0.049) and positively with fasting insulin (P =0.001). Thus obesity driven inflammation in pregnancy could be implicated in pathogenesis of pre-eclampsia and gestational diabetes. Indeed, some suggest that pre-eclampsia is a disease of inflammation [94 109 110] with endothelial cells in maternal systemic vessels becoming activated and damaged by circulating factors.

As expected concentrations of leptin, an adipocyte derived molecule[179 188], were more than two-fold higher in the obese women. Leptin is also produced by the placenta [189], so it is not entirely clear from our data to what extent the high leptin reflects adipose tissue-derived or placental leptin. However, as leptin independently correlated with both C-reactive protein and fasting insulin levels in our cohort, we suggest a dominant role for adiposity in the elevated leptin levels

in obese women. In line with this, we have suggested previously that elevated leptin concentrations in pregnancy could be linked to changes in maternal fat mobilisation [190]. These combined observations further emphasise the importance of adipose tissue as an active metabolic organ even in pregnancy [58 191].

Finally, could the above metabolic and vascular disruption have adverse consequences for fetal programming? Firstly, altered maternal vascular function and dyslipidaemia may dysregulate blood and nutrient flow to the developing fetus. Secondly, a higher inflammatory burden may be damaging since a recent animal study demonstrated that offspring of rats injected with IL-6 throughout pregnancy had greater body fat and in male offspring reduced insulin sensitivity. [130] A pro-inflammatory phenotype is also linked to miscarriage [192]. Finally, data exists linking maternal obesity to cardiovascular and metabolic disease in her offspring. A population-based study from Finland [124] demonstrated a positive relationship in short mothers between maternal body mass index on admission to labour ward and future death rate from coronary heart disease in male offspring. Higher adult rates of type 2 diabetes have also been reported in offspring of mothers who were above average weight in pregnancy in a different population [125]. Clearly, the dysregulation in several risk factor pathways described herein may be relevant to this process and importantly such effects need not include glucose dysregulation. However, this suggestion remains speculative and more data linking maternal obesity with the health of offspring are needed.

We recognise that our study has limitations in that it is cross-sectional in nature

and therefore further prospective data are now required. However, strengths include a comprehensive assessment of a panel of classical and novel risk factors and robust methodology.

In conclusion, these comprehensive data demonstrate for the first time that, as in non-pregnant obese individuals, obesity in pregnancy is associated not only with marked hyperinsulinaemia (in advance of glucose dysregulation) and deranged lipids, but also impaired endothelial function and inflammatory up-regulation. Such perturbation may contribute to the risk for maternal complications in obese women and as a result may also be relevant to fetal programming of adult vascular disease. Clearly, these data provide further rationale to examine the potential benefits of pre-conceptual weight loss and antenatal exercise.

Chapter Five

Enhancement of endothelial function by pregnancy: inadequate response in women with type I diabetes.

Introduction

Type 1 diabetes is an established risk factor for the development of macro- and micro-vascular disease [193], complications that predispose to much of the increased morbidity and mortality associated with diabetes. Individuals with type 1 diabetes mellitus are recognised to be at extremely high risk for the development of cardiovascular disease and have a three to six fold increased risk of early cardiovascular death before the age of 60, as compared with non-diabetics. [194]

Normal pregnancy is characterised by a progressive increase in insulin resistance [81] and to meet the increasing demands of the feto-placental unit, glucose production also increases in late pregnancy. [82] These physiological adaptations of pregnancy may accelerate the development of microvascular complications in pregnant women with diabetes, potentially increasing the risk of maternal vascular disease such as pre-eclampsia [195 196], a condition also associated with insulin resistance. The fetus is also adversely affected in diabetic pregnancy. Maternal hyperglycaemia in early pregnancy is associated with an increased incidence of fetal anomalies and poor glycaemic control throughout pregnancy results in fetal hyperinsulinaemia and secondary macrosomia. [197]

A considerable body of evidence exists concerning potential mechanisms associated with the increased morbidity and mortality associated with diabetes. One potential unifying hypothesis is that of altered micro-vascular reactivity. This phenomenon has been described in pregnant women with gestational diabetes using wire myography [198], however to our knowledge such experiments have not

been performed in-vivo in diabetic women either antenatally or postpartum. Therefore, the aim of this investigation was to examine microvascular function using a well-tolerated in-vivo technique, in the antenatal and postpartum periods in women with type I diabetes and healthy controls. We also examined whether conventional (lipids) or novel vascular risk factors (inflammatory parameters) were perturbed in such patients. Our hypothesis was that microvascular function would be impaired in non-pregnant diabetic women as compared with healthy controls, and that this impairment would be evident even during pregnancy perhaps in association with deranged risk factors.

Subjects and Methods.

The study was divided into two portions comparing diabetic women and healthy controls in the antenatal and postnatal periods. All work was performed according to the Declaration of Helsinki, approval was granted by the institutional ethics committee and all patients gave written informed consent.

I. 15 women with type I diabetes (no microscopic proteinuria, retinopathy or ketonuria) and 30 pregnant controls, were recruited from antenatal clinics in the 3rd trimester of pregnancy. Women participating in the study were healthy and normotensive with no significant past medical history, such as peripheral vascular abnormalities or dermatological diseases and no relevant complications of pregnancy.

II. The above groups were invited to return at least 4 months after delivery. Of the original group, 9 of the diabetic and 16 of the control participants

attended for postpartum assessment. Exactly the same protocol was followed at the postpartum and antenatal assessments.

Clinical, laboratory and perfusion measurements

Controls attended for participation in the study after an overnight fast and underwent testing between 9 and 11 am. Women with type I diabetes were seen pre-midday meal due to difficulty enforcing an overnight fast in out-patients in whom glycaemic control is tight. Nevertheless, all diabetic women were instructed to have a light breakfast prior to 7am in the morning and examination was performed after 1200. Blood pressure was recorded using a standard sphygmomanometer and appropriately sized cuff and a blood sample was withdrawn for lipid profile, glycosylated haemoglobin (HbA1c), and C-reactive protein (CRP) as described in previous chapter. In addition, assessment of soluble vascular cell adhesion molecule (VCAM) (Parameter Human sVCAM-1 Immunoassay, R&D systems Inc., Oxon UK) was performed. In the diabetic women, random capillary glucose was recorded before testing. (One II One glucometer and reagent strips, Lifescan, Buckinghamshire, UK). Perfusion measurements were performed as previously described in methods chapters and chapter four. Units of perfusion were derived from multiplying the perfusion.time integral by the resitance.time integral as calculated by continous voltage monitorring as described on page 63. [178]

Statistical analyses

Dose response curves were expressed as means ± SEM and comparisons were by 2-way ANOVA. Other data are expressed as medians with the interquartile range as the measure of variability. For these data the Mann Whitney U test for comparisons between groups and Wilcoxon test for comparison within groups were used as appropriate. Linear relationships between variables were examined using the Pearson correlation coefficient after normalising skewed variables by log transformation. Regression analysis was used to test for independent associations between variables.

Results

Ia. ***Antenatal diabetic versus control***

Demographic characteristics are given in **Table 1**. Although all women were tested in the third trimester, gestation differed by three weeks in the two groups. Despite excellent glycaemic control the birth centile was significantly greater in the diabetic births (85 (64-98) vs 50 (25-75); p=0.0067).

	Antenatal diabetic (n=15)	Antenatal Control (n=30)	P value
Age (years)	29 (25-31)	29 (25-32)	0.99
Gestation (weeks)	32 (29-35)	35 (34-37)	0.001
BMI (kg/m^2)	26.5 (23.4-28)	26.7 (21.8-30.2)	0.8
Systolic (mmHg)	120 (110-130)	120 (110-130)	0.4
Diastolic (mmHg)	75 (67-80)	75 (69.5-80)	0.8
First trim Systolic (mmHg)	114 (100-120)	119 (112-126)	0.13
First trim diastolic (mmHg)	67 (60-72)	71 (62-77)	0.25
Primigravid	9	21	0.8^{+}
Smokers	2	5	0.959^{+}

Table 1 Demographic characteristics of study subjects.

All values are medians, (interquartile ranges). Statistical analysis performed using Mann-Whitney U test, $^{+}$ Chi sqared test. BMI = Body Mass Index. All diabetic women were treated with insulin and had no microalbuminuria.

Endothelial dependent vasodilatation

There was a significant difference in dose dependent perfusion responses to ACH (**Figure 1a**) between diabetic and control groups ($p<0.001$). Median response to ACH (as defined by corrected area under the perfusion.time curve (AUC)) was significantly lower in the diabetic group (8176 [3618-11434] vs 13235 PUMΩ.min [9083-16809], p=0.0017). This difference remained after adjusting for differences in gestation between the two groups (T=-2.39, p=0.022).

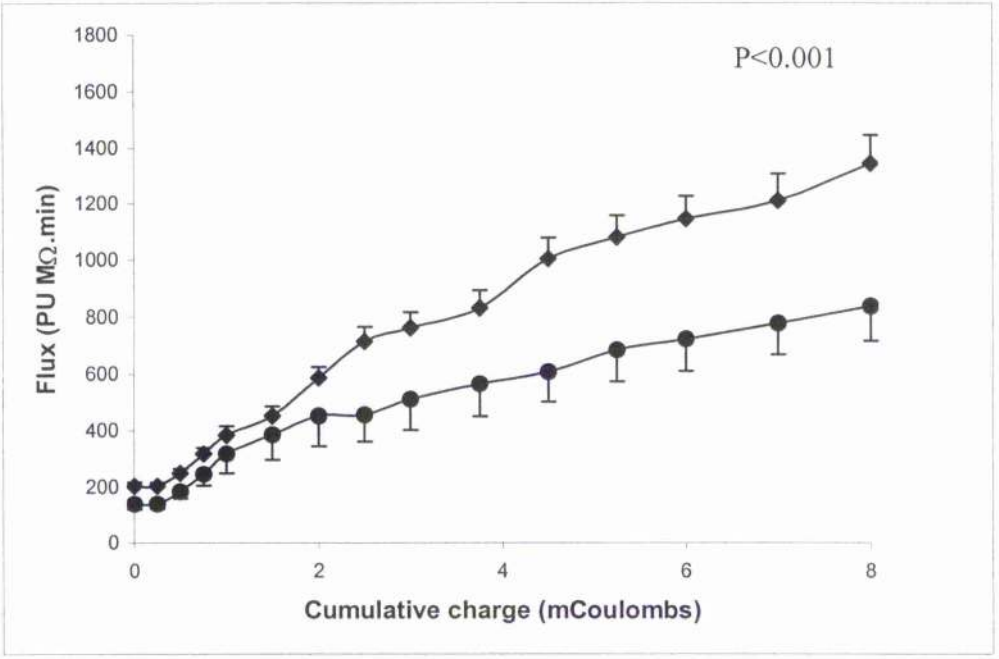

Figure 1a: Endothelial dependent (ACH) vasodilatation (pregnancy).

Dose dependent perfusion response to ACH in pregnant controls (n=30, ♦) versus diabetics (n=15, ●). Data are mean +/- standard error (SEM). P < 0.001, 2-way ANOVA.

Endothelial independent vasodilatation

Dose dependent perfusion responses to SNP were also lower in the pregnant diabetic women as compared with healthy pregnant controls. (**Figure 1b**, p=0<0.001) Median vascular response to SNP (AUC) was also significantly less in the diabetic (6339 [2712-7568] vs 9130 PUMΩ.min [7462-11489], p=0.0007). This difference remained after adjusting for gestation. (T=-2.83, p=0.007).

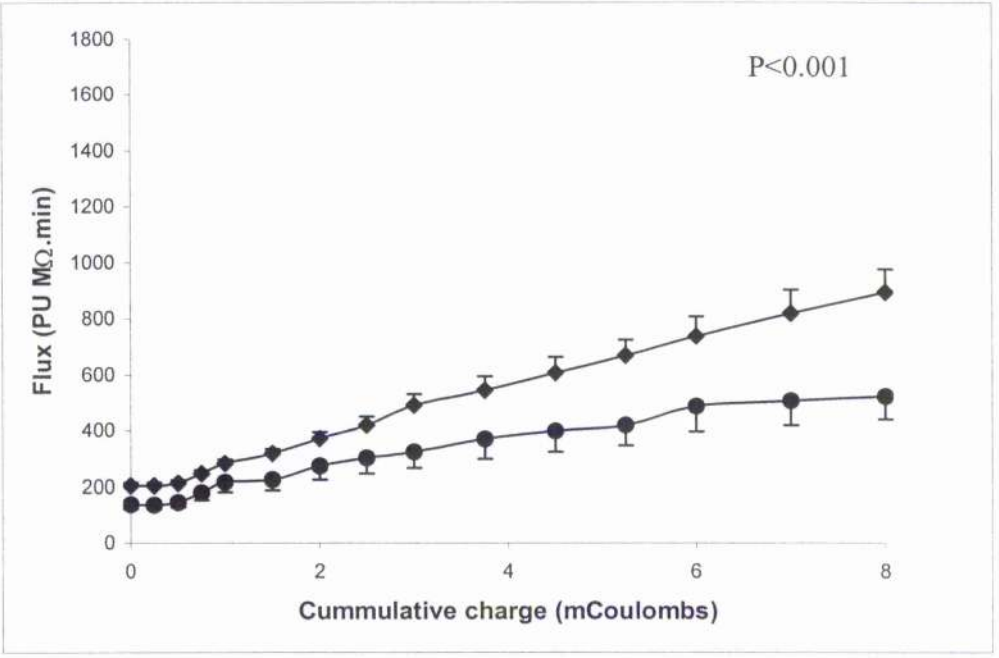

Figure 1b: Endothelial independent (SNP) vasodilatation (pregnancy).
Dose dependent perfusion response to SNP in pregnant controls (n=30, ♦) versus diabetics (n=15, ●). Data are mean +/- standard error (SEM). P < 0.001, 2-way ANOVA.

Plasma Analyses

Results of plasma measurements are displayed in **Table 2**. The concentration of HbA1c was greater in the women with diabetes than controls. The median capillary blood glucose in women with diabetes at the time of testing was 6.15mmol/l (IQ range 5.38-9.73). There was no correlation between AUC for ACH response in relation to capillary blood glucose (r=-0.069, p=0.815), although a non-significant inverse relationship was observed between HbA1c and ACH response (r=-0.384, p=0.16). Adjustment for HbA1c resulted in a loss of significance in ACH response between cases and controls. (T=-0.64, p=0.523). There were no differences in plasma lipoproteins in the antenatal period between

the groups. Soluble VCAM-1 concentration was similar in both groups. Compared with controls, CRP concentrations were significantly higher in diabetic women in the antenatal period, although this observation did not persist after adjustment for gestation (T=1.43, p=0.162). CRP did correlate with AUC for ACH response in the control group (r= -0.37, p=0.043) but not in the women with diabetes (r=0.041, p=0.885). Adjustment for CRP did not attenuate the case-control difference in ACH response. (T=-2.98, p=0.005).

	Antenatal diabetic (n=15)	Antenatal control (n=30)	P value	P value*
Cholesterol (mmol/l)	6.05 (5-6.95)	6.47 (5.7-7.2)	0.25	0.44
Triglyceride (mmol/l)	2.45 (2-2.95)	2.37 (2.05-3)	0.66	0.59
VLDL-C (mmol/l)	0.55 (0.25-0.8)	0.6 (0.34-0.8)	0.64	0.84
LDL-C (mmol/l)	3.75 (2.5-4.4)	4.05 (3.5-5.07)	0.15	0.24
HDL-C (mmol/l)	1.8 (1.65-2.0)	1.55 (1.4-1.8)	0.06	0.32
HbA1c (%)	5.8 (5.5-6.1)	4.55 (4.3-4.7)	<0.001	<0.001
CRP (mg/ml)	3.81 (2.47-8.15)	2.74 (1.53-4.4)	0.03	0.162
VCAM (ng/ml)	323 (254-370)	330 (249-390)	0.82	0.37

Table 2 Results of lipid profiles, HbA1c, C-reactive protein (CRP) and soluble VCAM. Pregnant diabetic women versus controls. All values are medians, (interquartile ranges). Statistical analysis performed using Mann-Whitney U test. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. HbA1c = Glycosylated Haemoglobin. CRP = C-reactive protein. VCAM = vascular cellular adhesion molecule. P value* = after adjustment for gestation.

1b. ***Postnatal results: diabetic versus control.***

Postnatal comparison of control and diabetic women showed no significant difference in BMI (22.7 versus 25.6kg/m^2, p = 0.28), blood pressure (Systolic: 115 versus 112mmHg, p=0.6; diastolic: 76 versus 76mmHg, p=0.98) or weeks elapsed since delivery (31.5 versus 28, p=0.23). The proportion of subjects breast-feeding (3 (18.8%) versus 3 (30%), chi squared p=0.412) or taking oral contraceptives (4 (25%) and 2 (22.2%), chi squared p=0.88) were also not significantly different.

Endothelial dependent and independent vasodilatation

Dose dependent perfusion responses to ACH and SNP were significant less in the women with diabetes than the control group, both p<0.001 (**Figures 2a and 2b**).

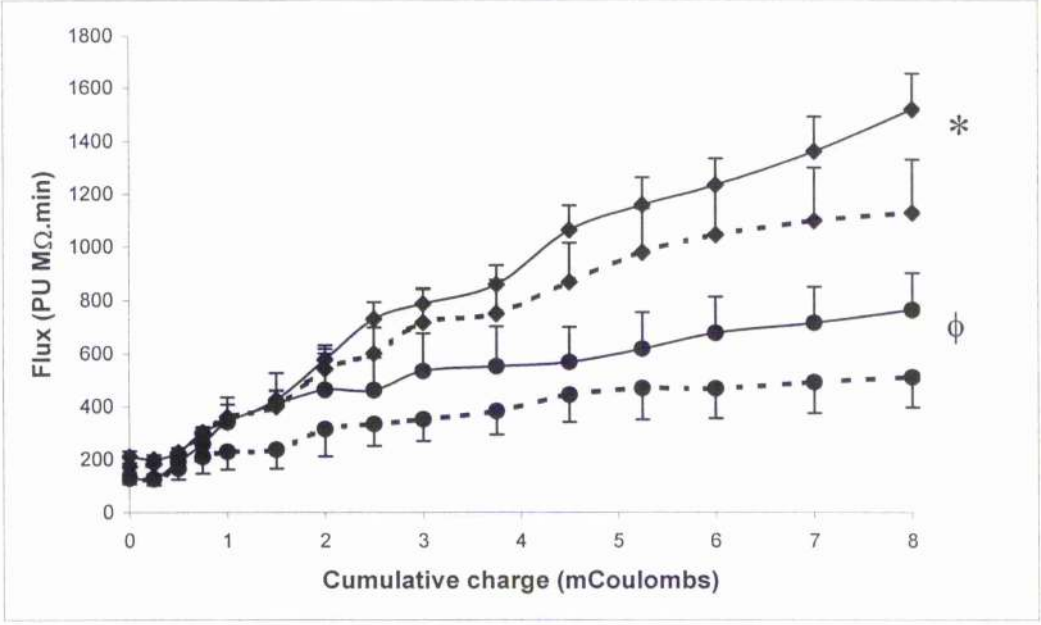

Figure 2a: Endothelial dependent (ACH) vasodilatation (control and diabetic): Antenatal versus postnatal. Dose dependent perfusion response to ACH in control women: antenatal (n=16; ♦) versus postnatal (n=16; dashed line ♦) and diabetic women: antenatal (n=9; •) versus postnatal (n=9, dashed line •). Data are mean +/- standard error (SEM). ∴ = control antenatal versus postnatal: P = 0.018; ϕ = diabetic antenatal versus postnatal: P = 0.029, 2-way ANOVA.

II. *Antenatal versus postnatal in diabetic and control groups.*

Endothelial dependent vasodilatation

There was a significantly greater response to ACH in pregnancy compared with the postpartum period as represented by dose dependent perfusion responses in both control (p=0.018) and diabetic (p=0.029) groups (Figure 2a).

Endothelial independent vasodilatation

In healthy controls a greater dose dependent perfusion response to SNP was observed in pregnancy as compared with the postpartum period (p=0.01). In the diabetic women this effect was also observed but was smaller in magnitude and did not attain significance (p=0.105) (Figure 2b).

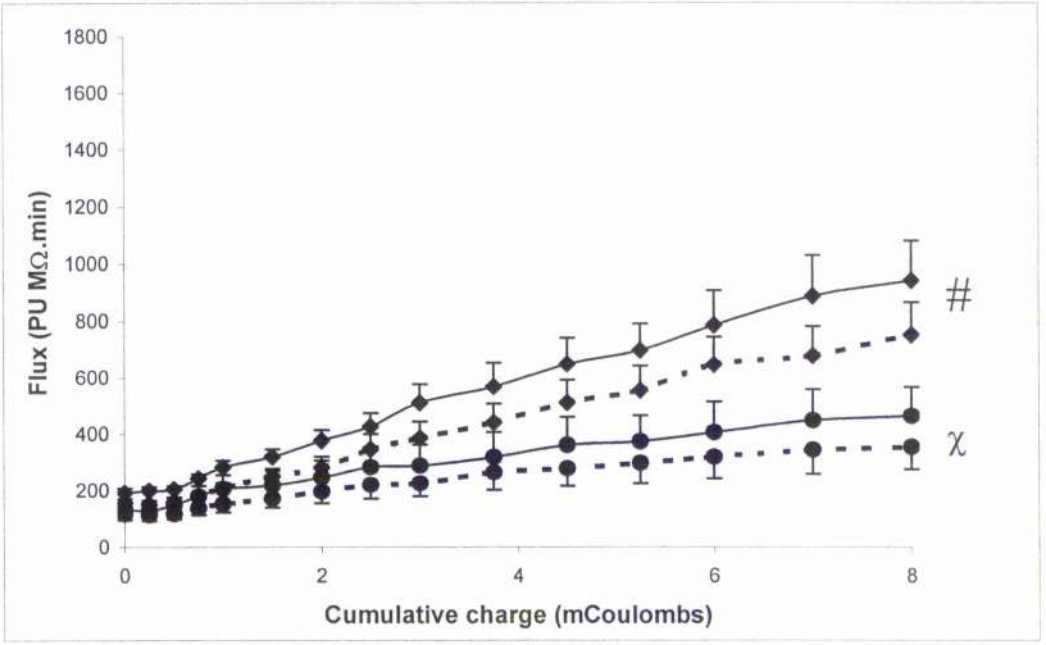

Figure 2b: Endothelial independent (SNP) vasodilatation (control and diabetic): Antenatal versus postnatal. Dose dependent perfusion response to SNP in control women: antenatal (n=16; ♦) versus postnatal (n=16; dashed line ♦) and diabetic women: antenatal (n=9; •) versus postnatal (n=9, dashed line •). Data are mean +/- standard error (SEM). # = control antenatal versus postnatal: P = 0.01; χ = diabetic antenatal versus postnatal: P = 0.105, 2-way ANOVA.

Plasma Analyses (**Table 3**)

In both groups, lipid and lipoprotein concentrations were significantly elevated in pregnancy in comparison with the postpartum period. CRP concentrations demonstrated a trend to be elevated during pregnancy in patients with diabetes ($p<0.10$). HbA1c results were similar in the control group during and after pregnancy but a marked increase was noted in women with diabetes post pregnancy. Postpartum VCAM-1 concentrations tended to increase in the women with diabetes and fell in the control subjects. However, VCAM-1 levels were higher in the diabetic women than controls in the postpartum period ($p<0.005$).

	Controls			Type 1 diabetes			
	Antenatal (n=16)	Postnatal (n=16)	P value	Antenatal (n=9)	Postnatal (n=9)	P value	P Control v diabetic at post natal visit
Lipoproteins							
Cholesterol	6.6	4.8	<0.001	6.05	4.4	0.009	0.46
(mmol/l)	(5.8-7.5)	(3.8-5)		(5.2-6.5)	(3.9-5.0)		
Triglyceride	2.22	0.85	<0.001	2.3	0.85	0.013	0.75
(mmol/l)	(1.9-3)	(0.7-1.1)		(1.9-3.3)	(0.7-1.6)		
VLDL-C	0.6	0.22	0.001	0.55	0.25	0.08	0.55
(mmol/l)	(0.3-0.9)	(0.1-0.4)		(0.3-0.7)	(0.1-0.5)		
LDL-C	4.32	2.95	0.001	3.75	2.5	0.009	0.25
(mmol/l)	(3.5-5.2)	(2-3.5)		(2.7-4.1)	(2.1-2.8)		
HDL-C	1.52	1.32	0.01	1.8	1.45	0.009	0.73
(mmol/l)	(1.2-1.9)	(1.1-1.7)		(1.7-2.0)	(1.2-1.7)		
Inflammation							
CRP	2.13	1.15	0.48	3.44	2.95	0.076	0.174
(mg/ml)	(0.9-3.9)	(0.3-3.3)		(2.3-7.6)	(0.9-6.7)		
VCAM-1	369	344	0.103	351	419	0.124	0.004
(ng/ml)	(321-424)	(285-393)		(299-391)	(385-476)		
Glucose control							
HbA1c	4.6	4.7	1.00	5.8	7.6	0.033	<0.001
(%)	(4.4-4.8)	(4.4-4.9)		(5.3-5.9)	(5.9-7.7)		

Table 3 Results of lipid profiles, HbA1c, soluble VCAM and CRP. Antenatal versus postnatal (control and diabetic).All values are medians (interquartile rauges). Statistical analysis performed using Mann-Whitney U test. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. HbA1c = Glycosylated Haemoglobin. VCAM = vascular cellular adhesion molecule. CRP = C-reactive protein

Enhancement of endothelial function by pregnancy.

In the control group, women with lowest responses to ACH postpartum showed the greatest improvement during pregnancy, whereas those with the highest vasodilator responses postpartum showed the least change with pregnancy. This was demonstrated by correlating the change in response to ACH (AUC) between postpartum and antenatal time points with the ACH response postpartum (r^2= 0.84 P <0.001). (**Figure 3a**).

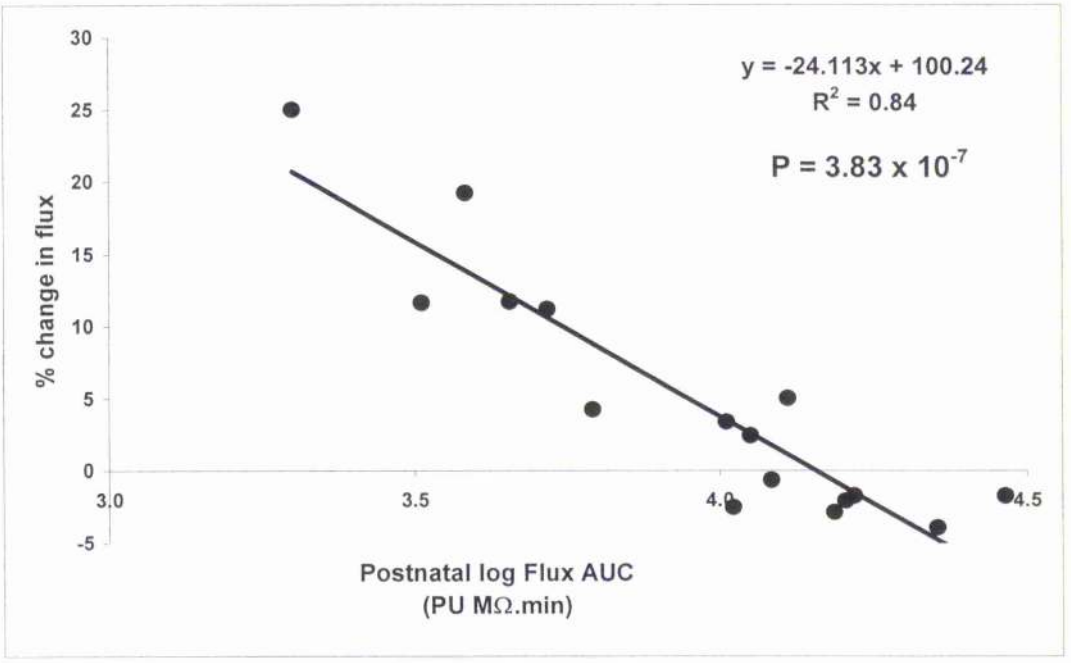

Figure 3a: Relationship of change in endothelial function (ACH response) in pregnancy to postnatal response (control). Inverse correlation between the percentage change in non-pregnant to pregnant log transformed endothelial dependent (ACH) perfusion and postpartum log transformed endothelial dependent (ACH) perfusion response in the control group. (n = 16) r^2 = 0.82, p=3.83x10^{-7}.

This relationship was also near significant in women with diabetes ($r^2 = 0.40$, P = 0.065) showing a similar gradient of association (**Figure 3b**).

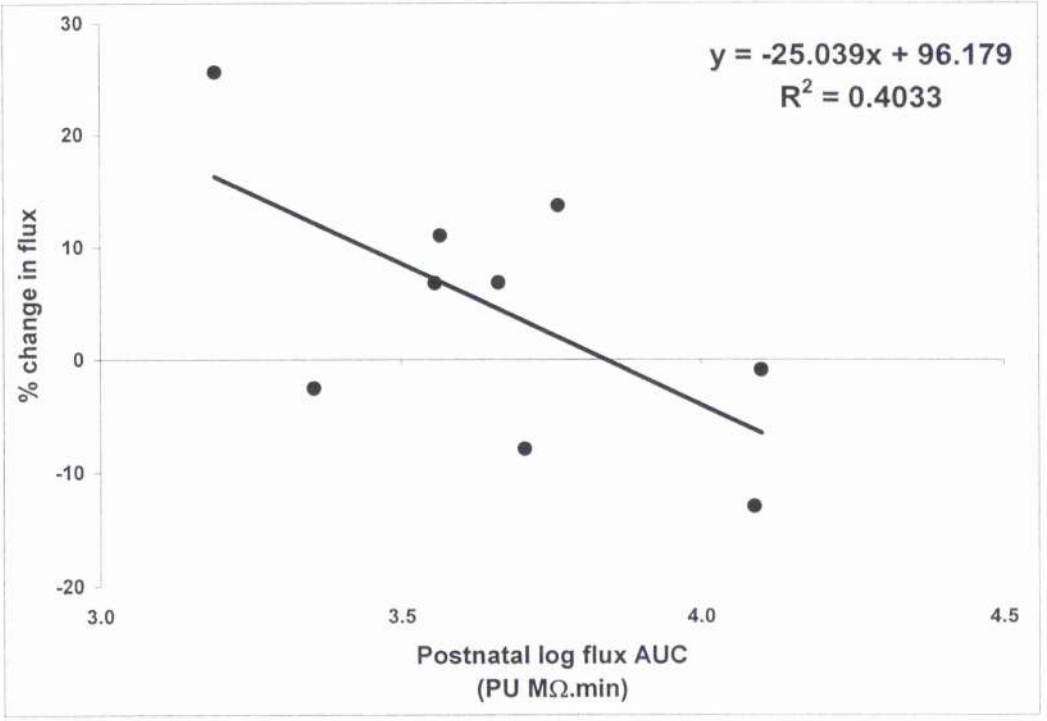

Figure 3b: Relationship of change in endothelial function in pregnancy to postnatal response (diabetic). Inverse correlation between the percentage change in non-pregnant to pregnant log transformed endothelial dependent (ACH) perfusion and postpartum log transformed endothelial dependent (ACH) perfusion response in the diabetic group. (n = 9) $r^2 = 0.4$, p=0.07.

Discussion

To the best of our knowledge, we have demonstrated for the first time using an in-vivo technique, the presence of microvascular dysfunction in the forearm skin resistance vessels of pregnant women with type I diabetes and no clinical evidence of microvascular complications. We demonstrate reduced vasodilator

responses to both endothelial dependent and independent stimuli, in contrast to previously reported work in diabetic pregnancy. [198] This finding was observed using a well tolerated non-invasive method of assessment, ideal for the pregnant patient. We have also been able to provide data concerning microvascular function in the non-pregnant state. In both groups the magnitude of improvement in ACH response with pregnancy correlated inversely with responses postpartum. In other words, our results indicate that pregnancy optimises vascular function in both healthy women and in those with diabetes. However, in the women with diabetes this improvement in vascular function is insufficient even to attain responses seen in healthy non-pregnant women. This finding suggests either a persistent vascular defect in young women with type I diabetes or the presence of toxic circulating factors. Our data, which considered circulating lipids, CRP and HBA1c, suggest that even a modest level of chronic hyperglycaemia is detrimental to vascular function.

Unlike previous hypotheses concerning the relationship of advancing gestation and endothelial function [75], pregnancy may not indeterminately improve endothelial function in all women. We propose there exists a gradient of vascular responses by which pregnancy can enhance vascular function, conferring most benefit to those with the most inadequate non-pregnant endothelium-dependent vasodilator mechanisms (Figures 3a and 3b). It is notable that sVCAM-1 concentration was significantly greater in the diabetic women in comparison to controls in the postnatal period, whereas during pregnancy the concentrations

were similar. Thus, we suggest that circulating markers of vascular function do not necessarily provide a sensitive examination of vascular function.

Previous investigations of microvascular function in non-pregnant type I diabetes have proposed endothelial dysfunction as a mechanism contributing to an increased incidence of cardiovascular complications within this group. [45] However, we have described not only a reduced response to an endothelial dependent vasodilator, but also reduced responses to an endothelial independent vasodilator in diabetic women, thus excluding any presumption concerning the function or integrity of the endothelial cell. These results concur with similar findings in type I diabetic subjects and their healthy relatives by other groups [36] [163] [149]. There may or may not be impaired production of endothelial derived nitric oxide (NO), however our results suggest alterations further downstream in the microvascular tree at the level of the vascular smooth muscle. This may indicate a problem with inactivation of endothelial derived NO or impaired function at the level of the vascular smooth muscle cell. In many studies of diabetic vascular function, hyperglycaemia is proposed as the main protagonist in the mediation of complications. Hyperglycaemia is believed to induce multiple changes in intracellular metabolism such as activation of the polyol pathway. Also, hyperglycaemia results in non-enzymatic glycation of circulating cells and proteins resulting in production of advanced glycation end products (AGEs). [194] [199] These molecules quench NO and in combination with free radicals such as superoxide anions, which directly inactivate NO, are responsible for reducing the

bio-availability of NO. [45] In our subjects, the difference between ACH responses in women with diabetes and controls was eliminated after adjustment for HbA1c. Our observation of a combined reduction in responses to endothelial dependent and independent vasodilatation may also reflect micro-vascular sclerosis, a more physical loss of vasodilator reserve. [47] In the early stages of diabetes some groups have suggested that increased microvascular flow and consequent increased capillary pressure results in up-regulated tissue perfusion, which may result in further shear stress applied to the vessel wall and consequent extra-vascular matrix proteins production as an injury response. The resultant effect may be micro-vascular sclerosis and impairment in vasodilatory reserve and autoregulation. [47]

We observed that the lipoprotein pattern was not perturbed in pregnant women with type 1 diabetes and if anything, HDL-cholesterol concentration was higher. This finding broadly concurs with existing data from non-pregnant populations in which the lipoprotein pattern appears anti-atherogenic in individuals with type I diabetes with optimal glycemic control [200]. Such a 'normal' lipid pattern in diabetic individuals is proposed to reflect insulin upregulation of key lipid-related enzymes such as lipoprotein lipase, responsible for breakdown of triglyceride-rich lipoproteins. Of note, a recent study of non-pregnant patients with type I diabetes reports elevated CRP concentrations in association with elevated VCAM-1 levels implicating a potential role for inflammatory mediators in the accelerated vascular disease of this group [201]. Inflammatory proteins such as CRP have also been

independently and strongly correlated with impaired endothelial function in both healthy non-pregnant individuals [56] and those with existing vascular disease [202]. In our cohort, CRP was correlated with ACH responses in the control subjects, and has been linked to endothelial function in other diseases. However, although higher in cases, the difference in CRP was not significant following adjustment for gestation and in addition, CRP did not attenuate the significant case-control difference in ACH responses. Clearly, larger studies are needed to address CRP changes in pre-gestational diabetes.

In conclusion, using an in vivo method of microvascular assessment, we have demonstrated that pregnancy optimises vascular function in both healthy women and in those with diabetes. However, in women with diabetes this improvement is insufficient even to attain responses seen in healthy non-pregnant women. Our data suggest a key role for the chronic effects of hyperglycaemia in the impaired vascular responsiveness in such women. These important observations provide an insight into mechanisms underlying pregnancy-associated and long-term vascular risk in women with type 1 diabetes.

Chapter Six

Metabolic, inflammatory and vascular derangement in pre-eclampsia and intrauterine growth restriction: adverse effects of adiposity and leptin.

Introduction

Pre-eclampsia is a pathological condition associated specifically with human pregnancy. It is characterised by a triad of symptoms, namely elevated blood pressure, proteinuria and oedema and its associated complications contribute significantly to maternal mortality and morbidity [203]. At present the only definitive treatment is termination of pregnancy with a consequent contribution of 40% to the iatrogenic premature delivery rate and associated perinatal morbidity and mortality. [204]

Pre-eclampsia is a multisystem disorder of which the patho-physiological mechanisms are poorly understood. Healthy pregnancy is associated with a significant drop in peripheral vascular resistance reaching a nadir around 20 weeks gestation before climbing to non-pregnant levels by the end of the 3rd trumester, despite increasing cardiac output and blood volume [60]. This physiological mechanism is believed to result from an increase in the production of endothelial cell derived nitric oxide. Ex-vivo experiments have demonstrated augmented endothelial dependent vasodilation and increased intraluminal flow in response to shear stress in small resistance vessels from pregnant women. [73 74] In-vivo assessment of endothelial dependent vasodilator mechanisms using both venous occlusion plethysmography and brachial artery Doppler ultrasound assessment of flow-mediated vasodilatation have confirmed these observations in pregnant subjects and suggest that endothelial dependent vasodilatation correlates

positively with gestation. [75 205] Pre-eclampsia, in terms of the maternal manifestation of vasomotor dysfunction/hypertension, has been proposed to result secondary to a failure in these mechanisms.

Endothelial activation and dysfunction are suggested as important mechanisms in the pathophysiology of pre-eclampsia. Increased sensitivity of maternal vasculature to angiotensin II can be demonstrated before hypertension develops [61]. Markers of increased coagulation activation can be demonstrated weeks to months before the development of the clinical disorder. [97 98 99] Several ex-vivo techniques have also supplied direct evidence of altered vascular reactivity in pre-eclampsia. Myographic techniques have demonstrated reduced endothelial dependent vasodilatation in isolated subcutaneous resistance vessels from women with pre-eclampsia [73 74 111]. However the mechanism by which this state arises is as yet elusive. One theory proposes impaired trophoblast invasion of the spiral arterioles to result in abnormal placentation and a high resistance circulation. This theory is also proposed in the growth restricted pregnancy despite the absence of maternal effect. This may result in episodes of hypoxia and ischaemia liberating oxidised molecules and inflammatory cytokines, suggesting pre-eclampsia to be a disease of inflammation [83]. These circulating molecules may pass into the maternal circulation to activate and damage endothelial cells.

Pre-eclampsia is also associated with significant metabolic derangement. Fasting triglyceride concentrations are doubled compared with normal pregnancy and a three-fold increase in VLDL and LDL-III concentrations are also observed. [102 206]

Small dense LDL III molecules are known to be highly atherogenic and can induce endothelial dysfunction and insulin resistance, also associated with pre-eclampsia. [93] It is of note that the specific vascular lesion of pre-eclampsia 'acute atherosis with lipid laden foam cells' as observed in spiral arterioles of the placental bed, is similar to that seen in atherosclerosis in the non-pregnant [77]. Using myographic techniques, Hayman et al related lipid concentrations, specifically reduced apo-AI (the major lipoprotein in HDL particles), to impaired endothelial function. Myometrial vessels from healthy pregnant women, incubated with plasma from pre-eclamptic women demonstrated reduced endothelial dependent vasodilatation providing evidence of the detrimental effect of a circulating factor upon endothelial function. [112]

Of interest, adiposity has been proposed to be a predictor of morbidity and mortality in cardiovascular disease and is associated, similarly to pre-eclampsia, with hyperinsulinamia, hypertriglyceridaemia, reduced HDL concentrations, impaired endothelial function and hypertension. [50] Obesity is also established as a significant risk factor in the aetiology of pre-eclampsia. [91] Most published work concerning endothelial function in pre-eclampsia has not considered or controlled for the effects of body mass index (BMI).

In-vivo techniques for assessment of microvascular function have not, as yet, demonstrated impaired endothelial cell function in pre-eclampsia. [207 208 76 209 210] We took advantage of developments in laser Doppler imaging technology, a non-

invasive technique used in combination with iontophoresis of endothelial dependent and independent vasodilators, to investigate cutaneous microvascular function in pre-eclamptic women. We also compared vascular function with circulating markers of endothelial damage and with simultaneously measured lipid and cytokine levels. We considered pre-eclamptic women in relation to their first trimester BMI and to BMI matched healthy pregnant controls. As pre-eclampsia and pregnancies complicated with intra-uterine growth restriction (IUGR) are proposed to arise from similarly impaired placentation, we included a cohort of women with IUGR.

Our aims were twofold. Firstly, to examine if microvascular endothelial function is altered in women with PE and in women with growth restricted pregnancies compared to healthy controls. Secondly, to comprehensively characterise candidate pathways proposed to mediate systemic disease simultaneously in women with PE and IUGR.

Subjects and Methods.

1. 15 women with PE were consecutively recruited. PE was defined as a blood pressure of greater than 140/90 mmHg on two separate occasions 4 hours apart or a single recording of a diastolic pressure of 110 mmHg, both in association with proteinuria consistent with or greater than two pluses on dipstick testing. Of the 15 pre-eclamptic women only 12 agreed to be tested with the LDI.
2. 16 women with pregnancies complicated with IUGR were also recruited. IUGR

was defined primarily by an ultrasound diagnosis of fetal abdominal circumference of less than the 5th centile. In addition 6 women had an amniotic fluid index of less than 6cm and/or abnormal end diastolic flow on umbilical artery Doppler waveform.

3. 30 normotensive control subjects with similar BMI to the PE group were recruited. A further subgroup of 16 controls with similar BMI to the women with IUGR was also defined.

Women participating in the study had no significant past medical history, such as peripheral vascular abnormalities, dermatological diseases or systemic disease processes such as diabetes mellitus. The study was performed according to the Declaration of Helsinki, approval was granted by the institutional ethics committee and all patients gave written informed consent. Clinical and laboratory measurements were performed as described in previous chapters after an overnight fast. In addition analysis of soluble adhesion molecules, ICAM-1 and e-selectin were performed. (Parameter Human sICAM-1 and e-selectin Immunoassay, R&D systems Inc., Oxon UK) Of the 15 pre-eclamptic women only 12 agreed to be tested with the laser Doppler perfusion imager. Perfusion measurements were performed as described in previous chapters using the low charge, incremental current delivery iontophoresis protocol. [178] An assessment of the overall response to drugs was obtained by taking the area under the perfusion.time curve (AUC). Units of perfusion were derived from multiplying

the perfusion.time integral by the resitance.time integral as calculated by continous voltage monitorring as described on page 63.

Statistical analyses

Dose response curves were expressed as means ± SEM and compared using 2-way ANOVA. Metabolic parameters were compared using the Mann Whitney U test. Univariate analysis relationships were expressed as Pearson's correlation co-efficients. Distribution of plasma results were assessed for normality and where skewed, considered as their log transformed integral.

Results

Demographic characteristics of each subject group are given in Table 1. There was no significant difference in BMI, age, smoking history, parity and gestation of examination between controls and PE women. Women with IUGR were found to be significantly leaner than PE women (IUGR: 22.2 (20-25.4) kg/m^2 Vs PE: 26(23.5-27.8) kg/m^2, p=0.044) therefore they were matched directly for BMI with a subgroup of the controls. Compared to this group women with IUGR had a higher proportion of smokers and were examined at an earlier gestation, reflecting the high-risk nature and pre-term identification and delivery of these women. Gestation of subsequent delivery was significantly less in the PE women and women with IUGR as compared with controls. Median birthweight centile of babies born to mothers with PE was significantly less than controls (50 vs 25, p=0.024), and as expected women with suspected antenatal IUGR, gave birth to

babies with weights less than the fifth percentile for gestational age. Only 50% of women with PE and IUGR achieved vaginal deliveries, which was significantly less than that seen in controls (80%). Of interest the section rate in the lean control group was only 9% compared with 20% in the overweight control group.

Table 1: Demographic characteristics of study subjects.

	Control (n=30)	PE (n=15)	Control (n=16)	IUGR (n=16)
Booking BMI (kg/m^2)	26.8 (21.8-30.2)	26.(24-27.8)	22.4 (20-24.6)	22.2 (20-25.4)
Age (years)	29 (25-32)	27 (22-29) *	26 (21-32)	25 (20.2-30.0)
Smokers	5 (16.7%)	5 (33.3%)	1 (6.2%)	7 (44%) †
Parity (primigravidae)	21 (70%)	13 (87%)	10 (62.5%)	7 (44%)
Gestation (weeks)	35 (34-37)	36 (32-37)	35 (35-37)	35 (32-35) †
Systolic BP (mmHg)	120 (110-130)	160 (150-169) ‡	110 (110-120)	116 (110-120)
Diastolic BP (mmHg)	75 (70-80)	100 (95-105) ‡	70 (60-80)	70 (60-80)
Booking systolic (mmHg)	120 (112-126)	117 (102-126)	118 (110-126)	110 (98-120.7)
Booking diastolic (mmHg)	71 (66.2-77.2)	70 (66-76.5)	64 (60-73)	60 (60-69.2)
Gestation of delivery (wks)	39 (38-40)	36 (33.7-37) ‡	40 (39-40)	37 (35-38) ‡
Birthweight centile	50 (30-75)	25 (8.25-50) †	40 (10-60)	2.5 (1-10) ‡
Caesarean Section	20%	50% †	9%	50% ‡

All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test and Chi-squared test. PE = Pre-eclampsia. BMI = Body Mass Index. BP=blood pressure. Statistical comparison of PE versus control and IUGR versus control: * $p<0.1$, † $p<0.05$, ‡ $p<0.001$

Pre-eclampsia

Results of fasting plasma lipoproteins, leptin, IL-6, CRP, insulin HbA1c, and soluble adhesion molecule concentrations are displayed in **Table 2.**

Metabolic parameters

Fasting plasma triglyceride and VLDL-C concentrations in the pre-eclamptic women were significantly higher than controls, as was fasting leptin, which was twice as high in pre-eclamptic women as compared with controls ($P<0.0001$) (**Table 2**). Despite this, there was no significant difference in fasting insulin concentration between women with PE and controls. These differences persisted after adjustment for differences in age, parity and smoking history.

Inflammatory parameters

IL-6 concentration and adhesion molecules, sVCAM-1 and E-selectin were significantly greater in PE women as compared with controls. Again these differences persisted after correction for age, parity and smoking history. Using univariate analysis, log leptin was found to correlate with log sVCAM-1 concentrations ($r=0.54$, $p=0.036$), and log IL-6 with log e-selectin ($r=0.66$, $p=0.014$) both independently of BMI (Leptin $T=4.18$, $p=0.001$; IL-6 $T=2.80$, $p=0.019$). No relationship existed between leptin and adhesion molecules in the control subjects ($r=-0.102$, $p=0.59$).

RESULTS	Control (n=30)	PE (n=15)	P value	Adjusted P values
Lipoproteins				
Cholesterol (mmol/l)	6.48 (5.68-7.28)	6.15 (5.3-7.65)	0.92	0.92
Triglyceride (mmol/l)	2.38 (2.05-2.95)	3.2 (2.75-4.75)	**0.002**	**0.001**
VLDL-C (mmol/l)	0.60 (0.34-0.85)	0.85 (0.65-1.15)	**0.02**	**0.03**
LDL-C (mmol/l)	4.05 (3.49-5.08)	3.5 (3.05-4.65)	0.31	0.51
HDL-C (mmol/l)	1.55 (1.40-1.80)	1.55 (1.25-1.8)	0.89	0.85
Inflammation				
Leptin (ng/ml)	40.5 (22.5-57)	74 (55.6-95)	**0.0001**	**0.001**
IL-6 (pcg/ml)	2.40 (1.76-3.09)	3.37 (1.7-8.5)	**0.03**	**0.02**
CRP (mg/ml)	2.74 (1.53-4.36)	3.45 (1.95-6.4)	0.30	0.21
VCAM-1 (ng/ml)	330 (249-391)	431 (321-526.3)	**0.02**	**0.02**
ICAM-1(ng/ml)	219 (179-235)	233.9 (168-258)	0.67	0.85
E-selectin (ng/ml)	51.3 (30.8-71.8)	55.98 (41-74.2)	0.15	0.06
Insulin sensitivity				
Insulin (mu/l)	9.8 (5.6-21.2)	14.5 (9.5-35)	0.09	0.21
HbA1c (%)	4.6 (4.3-4.7)	4.6 (4.5-4.9)	0.15	0.12

Table 2: Metabolic, and inflammatory parameters in pre-eclampsia.

All values are medians, (interquartile ranges). Statistical analysis performed using Mann-Whitney U test. PE = pre-eclampsia. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. HbA1c = Glycosylated Haemoglobin. IL= interleukin, CRP = C-reactive protein. ICAM = Intercellular adhesion molecule. VCAM = vascular cellular adhesion molecule. P values adjusted for differences in age, parity and smoking history.

Endothelial dependent vasodilatation

Twelve of the PE women, with a median BMI of 26 (IQ range 23.5-27.8), had perfusion measurements performed. Of those twelve, seven were taking anti-hypertensives; six taking labetalol and one taking nifedipine. Dose dependent perfusion response to ACH was not significantly different in controls as compared with pre-eclamptic women. **(Figure1)** Corrected area under the perfusion.time curve (AUC) for ACH response was also calculated for each individual woman and used in simple linear regression. Perfusion results were examined in relation to anti-hypertensive use. The seven women taking anti-hypertensives were found to have significantly worse endothelial dependent perfusion responses (ACH/AUC) than women not taking drugs (10304 (4727-16281) versus 23737 (13778-35039) PU MΩ.min, $p = 0.05$). Both systolic and diastolic blood pressures were found to correlate inversely with endothelial dependent perfusion responses (ACH/AUC). (Systolic BP v ACH: $r = -0.721$, $p = 0.008$; Diastolic BP v ACH: $r = -0.571$, $p = 0.05$).

Endothelial independent vasodilatation

There was no significant difference observed between control and pre-eclamptic women for dose dependent perfusion responses to SNP (P=0.69).

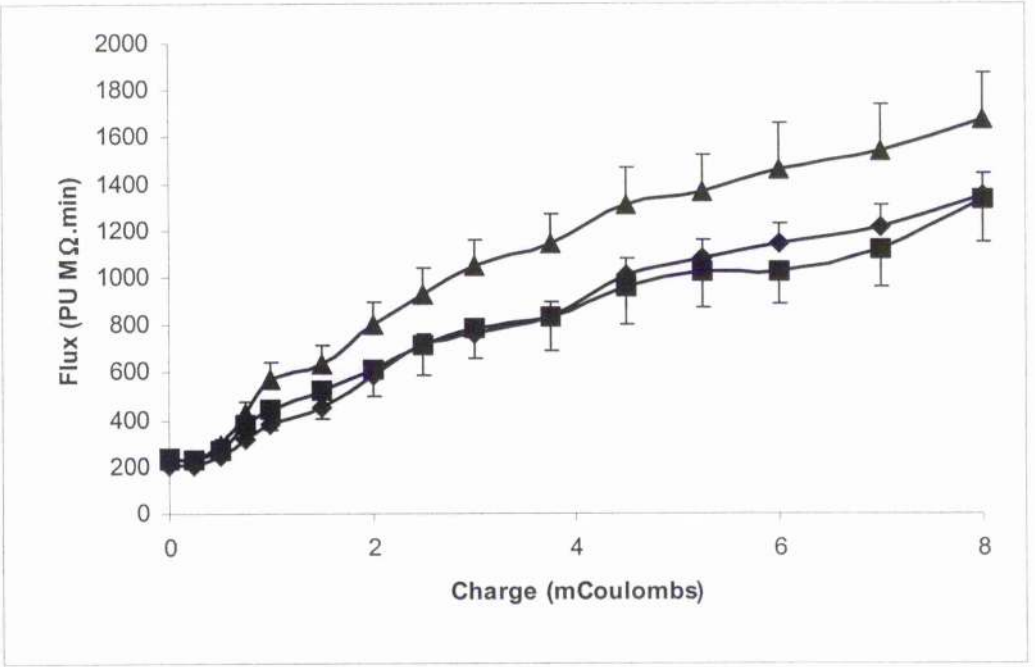

Figure 1: Endothelial dependent (ACH) vasodilatation.

Endothelial dependent (ACH) vasodilatation. Dose dependent perfusion response to ACH in control women (n=30, υ), pre-eclamptic (n=12, ν) and intrauterine growth restriction (n=16, σ). Data are mean +/- standard error (SEM). Control versus IUGR: $P < 0.01$; control versus pre-eclampsia: $P = 0.5$, 2-way ANOVA.

Intra-uterine growth restriction

Metabolic and Inflammatory parameters

LDL-C concentrations were significantly lower (3.0 (2.44-3.95) vs 4.0 (3.75-4.76) mmol/l, p=0.008) in the women with IUGR in comparison to controls following adjustment for differences in parity, gestation and smoking history **(Table 3).** Leptin was increased in the women with IUGR following similar adjustment but

this did not achieve statistical significance (P=0.1). No other significant differences were seen between the groups. Leptin did not correlate with any adhesion molecules.

RESULTS	IUGR (n=16)	Lean (n=16)	P value	Adjusted P value
Lipoproteins				
Cholesterol (mmol/l)	5.62 (4.9-6.5)	6.35 (5.8-6.8)	**0.03**	0.254
Triglyceride (mmol/l)	2.27 (1.56-3.07)	2.2 (1.98-2.56)	0.79	NS
VLDL-C (mmol/l)	0.62 (0.5-0.95)	0.55 (0.34-0.7)	0.16	NS
LDL-C (mmol/l)	3 (2.44-3.95)	4.05 (3.7-4.76)	**0.0019**	**0.021**
HDL-C (mmol/l)	1.67 (1.35-2.44)	1.725 (1.44-	0.95	NS
Inflammation				
Leptin (ng/ml)	27.1 (15.1-70.8)	23.8 (13.2-35.2)	0.17	0.1
IL-6 (pcg/ml)	2.07 (1.22-4.3)	2.1 (1.73-2.85)	0.74	NS
CRP (mg/ml)	3.61 (2.22-5.3)	2.25 (0.92-3.65)	0.07	0.176
VCAM-1 (ng/ml)	323 (289-415)	321 (276.3-	0.66	NS
ICAM-1 (ng/ml)	202	189	0.6	NS
E-selectin (ng/ml)	47.5	55	0.07	0.04
Insulin sensitivity				
Insulin (mu/l)	6.9 (3.8-15.5)	6.5 (4.6-9.7)	0.45	NS
HbA1c (%)	4.5 (4.3-4.6)	4.4 (4.2-4.7)	0.5	NS

Table 3: Metabolic, and inflammatory parameters.

All values are medians, (interquartile ranges). Statistical analysis performed using Mann-Whitney U test. PET = pre-eclampsia. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. HbA1c = Glycosylated Haemoglobin. IL= interleukin, CRP = C-reactive protein. VCAM = vascular cellular

adhesion molecule. Adjusted P value = adjustment for BMI, age, parity, gestation and smoking history. NS = not significant.

Endothelial dependent vasodilatation

Dose dependent perfusion response to ACH was significantly greater in women with IUGR (BMI 22kg/m^2) as compared with overweight controls (BMI 26kg/m^2). **(Figure 1;** $P < 0.01$**)**

Endothelial independent vasodilatation

There were no significant differences in dose -dependent perfusion response to SNP observed between women with pregnancies complicated with IUGR and controls ($p = 0.5$).

Discussion

This is one of the first studies to examine metabolic, inflammatory and vascular function simultaneously in groups of healthy pregnant women, women with pre-eclampsia and women with IUGR. We demonstrate significantly elevated triglyceride and VLDL-C concentrations in pre-eclamptic women compared to BMI matched controls. By comparison, women with IUGR alone had a specific reduction in LDL-C concentrations, confirming our earlier report. [121] We also noted a 4 unit lower median first trimester BMI in women who went on to develop a growth-restricted fetus as compared to those who developed PE (IUGR: 22.2 (20-25.4) kg/m^2 Vs PE: 26(23.5-27.8) kg/m^2, p=0.044). This 17% greater total body weight in women destined for PE may explain in part why systemic effects occur in PE but not those with IUGR alone. For example, one may speculate that

molecules liberated from a hypoxic placenta require the additional burden of a high maternal adipocyte mass to express their systemic influence to the full.

Leptin effects

Leptin concentration was nearly double in women with PE compared to BMI matched controls, suggesting elevation independent of total adiposity. Women with IUGR when considered in relation to BMI matched controls, had a trend towards a small elevation in leptin concentrations (27.1 (15.1-70.8) vs 23.8 (13.2-35.2) ng/ml, p = 0.001). Leptin is a 16kDA adipocyte-derived protein that plays an important role as an endocrine hormone regulating adipose tissue mass. Leptin is also manufactured in the placenta [211 212] and has recently been identified as an angiogenic growth factor in animal models. [213] Therefore its production by the placenta may play a role as a paracrine growth factor stimulating neovascularisation. Other groups have confirmed our findings of elevated concentrations of leptin in pre-eclamptic pregnancies [214] and this elevation pre-dates the clinical signs of pre-eclampsia [215] perhaps secondary to ischaemia. In support of this, upregulation of leptin production in cultured BeWo cells have been demonstrated in response to hypoxia [216 217] **(Figure 2)**.

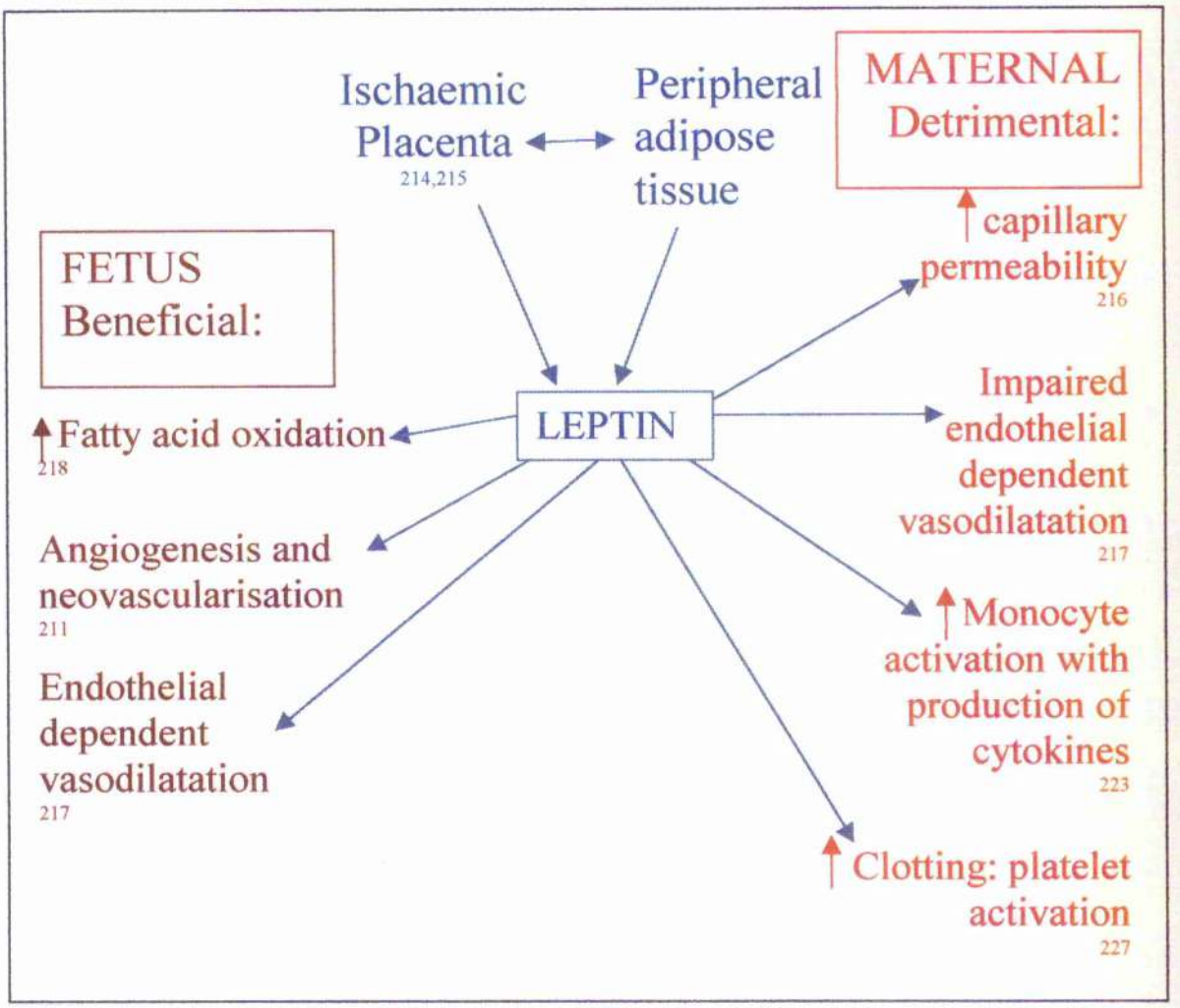

Figure 2 Potential mechanisms by which an excessive elevation in placental and potentially peripherally derived maternal leptin may effect the clinical syndrome of pre-eclampsia. A failure of this response to placental ischaemia may favour the clinical picture of IUGR, with maternal sparing from systemic effects.

The potential link between leptin and the maternal syndrome of pre-eclampsia is unclear. As described above, leptin plays a role as an angiogenic growth factor

The potential link between leptin and the maternal syndrome of pre-eclampsia is unclear. As described above, leptin plays a role as an angiogenic growth factor and Cao et al [218] have recently demonstrated in mice that new, leptin induced capillaries are fenestrated, with a rapid increase in vascular permeability observed in response to intradermal leptin administration. Certainly the clincial features of severe pre-eclampsia such as proteinuria, pulmonary and peripheral oedema, are thought to represent an increase in capillary permeability. In the leptin deficient ob/ob mouse, abnormalities in endothelial dependent vasodilatation are observed which administration of leptin or pre-incubation of endothelial cells with leptin can correct. [219] Therefore it may be that leptin acts somehow to produce endothelial dependent vasodilatation and the impaired vasodilatory effect observed in pre-eclampsia may be secondary to an exaggerated leptin resistance, such as that seen in the obese and/or insulin-resistant individual. In addition leptin induced angiogenesis may increase fatty acid oxidation in an attempt to maintain an appropriate balance between blood supply and fat deposits. This was demonstrated by *Yamagishi et al* [220] in association with induced reactive oxygen species in cultured endothelial cells. It is well established that oxidised molecules are associated with endothelial cell activation and damage [7] and this process is also implicated in the pathophysiology of pre-eclampsia [204]. In support of this, we have demonstrated a significant relationship between leptin and the soluble marker of endothelial activation VCAM-1 (leptin versus log VCAM: $r = 0.284$, $p = 0.028$; adjustment for BMI: $p=0.022$) We also demonstrate that although a small elevation in concentration of leptin in women with IUGR was noted, this was

such as leptin. Studies evaluating cord plasma concentrations of leptin have suggested that leptin concentration, when considered per kg. fetal weight, is significantly greater in growth restricted fetuses, perhaps consequent to abnormal placentation and resultant hypoxia. [221] [222] [223] We noted a significant inverse relationship between maternal third trimester leptin and subsequent birth centile of offspring (r = -0.40, adjusted p=0.016) **(Figure 3).** Potential mechanisms relating to this association are unclear although it is relevant that leptin enhances fatty acid oxidation, and thus efficient utilisation of maternal fatty acid stores (**Figure 2**). Therefore it may be that IUGR results partly due to an inadequate maternal response to placental ischaemia.

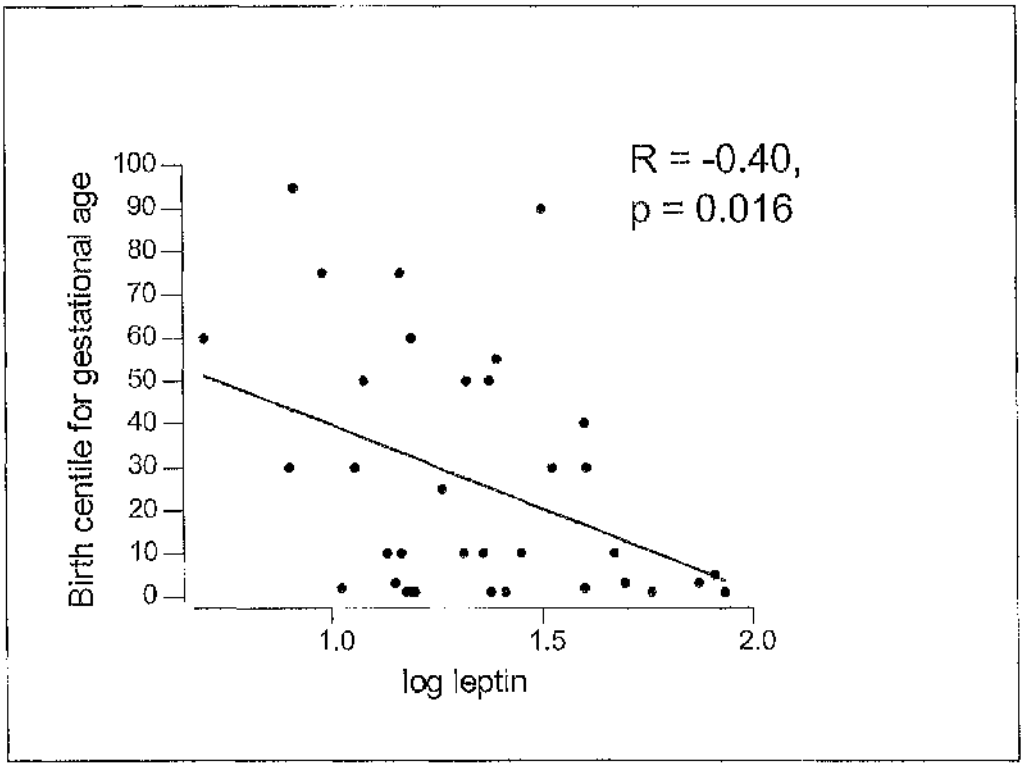

Figure 3: Relationship between maternal log transformed concentrations of leptin in third trimester and birth centile for gestational age, appropriate for sex of offspring.

Figure 3: Relationship between maternal log transformed concentrations of leptin in third trimester and birth centile for gestational age, appropriate for sex of offspring.

Inflammation

We observed an inflammatory phenotype with elevated circulating IL-6 concentrations in women with PE, but not in IUGR. VCAM-1 and E-selectin are glycoproteins expressed on the endothelial cell surface in response to an inflammatory, usually cytokine or oxidant mediated challenge. Their purposes are to recruit leukocytes and mediate leukocyte rolling and platelet-leukocyte aggregation, resulting in endothelial dysfunction and increased capillary permeability. [224] VCAM-1 and E-selectin were significantly elevated in pre-eclamptic women as compared with obese controls. In contrast, in women with IUGR, such parameters were not elevated and indeed after adjustment for differences in demographic factors, E-selectin concentrations in the women with IUGR were significantly less than controls (p=0.04). These data further support the hypothesis of an absence of maternal systemic inflammation in women with IUGR.

Evidence from existing literature demonstrates activated inflammatory cells such as granulocytes and monocytes in women with pre-eclampsia with release of pro-inflammatory cytokines such as TNFα, IL-6 and soluble phospholipase A2. [94 107] Human blood leukocytes express the leptin receptor, and the predominant leptin receptor-expressing cell type is the monocyte. In vitro, leptin has been shown to modulate the immune response, inducing the production of inflammatory cytokines, particularly TNF-α and IL-6. [225] **(Figure 2)** Also, an increase in serum

concentrations of both VCAM-1 and ICAM-1 have been demonstrated previously [108] [109]. Heyl W et al [226] noted a similar pattern of elevated E-selectin and VCAM-1 but not ICAM-1 in their investigation of serum from pre-eclamptic women. Greer et al [110] also noted a significant correlation between IL-6 and VCAM-1 ($r=0.539$, $p<0.005$) in women with pre-eclampsia. This relationship was confirmed in our subjects (log IL-6 versus log VCAM; $r = 0.268$, $p = 0.038$). The increase in VCAM is of particular interest in the metabolic context as VCAM is critical for monocyte activation to macrophages. The combination of monocyte activation and high triglyceride levels will lead to lipid laden marrophages in the vessel wall, which is a feature of the characteristic vascular lesion seen in pre-eclampsia.

In-vivo endothelial function

We did not demonstrate in-vivo evidence of microvascular dysfunction in women with pre-eclampsia. To our knowledge, all previous ex vivo studies demonstrating endothelial dysfunction in PET using dynamic tests have not considered nor controlled for BMI[73] [74] [111]. By contrast, in-vivo findings [207] [208] [76] [210] have consistently failed to demonstrate endothelial dysfunction in PET, and in general have controlled for BMI. Therefore, previous ex-vivo evidence of endothelial dysfunction in PET may reflect the inadvertent use of a lean control group.

These findings are obviously somewhat confusing and may be interpreted in different ways. Firstly, it may be that observed microvascular endothelial dysfunction may relate predominantly to total adiposity. Obesity is a risk factor

for cardiovascular disease [10] and particularly pre-eclampsia [90] and is associated with hyperleptinaemia, deranged lipid profiles, insulin resistance and inflammation [178].

Secondly, elevation in leptin in pre-eclamptic women is much greater than that explained by raised BMI alone and placental ischaemia has been implicated. As discussed above leptin may increase capillary permeability as seen in severe pre-eclampsia, independently of impaired endothelial dependent vasodilatation. In support of this, a recent study by Wang et al has demonstrated increased endothelial monolayer permeability in cells from pre-eclamptic women. [227] **(Figure 2)** Our technique for microvascular assessment of LDI in combination with iothophoresis of ACH and SNP does not detect endothelial permeability.

A third explanation may be that the technique of LDI in combination with iontophoresis does not examine the biologically appropriate vascular beds. We have examined the skin microvasculature on the assumption that these small vessels represent resistance vessels that in other experiments out-with pregnancy have been implicated in the development of cardiovascular disease. Reduced responsiveness to iontophoretic administration of ACH has been observed in diabetes [36 148 149] and hypercholesterolaemia [152] and in both these conditions there is a parallel reduction of the ACH response in the forearm circulation (a predominantly skeletal muscle vascular bed) assessed by venous occlusion plethysmography. [153 154] These observations were independent of BMI and

therefore in groups with elevated vascular risk this technique is adequate to demonstrate an effect. Vollebregt et al also failed to demonstrate endothelial dysfunction in pre-eclamptic women using laser Doppler fluxmetry but did demonstrate impaired local veno-arteriolar reflex in the capillaries of the skin [210]. It may be that in-vivo, the capillary beds, rather than the arterioles, manifest the increased endothelial permeability and dysfunction associated with the clinical syndrome of pre-eclampsia.

Finally, the syndrome of pre-eclampsia is a notoriously heterogeneous condition with many studies diluted by differing definitions of the condition. It has been suggested that mild disease at term is significantly different from severe premature disease requiring interventional delivery. Due to the bulky nature of our equipment, subjects had to come to the test area rather than the investigation being performed at the bedside. As a result, a less severe group of women were examined. The relationship between gestation of onset of disease and the response to ACH as determined by the area under the perfusion time curve (AUC), demonstrated an indirect correlation, that is the worst endothelial dependent responses were associated with earliest gestation of disease onset. ($r = 0.513$, $p = 0.088$) This may imply that our pre-eclamptic cohort may be diluted to some degree, with our median gestation of testing being 36weeks. The fact that over half our pre-eclamptic subjects were taking antihypertensives did not seem to adversely affect our perfusion results. One may predict that the more clinically severe cases would be those women where anti-hypertensives were necessary and

this is reflected in our results with the seven women taking medication demonstrating significantly worse responses to ACH. Also despite a significant proportion of the women taking anti-hypertensives, the blood pressure at the time of testing was found to demonstrate a significant inverse correlation with ACH response.

Therefore, in summary, we demonstrate elevated triglyceride and near double leptin levels in PE independent of BMI. By contrast, BMI and lipid (LDL-C) concentrations in women with IUGR were significantly reduced, suggesting a possible protective role for "leanness" with regard to maternal systemic effect. Women with PE but not IUGR also demonstrate elevations in circulating cytokine and adhesion molecules. Despite these findings, in vivo evidence of impaired endothelial-dependent vasodilatation in skin microvasculature was lacking in women with PE in comparison to a BMI matched group of healthy women. We propose that demonstrable in-vivo endothelial dysfunction may not represent the entire pathophysiological mechanism of PET and inflammation and increased capillary permeability, perhaps secondary to massively elevated levels of placentally derived leptin, may result in much of the clinical syndrome of PET (**Figure 2**).

Chapter 7

Pre-eclampsia and maternal coronary heart disease may be linked through inflammation and insulin resistance.

Introduction

Epidemiological studies have recently demonstrated a relationship between pre-eclampsia and an increased risk of maternal coronary heart disease in later life. [228] [88] However, there are few data available to explain any underlying mechanism. Pre-eclampsia shares common pathological features with atherosclerosis, both biochemical (insulin resistance, hypertryglyceridaemia, thrombotic and pro-inflammatory changes) [89] [229] and biological (endothelial dysfunction) [73] [228] [113]. Thus common risk factors, either genotypic or phenotypic, may underlie both pre-eclampsia and coronary heart disease.

Only one small study examining insulin sensitivity [89] has assessed women at a considerable interval (17 years) after a pre-eclamptic pregnancy; an age when risk factors for coronary heart disease may be more relevant. Furthermore, despite the predictive value of inflammatory markers for vascular disease in women [13] there are limited data available in relation to women with previous pre-eclampsia. The endpoint of these metabolic and inflammatory disruptions is believed to be endothelial dysfunction and this has been proposed as a mechanism preceding the development of coronary artery disease. [229] [143] Endothelial dysfunction has been demonstrated in vessels from pre-eclamptic women during pregnancy and recently Chambers et al demonstrated in-vivo, impaired endothelial function at least 3 months (median 3 years) postpartum. [2301] Our aim in this study therefore was firstly, to test the hypothesis that insulin resistance, inflammation, and hyperlipidaemia would persist in women with a history of pre-eclampsia up to 15-

25 years following pregnancy and secondly that within this group there would exist demonstrable endothelial dysfunction.

Methods

This study was carried out in two sections. All work was performed according to the Declaration of Helsinki, approval was granted by the institutional ethics committee and all patients gave written informed consent.

1. ***Indirect examination of cardiovascular risk factors***

   (serum lipids, markers of inflammation and glycaemic control)

   Primigravidae delivering between 1975 and 1985 with proteinuric pre-eclampsia and controls, matched as a group for time since index pregnancy, parity and current body mass index, were identified from medical records. Forty women with a history of pre-eclampsia and 40 controls were recruited. Blood pressure, body mass index, and abdominal circumference were measured. Venous blood was taken after an overnight fast and analysis performed as previously described in preceding chapters.

2. *Direct examination of cardiovascular risk factors.*

   (In-vivo microvascular function)

   Of the original cohort, 10 cases and 10 controls were invited to re-attend for in-vivo assessment of microvascular endothelial function using laser Doppler perfusion imaging and iontophoretic administration of microvascular vasodilators

acetylcholine (ACH; endothelial dependent) and sodium nitroprusside (SNP; endothelial independent) as previously described in chapter two. [178] Units of perfusion were derived from multiplying the perfusion.time integral by the resitance.time integral as calculated by continous voltage monitorring as described on page 63. This group was selected by writing to the women who participated in part one of the study and inviting them to reply if they were able to return for further examination. The first ten cases and controls to reply were investigated.

Statistical analyses

Dose response curves were expressed as means ± SEM and comparisons were by 2-way ANOVA. Other data are expressed as medians with the interquartile range as the measure of variability. For these data the Mann Whitney U test were used for comparisons between groups. Linear relationships between variables were examined using the Pearson correlation coefficient after normalising skewed variables by log transformation.

Results

1. *Indirect examination of cardiovascular risk factors*

   (serum lipids, markers of inflammation and glycaemic control)

   The demographic details of these groups are demonstrated in **Table 1.**

Characteristics	PET group *N=40*	Control group *n=40*	P-value
Age (years)	43 (40-47)	44 (43-47)	0.5
Time since index preg. (years)	20 (17.5-22)	20 (17-23)	0.96
BMI (kg/m^2)	27 (23-30)	26 (23-28)	0.5
Abdominal circ. (inches)	32 (30-36)	31.5 (28.7-34)	0.46
Parity (1, 2, >2)	12, 16, 12	12, 16, 12	0.94*
Systolic pressure (mmHg)	124 (114-136)	116 (108-130)	0.086
Diastolic pressure (mmHg)	83 (74-88)	76 (69-83)	0.035
Smokers (%)	9 (22.5%)	6 (15%)	0.39*
Hypertensive Rx	7 (17.5%)	2 (5%)	<0.08*
Dyslipidaemia Rx	2 (5%)	0 (0%)	0.152*
Hormone Rx (HRT or contraception)	13 (32.5%)	6 (15%)	0.008*
Menopausal (%)$^+$	15 (37.5%)	7 (17.5%)	0.045
Index pregnancy			
Age (years)	24 (21.2-26)	25 (21-27)	0.83
BMI (kg/m^2)	22 (21-24.5)	22 (20-23)	0.02
Smokers (%)	10 (25%)	11 (27.5%)	0.8*
Social class (DEPCAT score)	4 (2-7)	4 (2-6)	0.46
Gestation at delivery (weeks)	36 (33.2-38)	40 (38-41)	<0.001
Birth centile (weeks)	18 (5.2-64.2)	60 (27.5-90)	0.006

Table 1. Demographic characteristics (Part 1) All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test, $^+$Chi-squared test. PET = Pre-eclampsia. BMI = Body Mass Index. Preg = pregnancy, circ = circumference. HRT = hormone replacement therapy.

There were no significant differences in age, BMI, abdominal circumference, smoking status and parity between the two groups at the time of testing. Although normotensive, the women with a past history of pre-eclampsia were found to have higher blood pressure than the controls. (124/83 versus 116/76 mmHg). Of interest, a greater proportion of the women with a past history of pre-eclampsia

were taking anti-hypertensive, lipid-lowering and hormonal therapy (contraceptive and hormone replacement therapy) at the time of testing. In addition, despite similar ages, a significantly greater proportion of the women with a past history of PE were menopausal (P=0.045). Two of the women with a past history of pre-eclampsia were diabetic. With regards to the index pregnancy, the women with pre-eclampsia had a significantly greater BMI (p=0.02), earlier gestation of delivery (<0.001) and gave birth to babies with a lower birth weight centile (0.006). The social class distribution was significantly different at the time of the index pregnancy.

There was no difference in fasting lipid concentrations. (**Table 2**) VCAM-1 and ICAM-1 concentrations were significantly higher in the pre-eclampsia group, and the difference in ICAM-1 but not VCAM-1 concentrations persisted after adjustment for antihypertensive, lipid-lowering, and hormonal therapy. (ICAM: T=-2.77, p=0.007; VCAM: T=-1.67, p=0.1). The women with a past history of pre-eclampsia had higher fasting insulin and significantly greater HbA1c concentrations (p=0.004). Difference in HbA1c concentrations also persisted after adjustment for the above factors (T=-2.65 p=0.01) and on re-analysis after exclusion of the two diabetic women the statistical difference in HbA1c concentrations persisted (median 4.7% versus 4.5%, p=0.01). Smoking influenced ICAM concentration but the relationship with pre-eclampsia persisted on logistic regression (T=-2.15, p=0.035). Within the post pre-eclamptic women, log VCAM-1 correlated with BMI (r = 0.4, p=0.009), log leptin (r=0.33, p=0.04), and log

HBA1c (r=0.44, p=0.005). Log ICAM-1 correlated indirectly with log HDL (r=-0.38, p=0.01).

Variable	PET group *n=40*	Control group *n=40*	P-value
Lipids			
Cholesterol (mmol/L)	5.2 (4.4-5.6)	4.7 (4.0-5.6)	0.26
Triglyceride (mmol/L)	1.0 (0.7-1.3)	0.9 (0.7-1.2)	0.79
VLDL-C (mmol/l)	0.35 (0.2-0.4)	0.36 (0.2-0.5)	0.98
LDL-C (mmol/l)	2.85 (2.5-3.6)	2.81 (2.1-3.6)	0.41
HDL-C (mmol/l)	1.55 (1.3-1.8)	1.45 (1.2-1.8)	0.38
Inflammation			
ICAM-1 (ng/ml)	351 (249-449)	243 (205-315)	0.002
Nonsmokers	337 (235-393)	220 (204-268)	0.004
Smokers	452 (356-583)	336 (241-568)	0.26
VCAM-1 (ng/ml)	390 (322-460)	342 (272-417)	0.038
E-selectin (ng/ml)	59.6 (38.2-77.4)	49.8 (32.5-75)	0.44
CRP (mg/L)	1.42 (0.7-4.3)	1.19 (0.6-2.8)	0.25
Metabolic			
HBA1c (%)	4.7 (4.4-5.2)	4.5 (4.4-4.6)	0.004
Insulin (mIU/L)	8.35 (5.3-12.8)	6.4 (4.5-9.3)	0.08
Leptin (ng/ml)	33.7 (19.4-51.2)	25.2 (18-37)	0.13

Table 2 Inflammatory and metabolic markers (Part 1) All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test. ICAM = intracellular adhesion molecule. VLDL-C = Very Low Density Lipoprotein Cholesterol. LDL-C = Low Density Lipoprotein Cholesterol. HDL-C = High Density Lipoprotein Cholesterol. VCAM = vascular cellular adhesion molecule. CRP = C-reactive protein. HbA1c = glycosylated haemoglobin.

2) Direct examination of cardiovascular risk factors. (In-vivo microvascular function)

Demographic details of the subgroup investigated are displayed in **table 3.** There were no significant differences in age and BMI at the time of testing, however the post pre-eclamptic group did have a significantly higher diastolic blood pressure (84 v 68 mmHg, p =0.007) and a greater proportion of women taking antihypertensive therapy. As before, the BMI at the time of index pregnancy was greater in the pre-eclamptic women. (23 v 20 kg/m^2)

Characteristics	**PET group** *N=10*	**Control group** *n=10*	**P-value**
Age (years)	44.5 (38-47)	46 (42-48)	0.4
Time since index preg. (years)	22.5 (16-23)	20 (18-24)	0.7
BMI (kg/m^2)	24 (21-28)	23 (22-26)	0.6
Abdominal circ. (inches)	29 (28-32)	29.5 (26-32)	0.9
Systolic pressure (mmHg)	130 (117-136)	120 (98-131)	0.16
Diastolic pressure (mmHg)	84 (77-87)	68 (60-77)	0.007
Smokers	2	2	0.98*
Hormone therapy	5	3	0.463*
Antihypertensive therapy	3	0	0.07*
Index pregnancy			
Age (years)	24.5 (21-30)	25 (21-30)	0.7
BMI (kg/m^2)	23 (21-24)	20 (19-22)	0.038
Smokers	1	3	0.2*
Gestation at delivery (weeks)	36 (32-38)	39 (38-40)	0.01
Birth centile (weeks)	17 (4-69)	60 (34-85)	0.13

Table 3. Demographic characteristics (Part 2) All values are medians (interquartile ranges). Statistical analysis performed using Mann-Whitney U test, * Chi-squared test. PET = Pre-eclampsia. BMI = Body Mass Index. Preg = pregnancy. Circ = circumference

Endothelial dependent vasodilatation

Dose dependent perfusion response to ACH was significantly greater in the control as compared with post pre-eclamptic women (**Figure 1A**, P =0.005).

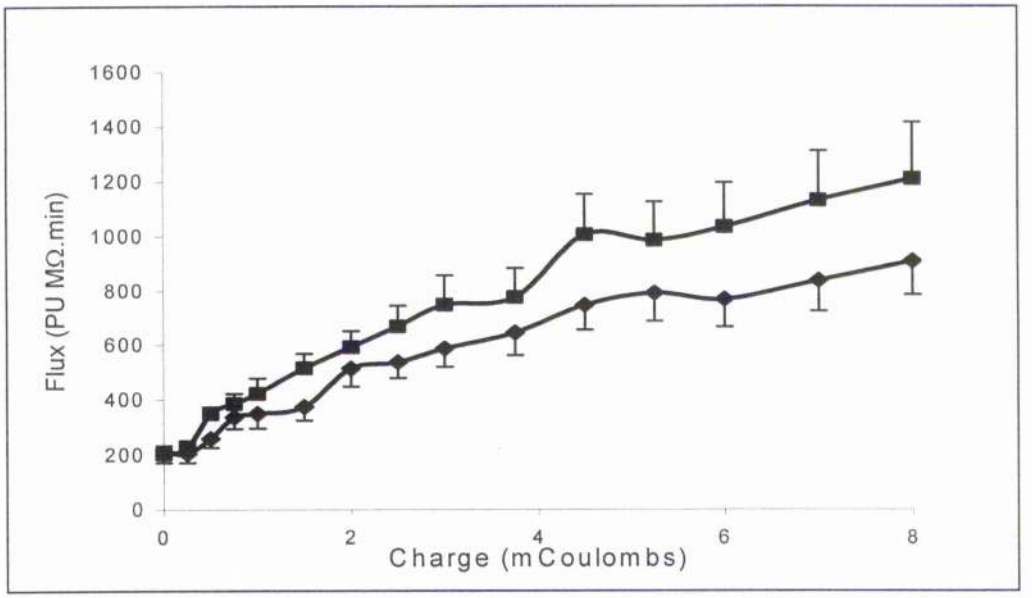

Figure 1: Endothelial dependent (ACH) vasodilatation.

A) Dose dependent perfusion response to ACH in post pre-eclamptic women (n=10; ♦) and control women (n=10; ■). Data are mean +/- standard error (SEM). P = 0.005, 2-way ANOVA.

Corrected area under the perfusion.time curve for ACH response was also calculated for each individual woman and reflected these observations with a significantly smaller response seen in the women with a past history of pre-eclampsia. (9358 (4020) v 14979 (6530) PU MΩ.min, p = 0.044 **Figure 1B**) This relationship persisted after correction for smoking history, overall parity, age, BMI and blood pressure at the time of testing. However after adjustment for differences in those women taking antihypertensives, the significance was reduced (T=1.86, p=0.08).

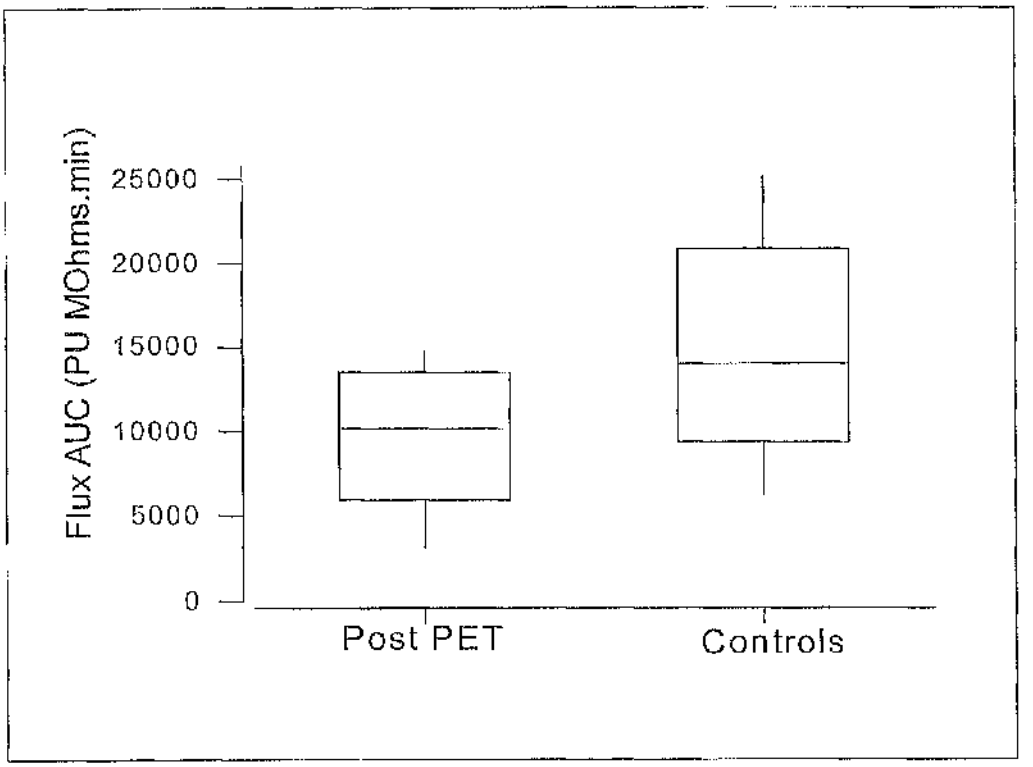

Figure 1: Endothelial dependent (ACH) vasodilatation.

B) Boxplot of area under the perfusion.time curve (AUC) for response to ACH in post pre-eclamptic women (n=10) and control women (n=10). P = 0.044, students t-test

Endothelial independent vasodilatation

Dose dependent perfusion response to SNP was significantly greater in the control as compared with post pre-eclamptic women (**Figure 2A**, P=0.023).

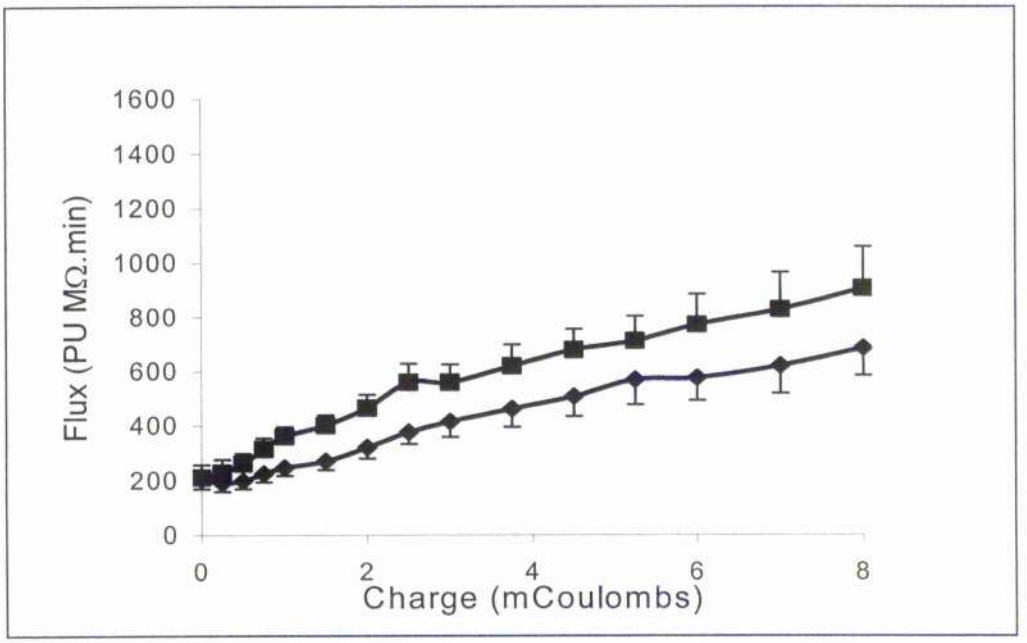

Figure 2: Endothelial independent (SNP) vasodilatation

A) Dose dependent perfusion response to SNP in post pre-eclamptic women (n=10; ♦) and control women (n=10; ■). Data are mean +/- standard error (SEM). P = 0.023, 2-way ANOVA.

Corrected area under the perfusion.time curve for SNP response was also calculated for each individual woman and again showed a significantly smaller response in women with a past history of pre-eclampsia. (7861 (3676) v 12136 (4620) PU MΩ.min, p = 0.043 **Figure 2B**) Again, this relationship persisted after correction for similar demographic factors as described above, including those women taking hypertensive therapy.

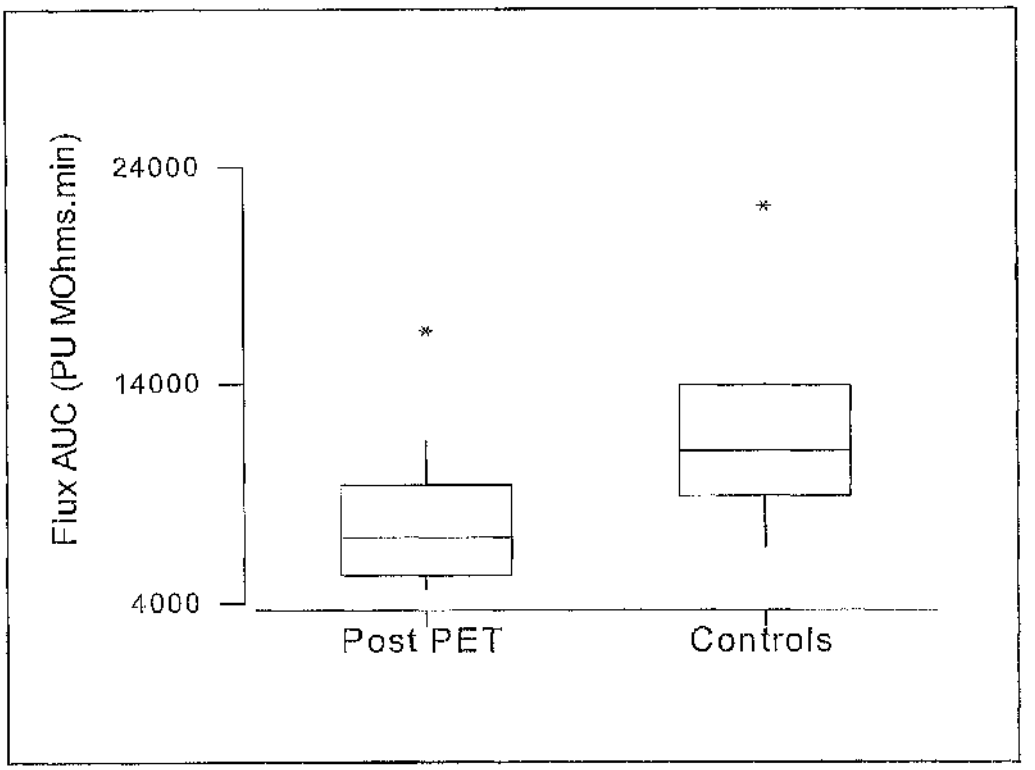

Figure 2: Endothelial independent (SNP) vasodilatation

B) Boxplot of area under the perfusion.time curve (AUC) for response to SNP in post pre-eclamptic women (n=10) and control women (n=10). P = 0.043, students t-test.

Discussion

These data show for the first time that women with a past history of pre-eclampsia have increased plasma concentrations of the inflammatory markers, VCAM-1 and ICAM-1, 15 years or more after the index pregnancy compared to controls. We have also demonstrated, using a novel, non-invasive, in–vivo technique, impaired endothelial dependent vasodilatation in women with a past history of pre-eclampsia. *Chambers et al* also examined these parameters in a group of women at least 3 months (median 3 years) postpartum. [230] In concordance with our results, they did not identify any significant difference in lipids between the post pre-

eclamptic women and controls but did not identify any elevation in concentration of adhesion molecules in women with a single episode of pre-eclampsia despite demonstrating, using a different in-vivo technique, impaired endothelial dysfunction. This may represent the short interval at which these factors were evaluated following the index pregnancy.

Adhesion molecules are expressed on the surface of vascular endothelial cells in response to inflammatory cytokines and are believed to encourage adhesion of circulating leukocytes and their subsequent trans-endothelial migration. This process is believed to be critical in the progress of early atherogenesis and cross-sectional data suggest that soluble forms of these proteins are elevated in patients with atherosclerosis. [231] [232] ICAM-1 in particular has recently been established to be independently predictive for coronary heart disease [12] and from the Atherosclerosis Risk In Communities (ARIC) study, those with baseline concentrations of sICAM-1 in the highest quartile, when followed up for five years, were found to have a five-fold increased risk of incident coronary heart disease (RR 5.5, 95%CI 2.5-12.2). [233] These data are proposed to support the hypothesis that endothelial activation and damage, or "inflammation" occur early in the atherosclerotic process.

Women with a past history of pre-eclampsia also have greater insulin and HBA1c concentrations, confirming observations from an earlier report [89]. We also demonstrated in our population that concentrations of soluble VCAM-1 correlate

with HBA1c and BMI. Serne et al [42] demonstrated an inverse relationship between blood pressure and insulin sensitivity in the non-pregnant individual as well as a strong positive relationship between microvascular function and insulin sensitivity. Consequently, this group propose abdominal fat to be a source of free fatty acids (FFA) and cytokines, which are secreted into the circulation. These circulating factors would promote vascular inflammation and endothelial dysfunction, and result in insulin resistance and hypertension as described above. Certainly, the deleterious effect of FFA and cytokines on endothelial function has been demonstrated previously. [11 43] One may hypothesise, therefore, that reduction in BMI, with consequent reduction in inflammation and improvement in insulin sensitivity may provide some hope of altering this predisposed risk of cardiovascular disease. *Ziccardi et al* have demonstrated in a group of obese pre-menopausal women that weight loss over one year was associated with a reduction in inflammatory cytokine concentrations, a reduction in adhesion molecule concentrations including ICAM-1 and an improvement in endothelial dependent vascular function. [234]

In conclusion therefore, these data suggest that the phenotype associated with pre-eclampsia is linked to novel mechanisms underlying coronary heart disease and may explain, in part, the epidemiological association between pre-eclampsia and coronary heart disease. This association may be genetically or phenotypically determined but evidence exists to suggest that such lifestyle modifications as weight loss may ameliorate this underlying cardiovascular disease risk.

Chapter Eight

Conclusions

The origins of atherosclerotic cardiovascular disease are believed to lie in the functionality of the microvascular endothelium. These cells provide not only a structural role but they also act as a highly complex endocrine and paracrine organ. They produce many vaso-active substances, such as vasodilators, nitric oxide, the eicosanoids, particularly prostacyclin, and endothelial derived hyperpolarising factor, as well as the vasoconstrictor endothelin, all of which play a role in the modulation of local and systemic blood flow and peripheral pressure. However, the endothelium and its products also provide a protective function for the vessel wall, inhibiting platelet aggregation, smooth muscle cell migration and proliferation, adhesion molecule expression and consequently monocyte adhesion, thus inhibiting the development of thrombosis and atheromatous plaques. The disease process involved in the activation and eventual damage of this cell layer is believed to arise secondary to an inflammatory stimulus. Some individuals will inherit a genetic tendency to be more predisposed to the effects of inflammation or to be more "pro-inflammatory" either through oxidative processes or through their production of inflammatory molecules. Acquired factors, such as older age and obesity, as well as environmental factors, for example cigarette smoking or social deprivation, will also contribute.

Many of the clinical risk factors for cardiovascular disease in later life are also shared by cardiovascular disease in pregnancy; that is pre-eclampsia. Epidemiologically, a past history of a pregnancy affected by pre-eclampsia confers an elevated risk of premature development of ischaemic heart disease,

hypertension and type two diabetes. At present, the exact mechanisms involved in the pathophysiology of pre-eclampsia are poorly understood. Also, little is known concerning the mechanisms involved in the association between such complications of pregnancy and cardiovascular disease in later life. Endothelial cell dysfunction would seem a reasonable hypothesis linking these two conditions and this has been demonstrated in vitro in vessels and cells from pre-eclamptic women.

Therefore, in chapter two and three we described how we devised a robust protocol for the in-vivo assessment of microvascular endothelial cell function, which could be utilised for the examination of pregnant women. Thus this technique required to be safe and well tolerated. We used laser Doppler imaging in combination with iontophoretic topical drug delivery for the assessment of the skin microvasculature. In chapter two we focused on drug delivery and noted that quantity of drug delivered is not only proportional to the amount of charge applied, as suggested from previous literature, but also to the individual electrical properties of the skin. We introduced continuous voltage monitoring during the procedure, which allowed us to calculate resistance using Ohm's law. We were able to demonstrate a strong inverse relationship between skin resistance and vasodilator effect and therefore were able to develop a correction factor, by multiplying the perfusion.time integral by the reciprocal of impedance or more simply by resistance.time integral. We studied vascular responses to the vasodilators, ACH (endothelial dependent) and SNP (endothelial independent)

and identified that response to ACH is unlikely to be mediated by prostanoids as vasodilatation secondary to this drug was not reduced by administration of cyclo-oygenase inhibition.

In chapter three we focused on the elimination of electrically-induced hyperaemia. Iontophoresis or essentially the passage of a small electrical charge through the microvasculature can result in a hyperaemic response independent of any drug delivery and therefore this would complicate the interpretation of any results. Again the use of continuous voltage monitoring enabled us to realise that the production of such a response is associated with high voltage secondary to high resistance or current. These situations can result consequent to use of a non-conductive vehicle such as distilled water or large amounts of charge either by using high currents or very small areas of current application. Therefore we were able to show that drugs in solution were associated with low voltages and therefore were unlikely to be complicated by this effect. We identified that use of large drug chambers, low charges and an ionic solution such as 0.5% NaCl for the vehicle, produced no electrically induced hyperaemia and was also associated with low voltages. We also identified that this effect appeared to be, at least in part, prostaglandin dependent, as cyclo-oxygenase inhibition reduced the response.

We then went on to utilise this now robust and well-tolerated in vivo technique for

the examination of microvascular function in pregnant women. As discussed above many clinical risk factors are shared between vascular diseases of pregnancy and later life. In chapter four and five, we examined pregnant women with such clinical risk factors in an attempt to elucidate potential mechanisms predisposing these women to an elevated risk of pre-eclampsia.

In chapter four we examined obese pregnant women in relation to lean. Out-with pregnancy, adipose tissue takes on a dynamic role, releasing many inflammatory molecules such as cytokines and free fatty acids known to result in microvascular dysfunction. We demonstrated that these associations persist in pregnancy, despite the beneficial effects of other pregnancy associated changes with regards to vascular function. Our data showed that obesity in pregnancy is associated not only with marked hyperinsulinaemia (without necessarily glucose dysregulation) and dyslipidaemia (elevated triglyceride an reduced HDL-C), but also impaired microvascular function, higher blood pressure and inflammatory up-regulation (elevated IL-6 and CRP concentrations). Such a spectrum of risk factors may contribute to maternal complications in obese women.

In chapter five we examined healthy women and women with type 1 diabetes in the third trimester of pregnancy and again in the postpartum period.
Microvascular function in both controls and diabetic women improved during pregnancy although responses in women with diabetes were significantly inferior

to controls during both these periods despite similar lipoprotein profiles. Moreover, in both groups the magnitude of improvement in ACH response with pregnancy correlated inversely with responses postpartum. The difference in vascular responsivity between cases and controls was significantly attenuated by adjustment for differences in HbA1c, but not CRP concentrations in the two groups. These data suggest that pregnancy enhances microvascular function, conferring the greatest improvement for those individuals with the worst microvascular function out-with pregnancy. However, in women with diabetes such improvements are insufficient to attain responses seen even in healthy, non-pregnant women. This suggests a persistent vascular defect in young women with type I diabetes that may contribute to adverse pregnancy outcome. Our data suggest a role for the chronic effects of hyperglycaemia in the impaired vascular responsiveness in such women.

As described previously, pre-eclampsia is proposed to arise secondary to an inflammatory insult, activating and damaging endothelial integrity. One hypothesis for the source of this insult is a factor released into the maternal systemic circulation from a poorly implanted and ischaemic placenta. In chapter six our technique for microvascular assessment did not confirm endothelial dysfunction as the main pathological factor in the mechanism of pre-eclampsia. We noted a stepwise decrease in endothelial function from lean (mean BMI 22 kg/m^2), to pre-eclamptic (mean BMI 26 kg/m^2) although worst results were seen in obese normotensive women (mean BMI 32 kg/m^2). We demonstrated elevated

concentrations of both fasting leptin and triglyceride in pre-eclamptic women in relation to obese controls, suggesting this effect to be independent of BMI. As suspected women with IUGR were found to have normal endothelial function comparable with lean individuals and had a significantly lower BMI at booking than pre-eclamptic women. We also observed reduced LDL-C concentrations in women with IUGR as compared with pre-eclamptic women and controls. This may suggest a potential protective role for "leanness" with regard to maternal systemic effect. We noted a stepwise increase from lean to obese to pre-eclamptic in the concentration of the inflammatory cytokine IL-6 consistent with the inflammatory theory of pre-eclampsia. In support of this we demonstrated a significant elevation in the circulating concentrations of the adhesion molecules, VCAM-1 and E-selectin in the pre-eclamptic cohort as compared with obese controls. We propose that demonstrable in-vivo endothelial dysfunction as measured by this technique, may not represent the entire pathophysiological mechanism of PET. However, inflammation has been indirectly demonstrated by elevated concentrations of soluble circulating markers and IL-6. We propose that secondary to placental hypoxia, massively elevated levels of placentally derived leptin and inflammatory cytokines may increase maternal systemic capillary permeability and result in some of the clinical syndrome of severe pre-eclampsia. In the obese individual this may be exaggerated by an increased adipocyte mass with elevated inflammatory cytokines, free fatty acids and oxidated lipoproteins, whereas in the lean individual with IUGR, the placental production of inflammatory molecules has less of a maternal systemic insult. Vascular

dysfunction resulting in hypertension and vascular lability may be demonstrable in other vascular beds. Therefore, in-vivo methods of microvascular function assessment, using LDI and iontophoresis, is not a sensitive or specific test for the diagnosis or prediction of pre-eclampsia and will have little impact in clinical management of this condition.

In chapter seven we demonstrated that healthy women with a past history of pre-eclampsia, 15-25 years after the index pregnancy, have increased plasma concentrations of the adhesion molecules and markers of endothelial damage, VCAM-1 and ICAM-1. We also demonstrated impaired endothelial dependent vasodilatation in women with a past history of pre-eclampsia. These data suggest that the phenotype associated with pre-eclampsia is linked to novel mechanisms underlying coronary heart disease and may explain, in part, the epidemiological association between pre-eclampsia and coronary heart disease. This association may be genetically or phenotypically determined but evidence exists to suggest that such lifestyle modifications as weight loss and regular exercise may improve endothelial function and metabolic factors such as insulin sensitivity and therefore may ameliorate this underlying cardiovascular disease risk.

Therefore in summary, we have developed a robust protocol for the in-vivo assessment of cutaneous microvascular function. This technique has not confirmed previous in-vitro observations that pre-eclampsia arises secondary to

endothelial dysfunction and in fact, obese women had most severe alterations in microvascular function and remained normotensive. Certainly endothelial dysfunction contributes to pre-eclampsia to some extent and is implicated by elevated concentrations of circulating soluble adhesion molecules. Also we have identified that this state does persist into later years and good evidence exists outwith pregnancy to suggest that this phenotype is associated with a significantly elevated risk of ischaemic heart disease. It may be that women with a pre-existing vascular and/or inflammatory abnormality, which could be either genetically inherited or acquired through "intra-uterine fetal programming" or environmental factors, going into pregnancy, have a greater chance of initial problems with placental implantation and then maternal systemic effect resulting in pre-eclampsia. Such groups are those women, as described above, with obesity or diabetes. Thereafter, further inflammatory insult arises from the ischaemic placenta and a state, similar to severe sepsis, arises with vascular dysfunction, increased capillary permeability and coagulation activation. This may be mediated in a positive feedback loop by inflammatory cytokines, free fatty acids and oxidised cells and lipoproteins, which could be further contributed to from peripheral tissues such as adipose. This state recovers after the removal of the placenta but the underlying phenotypic or genetic abnormality persists until the development of clinical cardiac disease in later life.

Therefore opportunities for future research include:

1. Prospective study of metabolic and inflammatory consequences of maternal obesity throughout pregnancy: to establish if pregnancy has any beneficial or detrimental effects on pre-existing vascular and metabolic abnormalities.

2. Relationship between maternal BMI and fetal indices of metabolic function (cord blood analyses of lipoprotein constituent, insulin, and inflammatory molecules): evidence for fetal programming effect of maternal obesity.

3. Follow up children and examine blood pressure and vascular function using laser Doppler imaging technique age 5.

4. Examine children of women 15-19 years postnatal (chapter seven) from pre-eclamptic offspring versus controls (vascular function, insulin sensitivity): again implications for fetal programming and genetic inheritance, i.e. cycling of risk factors through generations.

5. Case control study of intervention of moderate, regular exercise versus no exercise in pregnancy, firstly in relation to maternal vascular function and indices of insulin sensitivity and metabolic function and secondly, in relation to fetal indices (see point 2 above): role for exercise and weight loss to break the cycle of vascular risk.

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Appendix (i)

Publications arising from this thesis:

Ramsay JE, Stewart F, Sattar N, Greer IA. Endothelial dysfunction: a link between pre-eclampsia and maternal coronary heart disease. In press: Accepted BJOG March 2003.

Sattar N, **Ramsay JE**, Stewart A, Crawford L, Cheyne H, Greer IA. Classical and novel risk factor parameters in women with a history of preeclampsia In press: Accepted Hypertension. March 2003.

JE Ramsay, RJ Simms, WR Ferrell, L Crawford, IA Greer, MA Lumsden, & N Sattar. Enhancement of endothelial function by pregnancy: inadequate response in women with type I diabetes. Diabetes Care. 2003 Feb;26(2):475-9.

Jane E Ramsay, William R Ferrell, Lynne Crawford, A. Michael Wallace, Ian A Greer & Naveed Sattar. Maternal obesity is associated with dysregulation of metabolic, vascular and inflammatory pathways.
Journal of Clinical Endocrinology and Metabolism 2002 Sep;87(9):4231-7.

Ramsay JE, Ferrell WR, Greer IA, Sattar N. 2002 Factors critical to iontophoretic assessment of vascular reactivity: implications for clinical studies of endothelial dysfunction. J Cardiovasc Pharmacol. 39:9-17.

William R. Ferrell, **Jane E. Ramsay**, Naomi Brooks, John C. Lockhart, Sylvia Dickson, Grainne M. McNeece, Ian A. Greer and Naveed Sattar. Elimination of electrically induced iontophoretic artefacts: implications for non-invasive assessment of peripheral microvascular function.
Journal of Vascular Research. 2002 Sep-Oct;39(5):447-55.

Appendix (ii)

Oral presentations to learned societies and prizes awarded:

British Maternal and Fetal Medicine society. Cambridge, 2003. Leptin and body fat in pre-eclampsia and intrauterine growth restriction:
A protective role for leanness? Jane E Ramsay, N Sattar, IA Greer.

International Society for the Study of Hypertension in Pregnancy. Toronto 2002. Microvascular disease in pre-eclampsia: potential cardiovascular sequelae. Jane E Ramsay, F Stewart, N Sattar, IA Greer. **Young investigators prize.**

British Maternal and Fetal Medicine society. Cambridge, 2002. Microvascular disease in pre-eclampsia: potential cardiovascular sequelae. Jane E Ramsay, F Stewart, N Sattar, IA Greer.

Society of Gynaecological Investigation (SGI), Toronto, March 2001.
Maternal obesity is associated with impaired endothelial function in uncomplicated pregnancy.

Royal College of Obstetricians and Gynaecologists, Scottish Executive Meeting. 7-9th December 2000. Non-invasive assessment of endothelial function in normal pregnancy: detrimental effects of obesity. **First prize.**