

The assessment of endothelial function in pregnancy by flow-mediated dilatation.

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Abstract.

The endothelium plays a major role in the regulation of vascular homeostasis in pregnancy. Endothelial dysfunction has been implicated in the pathophysiology of many diseases, including hypertension and pre-eclampsia. The aim of this work was to assess endothelial function in pregnancy.

The ultrasound technique used was flow-mediated dilatation, a marker of stimulated endothelial function. The technique involves inducing reactive hyperaemia with a blood pressure cuff. Brachial artery diameter is measured pre and post cuff release and flow-mediated dilatation is the percentage increase in artery diameter.

First, a longitudinal study to develop a normal range of endothelial function in pregnancy and postpartum was performed and compared with non-pregnant women. The study demonstrated endothelial function did not vary throughout pregnancy until 36+ weeks gestation after which it decreased significantly. This decrease is in accordance with the normal physiology of pregnancy. No significant difference in endothelial function was demonstrated between non-pregnant women and pregnant/post-partum women.

Second, a study assessing the effect of smoking on the endothelium in pregnancy was completed. Smoking in pregnancy was found to induce endothelial dysfunction. When women smoked and had growth restricted babies their endothelial function was significantly decreased compared to women with normally grown fetuses.

The final study tested endothelial function in women who developed pre-eclampsia and gestational hypertension. Women with gestational hypertension had endothelial dysfunction. Endothelial function in the pre-eclamptic women was similar to the normal

range result. The women in this study were in the obese range and most had late pre-eclampsia. These results reinforce the message that pre-eclampsia may not be a homogeneous disorder.

In conclusion, endothelial function can be assessed non-invasively in pregnancy. Women with gestational hypertension and pre-eclampsia demonstrate differences in endothelial function. Smoking in pregnancy results in endothelial dysfunction. This work provides a basis for future research on endothelial function in pregnancy, especially the hypertensive disorders of pregnancy.

Preface

All the studies performed in this thesis are my own work. I recruited the volunteers, performed all the flow-mediated dilatation studies and carried out all the measurements and calculations for the various parameters. Each study was approved by the Institution's Research and Ethics Committee.

Publications arising from this work.

Publications.

1. Quinton AE, Peek MJ, Cook C-M, Kirby A. Flow-mediated dilatation assessment in women with preeclampsia compared to women with gestational hypertension. *Hypertens Pregnancy*. 2010; Dec 21 (Epub ahead of print).
2. Quinton AE, Cook C-M, Peek MJ. The relationship between cigarette smoking, endothelial function and intrauterine growth restriction in human pregnancy. *BJOG*. 2008; 115(6):780-784
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2. Quinton AE, Cook C-M, Peek MJ. Smoking in Pregnant Women: The Effect on Endothelial Function. *J Paediatr Child Health*. 2007; 43(Suppl 1):A47
3. Quinton AE, Cook C-M, Peek MJ. The Relationship between Cigarette Smoking and Endothelial Function in Human Pregnancy. *J Perinat Med*. 2007; 35(Suppl 2): S139
4. Peek M, Cook C-M, Quinton A. Ultrasound Assessment of Endothelial Cell Function in Normal Pregnancy. *Hypertens Pregnancy*. 2004; 23(Suppl. 1):23

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2. Endothelial function is different in women with pre-eclampsia compared to women with gestational hypertension. Peek MJ, Quinton AE, Cook C-M, Kirby A. Global Conference of Maternal and Infant Health, Barcelona Spain, 2010.
3. A comparison of different methodologies in flow-mediated dilatation in women with pre-eclampsia compared to women with gestational hypertension. Quinton AE, Peek MJ, Cook C-M, Kirby A. Australian Sonographers Association (ASA) National Conference, Melbourne Australia, 2010.
4. Endothelial function is different in women with pre-eclampsia compared to women with gestational hypertension. Quinton AE, Cook C-M, Peek MJ. Society of Obstetric Medicine of Australia and New Zealand (SOMANZ), Auckland New Zealand, 2009.
5. Quinton AE, Cook C-M, Peek MJ. Ultrasound assessment of endothelial function in women with pre-eclampsia compared to women with gestational hypertension. 12th World Congress of the World Federation for Ultrasound in Medicine and Biology (WFUMB), Sydney Australia, 2009.
6. Quinton AE, Cook C-M, Peek MJ. Differences in endothelial function in women with pre-eclampsia compared to women with gestational hypertension. Perinatal Society of Australia and New Zealand (PSANZ), Darwin Australia, 2009.
7. Quinton AE, Cook C-M, Peek MJ. Endothelial function is different in women with pre-eclampsia compared to women with gestational hypertension. 25th Fetus as a Patient, Sydney Australia, 2009.

8. Peek MJ, Quinton AE, Cook C-M. Smoking in Pregnant Women: The Effect on Endothelial Function and the Relationship with Intrauterine Growth Restriction. XVI World Congress of the International Society of Hypertension in Pregnancy (ISSHP) Washington DC USA, 2008.
9. Quinton AE, Cook C-M, Peek MJ. The relationship between cigarette smoking, endothelial function and intrauterine growth restriction in human pregnancy. Society of Obstetric Medicine of Australia and New Zealand (SOMANZ), Sydney Australia, 2007.
10. Quinton AE, Cook C-M, Peek MJ. Ultrasound assessment of the Effects of Smoking in Pregnancy on Endothelial Function. Australasian Society of Ultrasound in Medicine (ASUM) 37th Annual Scientific Meeting, Cairns Australia, 2007.
11. Quinton AE, Cook C-M, Peek MJ. Smoking in Pregnant Women: the Effect on Endothelial Function. Perinatal Society of Australia and New Zealand 11th Annual Congress (PSANZ), Melbourne Australia, 2007.
12. Quinton AE, Cook C-M, Peek MJ. Ultrasound Assessment of the Brachial Artery to Determine Endothelial Function in Pregnancy. Australasian Society of Ultrasound in Medicine (ASUM) 35th Annual Scientific Meeting, Adelaide Australia, 2005.
13. Quinton AE, Cook C-M, Peek MJ. Assessment of endothelial function in women with normal, pre-eclamptic and hypertensive pregnancy. Combined Annual Scientific Meetings of Australasian Diabetes in Pregnancy Society (ADIPS) and Society of Obstetric Medicine of Australia and New Zealand (SOMANZ), Darwin Australia, 2005.

14. Quinton AE, Cook C-M, Peek MJ. Assessment of Endothelial Function in Normal Human Pregnancy. Perinatal Society of Australia and New Zealand 9th Annual Congress (PSANZ), Adelaide Australia, 2005.
15. Quinton AE, Cook C-M, Peek MJ. Ultrasound Assessment of Endothelial Cell Function in Normal Pregnancy. XIV World Congress of the International Society for the Study of Hypertension in Pregnancy, Vienna Austria, 2004.
16. Quinton AE, Cook C-M, Peek MJ. Endothelial function in normal human pregnancy. The University of Sydney College of Health Sciences, Fourth Research Conference 2004 “From Cell to Society 4”, Leura Australia. 2004.

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1. A comparison of different methodologies in flow-mediated dilatation in women with pre-eclampsia compared to women with gestational hypertension. Quinton AE, Peek MJ, Cook C-M, Kirby A. Australian Sonographers Association (ASA) National Conference, Melbourne, Australia, 2010. Winner of Best Research Paper and Best Research Paper Grant.
2. Quinton AE, Cook C-M, Peek MJ. The relationship between cigarette smoking, endothelial function and intrauterine growth restriction in human pregnancy. Society of Obstetric Medicine of Australia and New Zealand (SOMANZ), Sydney, Australia, 2007. Winner of best scientific research presentation.
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Table of abbreviations.

2D	2 dimensional
ACE	angiotensin converting enzyme
ACh	acetylcholine
ACOG	American College of Obstetricians and Gynecologists
ADMA	asymmetric dimethylarginine
ALK5	activin receptor-like kinase 5
ANOVA	analysis of variance
AOR	adjusted odd ratio
ASH	American Society of Hypertension
ASSHP	Australasian Society for the Study of Hypertension in Pregnancy
AUC	area under the curve
BH ₄	tetrahydrobiopterin
BMI	body mass index
BP	blood pressure
bpm	beats per minute
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CRP	C-reactive protein
CVD	cardiovascular disease
dB	decibel
DNA	deoxyribonucleic acid
ECG	electrocardiograph
EDHF	endothelial-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor

eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FBF	forearm blood flow
FDIU	fetal death in-utero
Flt-1	<i>fms</i> -like tyrosine kinase vascular endothelial growth factor receptor-1
FMD	flow-mediated dilatation
FMD _{max}	flow-mediated dilatation-maximum
FMD _o	flow-mediated dilatation-original
FMN	flavin mononucleotide
GDM	gestational diabetes mellitus
GTN	glyceryl trinitrate
H ₂ O ₂	hydrogen peroxide
HDL	high-density lipoprotein
HELLP	haemolysis, elevated liver enzymes, low platelet count
HO	heme oxygenase
IBF	intervillous blood flow
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-2	interleukin-2
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-18	interleukin-18

iNOS	inducible nitric oxide synthase
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intra-uterine growth restriction
K1	Korotkoff phase 1
K5	Korotkoff phase V
L-ADMA	N ^W N ^W -dimethyl-L-arginine
LDL	low-density lipoprotein
L-FMC	low flow-mediated constriction
L-NAME	L-nitro-arginine-methyl ester
L-NMMA	N ^W -monomethyl-L-arginine
LPS	lipopolysaccharides
LR	likelihood ratio
m [IQR]	median and interquartile range
MAP	mean arterial pressure
MHz	megahertz
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NHBPWG	National High Blood Pressure Working Group
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO ₂	nitrogen dioxide
NOS	nitric oxide synthase
NO _x	nitrite/nitrate
NPV	negative predictive value
O ₂	oxygen

O ₂ ⁻	superoxide
ONOO ⁻	peroxynitrite
OR	odds ratio
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PCV	packed cell volume
PGI ₂	prostacyclin
PI	pulsatility index
PI-C	pulsatility index change
PIH	pregnancy induced hypertension
PIGF	placental growth factor
PO	post occlusion
pO ₂	oxygen partial pressure
PPV	positive predictive value
RCOG	Royal College of Obstetricians and Gynaecologists
RI	resistance index
RMANOV	repeated measures analysis of variance
RNA	ribonucleic acid
RNOS	reactive nitrogen oxide species
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
sEng	soluble endoglin
sFlt-1	soluble <i>fms</i> -like tyrosine kinase

SGA	small for gestational age
SNP/s	single nucleotide polymorphism/s
SOGC	Society of Obstetricians and Gynaecologists of Canada
SOMANZ	Society of Obstetric Medicine of Australia and New Zealand
SPE	superimposed pre-eclampsia
STBM	syncytiotrophoblast basement membrane
SVR	systemic vascular resistance
TGF- α	transforming growth factor alpha
TGF- β	transforming growth factor beta
TNF α	tumour necrosis factor- α
t-PA	tissue plasminogen activator
TTP	time to peak
TXA2	thromboxane A2
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR or Flt	vascular endothelial growth factor receptor
VTI	velocity time integral
WHO	World Health Organization

Chapter 1

1.0 Introduction.

The endothelium is a responsive paracrine organ that has wide ranging functions in humans. It plays a major role in the regulation of the inflammatory response, the exchange of nutrients and angiogenesis (1). This regulation is achieved by the endothelium functioning as a mechanical barrier to movement in and out of the vascular compartment, by synthesising substances that induce repair, producing vasodilatation and constriction of arteries and recruitment of inflammatory cells (2). Regulation, maintenance and control of vascular tone, haemodynamic responses and adaptations are mediated by the endothelium constitutively producing nitric oxide (NO) along with other vasoconstrictors and dilators (1, 3). An intact endothelium enables physiological stimulators such as different chemicals, hyperaemia and pulsatile flow to induce vasodilatation and/or constriction (1, 4).

The vasodilatation of arteries can result after the local flow regulatory mechanism, reactive hyperaemia, occurs. Reactive hyperaemia results after the blood supply to tissue in the body is blocked for either a few seconds or up to several hours and is then unblocked. The blood flow to the deprived tissue will then increase four to seven times that of normal, for either a few seconds or for many hours. This rapid increase in blood flow causes shear stress on the endothelial cells and contorts the endothelium in the direction of flow. Nitric oxide is then released causing the local artery wall to relax and dilate, resulting in dilatation of the larger upstream arterial vessels (5).

Normal pregnancy results in profound changes to the maternal cardiovascular system with the purpose of accommodating the developing fetus. It is a state of increased

vasodilatation with the endothelium playing a key role in the control of vascular tone. Multiple vasoconstrictors and vasodilators such as NO, prostaglandins, endothelium-derived relaxing factor, thromboxane-A and endothelin-1 are involved (6-8).

While the majority of pregnancies result in a good outcome for both mother and baby, complications can sometimes occur. One of the more serious and common groups of problems are the hypertensive disorders of pregnancy, in particular pre-eclampsia and gestational hypertension. Both disorders can result in severe morbidity to the mother and fetus. The onset of gestational hypertension in pregnancy means an increase in monitoring of both mother and fetus as severe gestational hypertension is not a benign disease (9). The endothelium has been implicated in the vascular dysfunction that occurs in hypertensive non-pregnant people (10) and in pre-eclampsia (11).

Pre-eclampsia usually presents as hypertension and proteinuria but will eventually progress to a multi-system disorder affecting the wellbeing of both mother and fetus (11). Although the aetiology of pre-eclampsia is unknown, placental ischaemia is thought to be the predisposing event with numerous other risk factors potentially contributing to the disease. The endpoint of the clinical syndrome affects the maternal endothelium with widespread endothelial dysfunction (11). The result of endothelial dysfunction is systemic vasospasm, decreased organ flow, platelet aggregation and the clinical picture of pre-eclampsia (12). The endothelium is the target for a variety of circulating factors such as angiogenic factors, cytokines, reactive oxygen species and hypoxia inducible factors (13, 14). It can also be affected by other extraneous influences such as differences in subject demographics and environmental factors such as caffeine, diet and cigarettes (15). Endothelial dysfunction

may present as modifications in the availability of various vasodilators and or vasoconstrictors (14) and as reduced vasodilatation in vessels (16).

Endothelial function and dysfunction can be studied non-invasively using high-resolution ultrasound of the brachial artery. This technique is called flow-mediated dilatation (FMD) and is a measure of stimulated endothelial function. Normal endothelial function results in dilatation of the artery whereas endothelial dysfunction is reported to present as reduced vasodilatation. The FMD technique involves inducing reactive hyperaemia by inflating a blood pressure cuff on the forearm for a specified number of minutes and releasing it. Flow-mediated dilatation is the percentage difference in the brachial artery diameter as measured before and after cuff release (16). It is a technique that is mainly NO mediated (17).

As the work in this thesis uses the NO mediated technique of FMD, the literature review will commence with an overview of NO and the endothelium. This will be followed by a discussion of the FMD technique, the pathophysiology of pre-eclampsia and gestational hypertension. The relationship between the endothelium and pregnancy concentrating on NO mediated vasodilatory function and the FMD literature in pregnancy will then be evaluated. Finally, as other environmental factors are known to interact with the endothelium, the relevant literature on pregnancy, smoking, pre-eclampsia and endothelial function will be reviewed.

1.1 Nitric oxide.

In 1980 Robert Furchgott and John Zawadzki discovered that inadvertent rubbing of the intimal surface of strips of rabbit aorta removed the endothelial cells. This resulted in a loss of relaxation of the vessels by acetylcholine (a potent vasodilator). They demonstrated that smooth muscle in vessel walls was relaxed by the vascular endothelium, and the endothelial cells released a substance they called endothelium-derived relaxing factor (EDRF) (18). In 1987 EDRF was shown to be NO by two independent research groups (19, 20). Further evidence that EDRF is NO was provided by directly measuring NO in human volunteers (21). In 1992 the American Association for the Advancement of Science called NO the “molecule of the year” and in 1998, Furchgott, R., Ignarro, L. and Murad F. were awarded the Nobel Prize for Physiology and Medicine for their role in the discovery and mechanisms of NO (22). Nitric oxide is thought to be one of the most important regulators of inter- and intracellular signalling systems and is a major player in the control of vascular homeostasis by the endothelium.

1.1.1 The synthesis of nitric oxide.

Endothelial derived NO is obtained from the terminal guanidino nitrogens of the essential amino acid L-arginine (23). This process involves enzymatic oxidation of L-arginine from an enzyme known as nitric oxide synthase (NOS) (24). Nitric oxide synthesis from arginine is a reaction involving a five step oxidation process to produce L-citrulline and NO. The reaction requires the presence of oxygen and the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH₄) (22) as well as the presence of calmodulin (25). Calmodulin is a Ca²⁺ binding protein. The process involves the incorporation of molecular oxygen (O₂) and NADPH which is an electron source for the synthesis of NO from L-arginine, plus the presence of BH₄. This results in an intermediate

species, N^W-hydroxyarginine being formed which is then oxidized to form citrulline and NO (25).

1.1.2 The action of nitric oxide on smooth muscle.

Nitric oxide produced in endothelial cell diffuses through the adjacent vascular smooth muscle membrane, binding to the haeme portion of the enzyme soluble guanylate cyclase. The activated guanylate cyclase then increases the intracellular concentration of cyclic guanosine monophosphate (cGMP). This increase in cGMP decreases the intracellular calcium level in the vascular smooth muscle, causing dephosphorylation of the myosin light chains and vasodilatation (26-28).

1.1.3 The enzymes known as nitric oxide synthases.

Nitric oxide synthases are part of a family of enzymes known as cytochrome P450 haem proteins. A cytochrome protein is an intracellular haemoprotein respiratory pigment enzyme that functions in electron transport as a carrier of electrons. The NOS molecule transfers electrons from one atom or molecule to another by the chemical reaction known as oxidation-reduction or redox (22). The NOSs are divided into two types according to their actions:

Type 1. This is a calcium independent NOS, also known as type II NOS or inducible NOS (iNOS).

Type 2. These are the constitutive (always present) calcium and calmodulin dependent NOSs of which there are two types:

- a) type I, also known as nNOS or the neuronal isoform
- b) type III, also known as eNOS or the endothelial isoform (22, 25).

1.1.4 Inducible nitric oxide synthase.

Inducible NOS is stimulated by cytokines to produce NO in macrophages. Bacterial lipopolysaccharides (LPS) stimulate macrophages and a superoxide anion with hydrogen peroxide (H₂O₂) molecules forming. This results in messenger ribonucleic acid (mRNA) synthesis of NOS with an increase in NO synthesis (22).

1.1.5 Neuronal nitric oxide synthase.

Neuronal NOS is found in the human brain, human skeletal muscle, peripheral neural systems and spinal cord. The synthesis of NO in a neuron occurs with nNOS in response to glutamate. Non-adrenergic, non-cholinergic nerves are thought to produce NO in the peripheral and central nervous system (25).

1.1.6 Endothelial nitric oxide synthase.

Endothelial NOS has been found in the vascular endothelium in all types of blood vessels in conduit arteries, micro-vessels or veins, in human lungs, liver and skin blood vessels (25). The synthesis of eNOS in the vascular endothelial cell is stimulated by acetylcholine (ACh). Acetylcholine stimulates a muscarinic receptor on an endothelial cell which then releases NO (28). Acetylcholine only causes vasodilation when it occurs in low doses and when there is an intact endothelium. Constriction of vessels results when ACh occurs in higher concentrations due to a direct effect on the surrounding smooth muscle (29). When NO synthesis is inhibited by the L-arginine analogue N^W-monomethyl-L-arginine (L-NMMA), “the dilator response to acetylcholine is lost and the constrictor response is enhanced” (30). The infusion of L-NMMA merely attenuates the dilator response to ACh, without completely blocking it. Muscarinic agonists, acted upon by ACh appear to induce NO independent dilatation (31).

1.1.7 Stimulators of nitric oxide synthesis on the endothelium.

Induction of NO synthesis occurs through a number of physiological stimulators. These include increased and/or pulsatile flow causing shear stress on the endothelial cells (32, 33), or a change in blood flow with a shift in tension stress and blood oxygen partial pressure (PO₂) (28). To elicit vasodilatation in response to increased flow it is essential to have a functionally intact endothelium (32). In an experiment on isolated canine arterial segments to study the effect of pulsatile flow on the release of prostaglandins and NO, Pohl and colleagues demonstrated that the release of these endothelial products is enhanced by pulsatile flow (4). When the endothelium was removed, absence of flow-induced dilatation in arterial segments occurred (33). Induced shear stress from the inflation and release of a blood pressure cuff placed over the brachial and/or radial artery in human volunteers also results in NO dependent vasodilatation from reactive hyperaemia (34). Numerous vasoactive compounds cause NO synthesis including ACh, adenosine triphosphate, adenosine diphosphate, bradykinin, calcitonin gene-related peptide, calcium ionophore, cholecystokinin, histamine, noradrenaline, serotonin, substance P, thrombin and vasoactive intestinal peptide (28).

1.1.8 Haemodynamic forces induce signalling cascades to control vascular function.

Endothelial cells are constantly exposed to haemodynamic forces such as fluid shear stress which play an important role in the maintenance of vascular homeostasis. The mechanical stimulus of shear stress activates a chemical signal which induces gene expression in the endothelial cell. The function of the endothelial cell is then modulated by the regulation of genes that encode for proteins which have different functions. Some of the functions of these proteins are vasodilatory and include proteins such as NOS, endothelin-1 and prostacyclin. Other proteins produced are adhesion molecules, coagulation molecules,

growth factors, anti-oxidants and proto-oncogenes. The activation of transcription factors (transcription is the deoxyribonucleic acid (DNA) directed synthesis of mRNA) occurs at the endothelial cell surface membrane and in signalling pathways inside the cytoplasm. Among the signalling molecules modulated in endothelial cells are membrane K^+ channels, intracellular Ca^+ , cGMP and cyclic adenosine monophosphate (cAMP) (35).

1.1.9 Inhibitors of nitric oxide synthesis.

Inhibitors of NOS are used to study NO synthesis in mammals. These inhibitors can affect different parts of the NOS reaction. The most commonly used inhibitor is L-NMMA which is competitive with L-arginine. Another common inhibitor is $N^W N^W$ -dimethyl-L-arginine (L-ADMA) and L-nitro-arginine-methyl ester (L-NAME). Both L-NMMA and L-ADMA are analogues of L-arginine and are naturally occurring compounds. They occur normally in plasma in extremely low concentrations, although the levels can increase in renal failure and inhibit NO synthesis (22, 25). Other types of NOS inhibitors can compete with NADPH, calmodulin or BH_4 in the NOS reaction. In fact as all three types of NOS use NADPH, FAD, FMN and BH_4 as cofactors, any agent that is a derivative of arginine, or binds with the haem part of the enzyme and inhibits the binding and biosynthesis of BH_4 , or substitutes for any part of the NO cycle can inhibit NO synthesis (22).

1.1.10 The toxic effects of nitric oxide.

As well as being a regulatory agent, NO can have cytotoxic effects. In the chemical biology of NO there are two distinct categories that cause direct or indirect effects. A direct effect is when NO reacts with a biological target in a chemical reaction. Direct effects encompass low concentrations of NO ($<1\mu\text{mol/L}$) in a rapid reaction involving haem proteins such as guanylate cyclase, cytochrome P450 and haemoglobin (36).

Indirect effects are divided into two types, effects that cause nitrosative stress or oxidative stress. Nitrosative stress or nitrosation involves modifying thiols and some amines in proteins. Oxidative stress results in lipid peroxidation, DNA strand breakage and protein oxidation and is derived via one of two chemical pathways, either the reactive oxygen species (ROS) or the reactive nitrogen oxide species (RNOS) pathway. Reactive nitrogen oxide species which are derived from NO metabolism mediate the indirect effects. For indirect effects to occur NO is first activated by superoxide (O_2^-) or oxygen to form a RNOS. Further reactions result with a biological target such as proteins, lipids and DNA causing a NO-mediated apoptosis. These reactions occur when local concentrations of NO are high ($>1\mu\text{mol/L}$) for a prolonged period of time and in the vicinity of cells such as macrophages that are stimulated by pathogenic products such as cytokines. A ROS is formed from O_2^- and H_2O_2 . Metals such as irons react with H_2O_2/O_2^- to form hydroxyl radicals, resulting in powerful oxidants that can cause cell death (22, 36).

The cytotoxic effects of NO are regulated by the cyclic conversion of NO and its by-products (nitrite, nitrate, nitrogen dioxide (NO_2) radicals, peroxynitrite ($ONOO^-$) and NO_2), the mechanism of which is called the nitric oxide cycle (37).

1.1.11 The biological actions of nitric oxide.

There are many varied actions of NO in biological systems. Nitric oxide is an immune regulator which is generated from iNOS in high levels and for sustained periods of time (over days to weeks) (38). By comparison NO is produced in short bursts at low concentrations by the constitutive eNOS for controlling vascular homeostasis (26). Nitric oxide has no surface cell receptors and enters cells indiscriminately. The selection of

targets depends on the concentration of NO and its reactivity with other molecules, how close the target cell is and the way the target cell is programmed to respond (38).

1.1.12 Endothelial nitric oxide regulates vascular homeostasis.

Nitric oxide derived from the endothelium has been shown to have important biological actions on the vascular system. Stable complexes are formed between nitric oxide and haemoglobin with NO binding to oxyhaemoglobin as part of the respiratory system. Oxygen delivery is then facilitated with the release of NO in resistance vessels which dilates the blood vessel (39, 40) and relaxes smooth muscle (18). Complexes are also formed between NO and serum albumin which circulates in plasma and regulates vascular tone (41). Endothelial NO is a regulator of leukocyte adhesion and prevents the adherence of neutrophils to the endothelium (42, 43).

The basal release of low levels of NO from the endothelium maintains vascular tone, causes relaxation of the smooth muscle cells and dilatation of the artery (18, 26). By injecting healthy volunteers with the NOS inhibitor L-NMMA, it was demonstrated that the regulation of systemic vascular resistance and therefore blood pressure is determined by the basal release of NO. An elevation in blood pressure occurs when NO synthesis is inhibited. Nitric oxide was also shown to regulate basal pulmonary vascular resistance (44). The targeted disruption of a gene that encodes for eNOS in mice caused hypertension, demonstrating eNOS controls basal vasodilatation (45).

Nitric oxide exerts an anti-platelet action. Nitric oxide produced by platelets and the endothelium can inhibit the aggregation of platelets and their adhesion to endothelial cells thereby playing a role in the regulation of platelet-vessel wall homeostasis (46, 47). In an

experiment designed to study the role of NO in haemostatic responses and platelet function, eNOS-mutant mice were used to examine the role of NO derived from platelets. Production of NO from platelets decreased bleeding time. This suggests a lack of platelet-derived NO enhances platelet activation and alters the regulation of haemostasis by increasing platelet recruitment (48).

1.2 The vascular endothelium.

The endothelium is a continuous layer of cells lining the entire cardiovascular system and is normally the only part of a vessel that is in contact with blood (49). It is a dynamic paracrine organ that in the adult human is composed of approximately $1-6 \times 10^{13}$ cells, weighs about one kilogram and has a surface area of between 1-7 square metres (50).

1.2.1 The role of the endothelium.

The endothelium regulates the flow of nutrients, different biologically active molecules and blood cells throughout the body. The membrane-bound receptors found on endothelial cells act as a gate-keeper for a range of molecules including proteins (coagulant and anticoagulant proteins and growth factors), lipid transporting particles (for example low-density lipoprotein) and metabolites such as NO and hormones (for example endothelin-1). Blood flow is regulated by the endothelial cells through the generation of an active anti-thrombin surface which moves plasma and other blood cells through the vessels. The secretion and uptake of vasoactive substances also regulates blood flow by constricting and dilating the endothelium (1).

1.2.2 The role of the endothelium as a nutrient regulator.

In its role as a nutrient regulator the endothelium has a number of different transport functions. One is to act as a barrier to the indiscriminate passage of molecules and cells from the blood to underlying tissue. Another function is to act as a selective barrier allowing passage of molecules through endothelial cell junctions. Particular metabolic needs are met by mechanisms that transport molecules (for example glucose and amino acids) across the endothelial cell to underlying tissues. The permeability of the vascular tree depends on tight junctions and caveolae. Caveolae, which are invaginations in the endothelial cell membrane are used in transcytosis (transcellular transport) of substances, for example albumin. Tight junctions are intercellular junctions that act as either a total barrier to the transport of molecules (for example the blood brain barrier) or a gate regulating the selective passage of molecules through these paracellular spaces (50).

1.2.3 The regulation of vascular tone by the endothelium.

Another important function of the vascular endothelium is the modulation of vascular tone (the control of blood pressure and blood flow) via the production of vasoactive substances which cause constriction and dilatation. These substances are the vasodilators NO, prostacyclin (PGI₂) and endothelial-derived hyperpolarizing factor (EDHF). The vasoconstrictors are endothelin-1 (ET-1), thromboxane A₂ (TXA₂) and platelet-activating factor (PAF). As was reviewed in the previous section, NO is constitutively secreted by endothelial cells with the production modulated by exogenous chemicals and physical stimuli. Nitric oxide maintains basal vessel tone by relaxing the vascular smooth muscle cells (1, 50). The following section will briefly discuss the other vasoregulators.

1.2.4 The vasodilator prostacyclin (PGI₂).

Prostacyclin is a short lived, powerful vasodilatory intercellular signalling molecule that is rapidly released from endothelial cells. Forces such as pulsatile pressure or chemical stimulants, for example bradykinin and thrombin released from plasma; or chemicals from stimulated platelets such as serotonin, platelet-derived growth factor, interleukin-1 and adenine nucleotides; result in PGI₂ production. Prostacyclin acts as a local hormone, preventing platelets from aggregating and depositing on the vessel wall. It also causes relaxation of the vascular smooth muscle surrounding the vessel lumen as vascular smooth muscles have a receptor for PGI₂, the IP receptor. However, PGI₂ does not regulate basal vessel tone because it is not constitutively produced (1, 51).

Compared with non-pregnant women, PGI₂ production is increased in normal pregnancy (52, 53). In pre-eclamptic pregnancy PGI₂ production is either unchanged (52) or decreased (53, 54) compared with normal pregnancy.

1.2.5 The vasodilator endothelial-derived hyperpolarizing factor (EDHF).

Endothelial-derived hyperpolarizing factor is involved in a vasodilatory process that requires the endothelium for vascular smooth muscle relaxation. Studies have shown that it is distinct from NO or cyclooxygenase (COX) metabolites such as PGI₂. Endothelial-derived hyperpolarizing factor causes dilatation by hyperpolarizing the vascular smooth muscle by potassium activation. Shear stress, pulsatile stretch and endothelial agonists all elicit a vasodilatory response by EDHF. There is limited knowledge regarding EDHF with controversy about how EDHF causes vasodilatation. Currently four major and other minor mechanisms are proposed among them the arachidonic acid metabolite pathway, the monovalent cation K⁺ channel mechanism, the use of gap junctions as a signal relay, or the

use of generated hydrogen peroxide to activate potassium channels. It has become apparent that EDHF is more prevalent in smaller arteries and arterioles with it contributing to the regulation of blood flow (55).

Endothelial-derived hyperpolarizing factor is up-regulated during pathological conditions such as ischaemia-reperfusion injuries, heart failure, coronary artery disease and traumatic injury. This up-regulation occurs more when NO production is attenuated by pathological conditions (55).

In normal pregnancy, EDHF along with NO has been shown to be implicated in endothelial dependent vasodilatation (56). In pre-eclampsia this EDHF mediated relaxation is reduced (57).

1.2.6 Platelet activating factor, a vasoconstrictor and vasodilator (PAF).

The vasoconstrictor PAF is a short lived intercellular signalling molecule that is synthesized from endothelial cells after mechanical or humoral stimulation. The stimulation is by receptor-mediated agonists such as thrombin, histamine, bradykinin or leukotriene C4/D4 or endothelial cell injury. Platelet activating factor promotes inflammation and thrombosis by acting in concert with another adhesion molecule expressed on the surface of endothelial cells called P-selectin. P-selectin tethers leukocytes to the endothelial surface, rolling them along until they are activated by PAF. Activation of the leukocyte by PAF stimulates the production of leukocyte thrombotic substances which then bind and modify platelets at the site of endothelial damage (58). Platelet activating factor causes either vasoconstriction or vasodilatation depending on the concentration and conditions at the time it is administered (59-62).

In newborn pig pial arterioles, PAF was found to be a potent vasoconstrictor (61). Venules and arterioles were also found to constrict in a dose dependent manner in hamsters (60). Others have found that PAF in rabbits causes vasodilatation at low concentrations and is a constrictor at high concentrations (62). In canine coronary arteries PAF causes constriction after ischaemia but dilatation under conditions of normal flow (59).

In the serum of normal pregnant women compared to non-pregnant women, there was no difference in PAF levels. In pre-eclampsia PAF levels are increased (63). In placental trophoblast, no difference was found in PAF levels between normal and pre-eclamptic pregnancies. Levels of PAF-acetylhydrolase (which degrades PAF) were increased in pre-eclampsia compared with normal pregnant women. This was thought to be a compensatory mechanism to regulate PAF levels in pre-eclampsia (64).

1.2.7 Endothelin-1 (ET-1), a vasoconstrictor and vasodilator.

Originally endothelin-1, produced by the vascular endothelium, was thought to function only as a potent vasoconstrictor. However it has been found that ET-1, an amino acid isopeptide, acts as a vasoconstrictor and a vasodilator through receptors named ET_A (found on smooth muscle cells) and ET_B (found on endothelial cells and smooth muscle cells). The ET_A receptors act as a promoter of smooth muscle growth and mediates smooth muscle contraction (65). Smooth muscle ET_B receptors induce contractions and endothelial cell ET_B receptors induce relaxation by producing NO and PGI₂. Nitric oxide and PGI₂ then act as a 'braking mechanism' against the overproduction of ET-1 induced contractions (66).

Serum from normal pregnancy produced more ET-1 compared with non-pregnant women. The serum from pre-eclamptic women decreased the production of ET-1 compared to normal pregnancy (52). Significant increases in plasma ET-1 has been shown in pre-eclampsia compared with non-pregnant and normal pregnant women (67, 68). Infusion of ET-1 into non-pregnant and normal pregnant women significantly decreased forearm blood flow as measured by plethysmography. No change in flow was found in pre-eclampsia. The study demonstrated in normal pregnancy ET-1 does not play a major role in the maintenance of vascular tone, and the endothelium does not respond to ET-1 in pre-eclampsia (69).

1.2.8 The vasoconstrictor thromboxane A₂ (TXA₂).

Thromboxane A₂ is a potent vasoconstrictor and also stimulates platelet aggregation. No differences in plasma levels of TXA₂ in women with normal pregnancy or mild pre-eclampsia were demonstrated, however in severe pre-eclampsia TXA₂ was significantly increased (70). The changes in TXA₂ were shown not to occur before the clinical onset of pre-eclampsia in a longitudinal study (71).

Thromboxane B₂ is a metabolite of TXA₂, and can be used to measure thromboxane levels. In severe pre-eclampsia and eclampsia thromboxane B₂ levels are increased antepartum, decreasing significantly after delivery (72).

The regulation of vascular tone by the endothelium is a complex interactive mechanism which involves a balancing act between dilators and constrictors. Increases or decreases in any of these factors in pregnancy may result in the endothelial dysfunction that occurs in pre-eclampsia (11).

1.3 Measures of endothelial function.

1.3.1 Tests assessing vascular tone.

As the endothelium has multiple functions, so there are multiple techniques in use to try and assess endothelial function and dysfunction. The work from this thesis has used the ultrasound technique of FMD (16) to assess endothelium dependent vasoreactivity. This technique will be described in detail in section 1.4 and section 2.2. A number of review articles have been published on the different methods employed to assess endothelial function (73-77). The following section will provide an overview of the other methods currently used to assess endothelial function.

1.3.2 Endothelium-independent vasodilatation of smooth muscle cells.

Vascular tone is dependent on the ability of the endothelium to produce vasoactive substances and the ability of the smooth muscle to respond to the stimulus. Smooth muscle function is assessed using endothelium-independent agents such as nitro-glycerine and sodium nitroprusside. These agents bypass the endothelium, acting directly on the smooth muscle cell, causing vasodilatation that is independent of the endothelium. This allows an investigator to differentiate between endothelium-dependent and –independent vasodilatation (73).

1.3.3 Plethysmography.

A method used to assess endothelial function is the forearm perfusion technique of plethysmography. With this technique a mercury strain gauge is placed around the forearm and a pharmacological agent such as ACh is infused into the brachial artery. As the venous outflow is obstructed with a blood pressure cuff placed on the wrist, a quantitative estimate of blood volume and flow to the forearm can be made and endothelial function investigated (7, 73, 74, 77).

1.3.4 Pulse wave analysis.

Pulse wave analysis or applanation tonometry involves placing a small pencil probe over an artery, usually the radial artery. Changes in endothelial function are reflected in the arterial waveform (73, 77, 78). A number of methods are used to analyse the pulse wave. With systolic waveform analysis the first waveform that is produced reflects left ventricular systole, the second peak quantifies the changes in arterial wall compliance. As the artery stiffens, the size of the second waveform increases (77). Diastolic waveform analysis uses radial pulse wave analysis to investigate pressure decay and arterial elasticity detected by pressure oscillations in diastole (77). Another method, pulse contour analysis, tests endothelial function by measuring changes in digital pulse volume using a photoplethysmograph (78).

1.3.5 Doppler laser flowmetry.

Doppler laser flowmetry of the skin uses reactive hyperaemia induced by either ACh, occluding blood flow to the finger, or by heat applied to the hand to measure endothelial function of the skin microvascular system. The technique has been shown to correlate well with FMD (79).

1.3.6 Radionuclide imaging.

A radionuclide technique has been validated for use in testing forearm blood flow as a measure of endothelial function. The amount of isotope tracer in the hyperaemic arm is compared to the normally perfused arm to obtain a ratio of hyperaemic response. This response ratio can distinguish between people with and without risk factors for coronary artery disease (76).

1.3.7 Brachial artery pulsatility index change.

Pulsatility index (PI) is a measure of downstream vascular resistance used in pulsed Doppler ultrasound (PI = peak systolic velocity-peak diastolic velocity/mean velocity) (80). The pulsatility index change (PI-C) is the difference in the Doppler PI measured at baseline and 60 seconds after 5 minute arm compression to induce reactive hyperaemia. This has been suggested as a measure of endothelial function because of the fall in downstream vascular resistance that occurs after hyperaemia due to arterial resistance vessel dilatation induced by the FMD technique (81).

A correlation between PI-C and FMD was demonstrated ($r = -0.66$ ($P < 0.01$)) (81). A later paper demonstrated that the PI-C had better reproducibility than the FMD technique in pregnant women (82).

1.3.8 Low flow-mediated constriction as an adjunct measure.

Low flow-mediated constriction (L-FMC) is measured when an inflated blood pressure cuff is placed around the radial artery, constricting blood supply and resulting in a decrease in radial artery size. It has been suggested as a measure of vascular endothelial function to be used in conjunction with FMD. To determine if L-FMC also occurs in the brachial artery a study was performed on healthy pregnant and non-pregnant women (83). Only in the radial (not brachial) artery did L-FMC occur. There was no correlation with shear rate reductions. A significant positive correlation was demonstrated between radial artery FMD and L-FMC. Further studies are required to determine if reduced L-FMC represents endothelial dysfunction.

1.3.9 Biochemical markers.

Substances produced by, or that act upon, or are metabolites of the vascular endothelium can be measured to reflect endothelial function. These include inflammatory markers, thrombosis markers, genetic markers, NO metabolites and endothelial progenitor cells. A widely used definition of endothelial dysfunction is an imbalance between a vasoconstrictor or vasodilator substance that affects endothelial cells. It is these substances that different groups have attempted to define and measure in their search for predictors or identifiers of endothelial dysfunction (74).

1.3.10 Inflammatory markers.

A theory regarding the aetiology of endothelial dysfunction involves the immune system with an inflammatory response. The first step in the process is thought to involve up-regulation of adhesion molecules in the vascular endothelium. This allows leucocytes and monocytes to adhere to the endothelial cell surface. The adhesion of cells allows penetration of monocytes and leucocytes into the sub-endothelial area because of endothelial adhesion up-regulation or activation, causing the production of cytokines (an adhesion is a surface antigen that enables cells to adhere to a host surface such as endothelial cells). This process contributes to vascular inflammation and a pro-inflammatory endothelial state. The markers of vascular inflammation and endothelial dysfunction can be measured *in vitro* and include adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, P-selectin and soluble CD40 ligand. These are mainly sourced from endothelial cells and cytokines such as interleukin-6 (IL-6), interleukin-18 (IL-18), TNF α and C-reactive protein (CRP) (74).

1.3.11 Thrombosis markers.

The endothelium is normally in an anti-thrombotic state. Regulation is achieved two ways. One is with inhibitors of coagulation or anti-coagulant factors that are produced by the endothelial cells such as thrombomodulin, protein S, heparin sulphate and tissue factor pathway inhibitor. The other method is by the production of pro-thrombotic mediators such as PGI₂, NO and surface-bound CD39. If endothelial function is disturbed, the endothelium changes from its anti-thrombogenic state to a pro-coagulation state. Plasma and/or serum levels of pro-coagulant mediators can be measured. These include von Willebrand factor which enhances thrombosis and the coagulation markers tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) (73).

1.3.12 Genetic markers.

Single or multiple genes can be studied to try and ascertain which genes are involved in the regulation of vascular biology. A genetic predisposition to hypertension is well documented as is the relationship between endothelial dysfunction and hypertension (84). For this reason candidate genes have been studied to try and identify any that may be related to endothelial dysfunction. The genes that may be involved are many and their number is growing as more sophisticated techniques are devised (74). Candidate genes include “angiotensin I converting enzyme, prepro endothelin, endothelin I converting enzyme, endothelin B receptor, eNOS, NF-kB, ICAM-1, VCAM-1, E-selectin, von Willebrand factor, adrenomedullin, C type natriuretic peptide, NADPH ox (p22phox), superoxide dimutase (SOD), leptin receptor, methylenetetrahydrofolate-reductase (MTHFR), α -adducin, caveolin, t-PA and PAI-1” (74).

Most of this work targets single nucleotide polymorphisms or SNPs which are variations that can occur in a DNA sequence when a single nucleotide (adenosine (A), thymine (T),

guanine (G) or cytosine (C)) is changed. If a sequenced DNA fragment from different individuals contains a difference in a single nucleotide it is said to have two alleles (an allele is one member of a pair of genes). To be called a SNP, a variation needs to occur in at least 1% of the population. The SNPs can be found in protein coding genes or non-coding regions of the genome. Not all SNPs affect cell function, however differences in SNPs may account for why some people are predisposed to certain diseases, or why they respond differently to drugs. Maps of SNPs are being used to try and identify markers in the human genome that are responsible for many things, among them vascular disease (85).

Rather than a single gene influencing endothelial function and dysfunction it is more likely multiple genes will be responsible. The technique of polymerase chain reaction (PCR) can be used to study the expression of multiple genes involved in a specific area of interest, for example vascular biology. Polymerase chain reaction involves denaturing (separating into single strands) DNA. The single strand of DNA is then multiplied by enzymatic replication so that approximately a million copies of a gene's DNA sequence are available for study. The PCR array is used to study a group of genes from a biological pathway or genes associated with a specified disease state. For example, PCR can be used to analyse which genes are involved in the regulation of NO signalling or assess which genes regulate the endothelial cell to control vascular tone and vessel diameter (86). Future studies will probably concentrate on multiple genes and their combined effect (73). By studying multiple variables, there is an increased risk of a type 1 error with associations between genes and endothelial function more likely to occur by chance (74).

A study was published (87) assessing the relationship between FMD and the eNOS genotype in healthy pregnant women at 12 weeks gestation when systemic vascular

resistance is reduced and differences in NO dependent genotypes may be more evident.

The polymorphism eNOS Glu298Asp was significantly correlated with the degree of FMD response suggesting a possible role for genetic factors in the vasodilatation that occurs in normal pregnancy.

1.3.13 Estimating nitric oxide production from the measurement of nitric oxide metabolites.

As discussed previously, the NO molecule is produced from the interaction of L-arginine and NOS. The oxidation of NO to nitrate by oxy-haemoglobin in erythrocytes then results in the production of the by-product nitrite. Both nitrate and nitrite are stable metabolites, excreted in urine after circulating in blood and can be measured in plasma, serum and urine. Nitrate (which is biologically inactive compared to nitrite) is the major oxidative metabolite of NO (88).

1.3.14 Endothelial progenitor cells.

Endothelial progenitor or precursor cells (EPC) are found in the circulating blood of adult humans. These cells are capable of differentiating into mature endothelial cells. The endothelium itself has a low capacity for regeneration and repair, so it is thought EPC circulate in the blood and are able to repair and/or patch injured areas of endothelium. With increased risk factors for cardiovascular disease, low levels of EPC have been found. Increased levels of EPC may reduce the risk of endothelial dysfunction (73, 77).

In normal pregnancy EPC increase with increasing gestation which was positively correlated with oestradiol levels (89). In pre-eclamptic pregnancy, no difference in EPC was found when compared with normal pregnancy (90).

As discussed there are many ways to assess endothelial function. The following section will discuss flow-mediated dilatation, a non-invasive ultrasound technique used to measure stimulated endothelial function.

1.4 Flow-Mediated Dilatation - a non-invasive method for the assessment of vascular endothelial function.

1.4.0 Introduction

In 1992 Professor David Celermajer from the University of Sydney first introduced the idea of assessing endothelial function in humans through the non-invasive ultrasound technique of FMD (16). A number of assumptions were made to validate this technique as a measure of endothelial function some of which have already been mentioned in the section on NO.

Briefly, it was known that when large arteries experience increased flow and subsequent shear stress, they dilate (91, 92). Animal studies on dogs had demonstrated an intact endothelium was necessary for this flow dependent dilatation to occur (32, 93). When this flow induced shear stress occurred in animals NO was produced (33). It was also known that basal blood flow was regulated in the human forearm by the continuous release of NO (94) and endothelial cells had ion channels that responded to shear stress (95). After the addition of a NO antagonist infusion (for example L-NMMA) (25), the degree of dilatation that occurred after the shear stress stimulus was decreased. Studies had also shown that NO was produced as a basal substance and was stimulated by the addition of various drugs (94). This knowledge applied along with high resolution ultrasound led to the seminal work on FMD for use in the detection of endothelial dysfunction (16).

As with all new techniques, knowledge has evolved regarding the methodology and what is considered best practice with complete agreement still to be reached. The following section will describe the ultrasound technique of FMD in detail. Baseline and post-occlusion volumetric flow and reactive hyperaemia and the errors encountered measuring these variables will then be addressed. Following this will be a discussion on shear stress and shear rate and how they relate to the technique of FMD.

1.4.1 The technique of FMD.

The technique of FMD originally published by Celermajer and colleagues involved inflating a blood pressure cuff placed on either the distal arm or leg at a pressure of 300mmHg for 4.5 minutes. Either the brachial artery superior to the elbow or the superficial femoral artery just inferior to the bifurcation of the common femoral artery was imaged with high resolution ultrasound (16).

After the subject had rested for at least 10 minutes, artery diameter was measured before cuff inflation, 45-60 seconds after cuff release and again after 15 minutes of rest when sublingual glyceryl trinitrate (GTN) spray was administered. The GTN spray was used to demonstrate the degree of endothelial-independent dilatation that occurred and to demonstrate the FMD technique was endothelial-dependent. The artery was imaged in a longitudinal section and the diameter was measured at end-diastole (determined from an electrocardiograph (ECG) waveform collected contemporaneously). Flow-mediated dilatation was calculated as the percentage difference in resting and post occlusion diameter (16).

Arterial Doppler waveforms were also collected at rest and directly after release of the cuff (for 15 seconds during reactive hyperaemia). Artery diameter and the Doppler information were used to calculate resting and post-occlusion volumetric blood flow. The degree of reactive hyperaemia was calculated as the difference in resting and maximum post-occlusion blood flow. The volunteers (n=100), a mixture of children, adults, smokers, non-smokers and people with and without a history of and/or established cardiovascular disease were grouped for analysis according to these categories. No mention was made about subject preparation (16).

In 2002 guidelines for the ultrasound assessment of endothelial dependent FMD were published (96). This included recommendations on subject preparation, the type of equipment necessary, image acquisition, analysis and a section on training and improving the quality of the test. By this stage it was appreciated that many factors could influence FMD and high end ultrasound equipment was necessary as were skilled people to perform the test. It was recommended that the artery should be greater than 2.5mm and less than 5.0mm in diameter. Images should also be collected when the artery was visualized in a longitudinal section. However, at this stage no consensus had been reached on whether it was better to place the blood pressure cuff on the upper or lower arm or whether 5 or 10 minute occlusion was preferable. The guidelines also stated that either the brachial, radial, axillary or superficial femoral arteries could be used (96).

In 2005 a review was published detailing updated guidelines (17). It was suggested that FMD was highly dependent on arterial diameter and the shear stress stimulus derived from reactive hyperaemia and questioned whether all FMD was NO dependent. To assess NO dependent vasodilatation, certain stimulus referred to as “the concept of stimulus response

specificity” (17) needed to be used. The stimulus technique that provided the most reliable NO dependent FMD involves placing the blood pressure cuff distal (on the lower arm) to the site of FMD measurement and inflating the cuff for five minutes only. Data should be collected from brachial or radial arteries only and ischaemic handgrip exercise should not be used (17).

Interestingly, a “Point: Counterpoint” article (and many letters to the editor) indicates that a consensus regarding whether FMD reflects NO mediated endothelial function is yet to be reached (97, 98). A tutorial article on FMD published in 2010 was still of the same opinion (99). Another guideline published recently (100) reiterated subject preparation and methodological guidelines although a consensus on all aspects of the technique is still to be reached. What is known is that FMD reflects NO dependent dilatation under very specific circumstances. The following section will discuss the technique of FMD and those circumstances.

1.4.2 The calculation of FMD.

The measurement and calculation of FMD, baseline and post-occlusion volumetric flow and reactive hyperaemia are intimately related. This next section will discuss the formula used in the calculation of FMD.

Flow-mediated dilatation is usually reported as the percentage change in arterial diameter after baseline and post occlusion arterial diameters have been measured. It is calculated from the formula: $FMD\% = [(post\ occlusion\ arterial\ diameter - baseline\ arterial\ diameter) / baseline\ arterial\ diameter] \times 100$ (16). This is the most common formula used. Another way to express the change in diameter is by the formula: Change = Maximum

diameter - baseline diameter (101). The recent guidelines suggest presenting the FMD results as both percentage increase and absolute change in millimetres (100). The technique of FMD will now be evaluated in more detail.

1.4.3 Ultrasound considerations for performing FMD, transducer frequency and resolution.

The resolution of ultrasound is highly dependent on the wavelength of the ultrasound pulse and consequently the frequency of the transducer. The axial resolution of an ultrasound system is approximately 0.3-0.5mm (102). For this reason it is necessary when scanning small structures such as the brachial artery to use a high frequency transducer (103). The minimum frequency for FMD should be 7MHz, and preferably a broad band linear array transducer with a frequency range between 7-12MHz (96, 100).

1.4.4 The ultrasound machine settings of dynamic range and gain.

Dynamic range (compresses low level echoes and affects the contrast of the image) and gain (amplifies the echoes and can increase or decrease image brightness) should not be changed throughout the FMD examination. The same settings should be replicated between subjects (104). An experiment investigating the effects of changes in dynamic range and overall gain on phantom arterial lumen measurements demonstrated that for every 5dB increase in dynamic range and gain, a significant decrease in lumen diameter occurred. Increasing the distance between probe and artery had the same effect. Gain and dynamic range increases had the effect of making the arterial wall leading edge appear thicker (104).

1.4.5 Measurement of baseline arterial diameter.

There have historically been three time points to measure baseline arterial diameter. The first is before cuff inflation (16), another is whilst the cuff is inflated during the first 10 seconds of inflation (101), the third is sometime during the last minute before deflation (105). A recent study (106) demonstrated a difference in baseline brachial artery diameters between the diameter obtained before cuff inflation and during cuff inflation in children and young adults. Artery diameter was significantly greater during cuff inflation, resulting in a decreased FMD. No difference was found in older people. The authors suggested using the pre-cuff inflation diameter as this measurement was not age dependent.

The most recent guidelines recommend using the pre-cuff inflation diameter for the basal measurement (100). When analysing FMD, adjustment for baseline artery diameter is also suggested when comparing groups as the percent change in FMD is a result of dividing the increase in vessel size by the baseline diameter (101).

The brachial and radial arteries are now the recommended arteries for assessing FMD (17). This is because the arm provides easy access for cuff placement and the arteries are readily found and scanned with ultrasound technology. Better occlusion and therefore increased stimulus occurs when the blood pressure cuff is placed on the arm (17). The brachial and radial arteries are also more likely to provide a measurement between the recommended 2.5-5.0mm (96). Celermajer's group demonstrated an inverse relationship between FMD and baseline artery diameter ($r = -0.81$, $P < 0.001$) and noted when an artery measures greater than 6.0mm, even in healthy volunteers, the degree of dilatation is small (16). When artery size is less than 2.5mm, the vessel walls are more difficult to resolve due to the increase in attenuation from the use of a high frequency transducer (80).

A study of 20 men, assessing FMD in a number of conduit arteries (radial, brachial, popliteal, superficial femoral and common femoral) using a within subjects method (measuring different arteries in the same person) also demonstrated an inverse correlation between FMD and baseline artery size (107). This relationship held when between artery correlations for FMD and baseline arterial diameter were calculated across all vessels ($r = -0.57$, $P < 0.001$). This means that smaller arteries produce a greater FMD response whilst larger arteries dilate less. The same study also demonstrated that smaller arteries reach peak dilatation faster than larger arteries. These findings suggest that baseline arterial diameter may reflect differences in the structure of arteries (107).

In a magnetic resonance imaging study (108) designed to assess why FMD is dependent on arterial size, it was found that smaller arteries have a greater shear stress and therefore greater stimulus placed upon them which is proportional to the radius squared. It was felt that increased FMD in smaller arteries did not necessarily reflect better endothelial function.

1.4.6 Where to measure arterial diameter.

Flow-mediated dilatation involves measuring the lumen diameter of the brachial artery, initially at rest and then post cuff release when maximum arterial dilatation has occurred.

In 1991 an elegant paper was published detailing the seven echo zones that can be visualized in the common carotid artery with ultrasound (103). These zones represent first the layers of the near vessel wall, the adventitia (zone 1), then the media (zone 2) and intima (zone 3). Next visualized is the lumen (zone 4-the anechoic space between the vessel walls). Finally the vessel wall furthest from the transducer is represented by the intima (zone 5), media (zone 6) and adventitia (zone 7). According to the anatomical

correlation performed in this study, to measure the lumen diameter of a vessel, it is necessary to measure from the leading edge of echo zone 3 to the leading edge of echo zone 5, that is from the media–intima interface to the intima–media complex (Appendix 1- Figure 1) (103).

Near wall interfaces are harder to resolve, especially in smaller arteries (109). For this reason it is important to measure from a leading edge interface (103) and to set the ultrasound focal zone at the vessel wall nearest to the transducer (16). The work by Wendelhag was based upon the larger common carotid artery (103). The carotid artery has a vein nearby to help delineate the layers of the vessel wall (110) which would improve the resolution of the arterial walls. Brachial artery diameter measurements are often made at the media-adventitia interface (“m-line”), in the near and far wall (16, 101, 111). In the “Guidelines for Measuring FMD” (96), although they state the artery measurement is done between the lumen-intima interface in the near and far wall, it is difficult to tell from their images where the actual measurement has been done and which interface was used.

Due to the difficulty in resolving the “m-line” with a 7.5MHz transducer, one group investigated the use of a 30MHz mechanical linear transducer and found improved resolution of the anterior and posterior wall “m-line” in 96% of cases (112). Because of the high frequency transducer and increased attenuation of the ultrasound beam, it was necessary to scan the radial artery instead of the brachial artery. When measuring lumen diameter choosing an interface from where to measure is important as is consistency in those measurements.

A longitudinal slice of the brachial artery is the imaging plane of choice. The transverse or cross-sectional slice is not recommended as the definition of the near and far walls of the vessel are inadequate (96). The cross-sectional plane provides very few specular echoes for determining the diameter (109). The length of the brachial artery is scanned and a section of artery that is at its widest point with well-defined walls and “strong specular echoes” (109) is identified. It is important to find a recognisable, reproducible anatomical point such as another vessel or fascia to use as a landmark (16, 111). This is so the same piece of artery is scanned throughout the examination as the brachial artery narrows as it travels distally and therefore varies in diameter. The use of a foam device to hold the arm in a fixed position and a stereotactic clamp to hold the transducer in position (74, 113) may help reduce error from inadvertently measuring a different section of artery pre and post occlusion.

Any section of the brachial artery is suitable for measuring. In a meta-regression analysis of 219 papers, the location of a brachial artery measurement, whether it was the ante-cubital fossa or upper arm was not related significantly to the variance in FMD (114). Another factor that may influence the measurement of an artery is its distensibility or compliance due to the pulsatility of the artery. The diameter of an artery changes throughout the cardiac cycle (115). For this reason during the FMD study, an ECG of the subject is taken so that the ECG is visible on the recorded brachial artery image. The brachial artery is then measured at the same time point, at end-diastole, which corresponds to the QRS complex of the cardiac cycle (16). A recent study found taking average measurements over the whole cardiac cycle (entire R-R interval) gave similar results to measurements taken at end-diastole (116). This approach was ratified by the recent guidelines (100).

1.4.7 Blood pressure cuff safety, pressure, placement and duration time of cuff occlusion and its effect on FMD.

The purpose of inflating a blood pressure (BP) cuff on the lower arm as part of the FMD technique is to occlude arterial blood flow to the limb distally. After cuff release, the resultant increase in blood flow induces NO mediated reactive hyperaemia. The cuff used most often is a pneumatic automatic tourniquet. Guidelines have been produced on the recommended practices for the use of pneumatic tourniquets (117). Among the recommended guidelines for safe practice are the following relevant statements. The rapid inflation of tourniquets results in simultaneous occlusion of arteries and veins. Whilst the ideal pressure for cuff inflation has not been determined pressure should be kept to a minimum. For upper extremities this is about 50-75mmHg above the person's systolic BP with one hour occlusion being the maximum time limit (117).

Experimental data have shown that injury from inflated tourniquets is related to the duration the cuff is inflated and the pressure used with injury more common after three hours inflation (118). A minimum time and pressure to occlude flow would therefore be preferable. Minimum pressure has been reported as systolic BP plus a safety margin. A systolic BP + 35mmHg has been reported as acceptable to occlude flow (119). Using a Doppler stethoscope, tourniquet pressures of 189.9 ± 24.1 mmHg (120) and 202.3 ± 34.2 mmHg (121) were found to be effective in achieving a bloodless operating field in the upper extremities. One article suggested a systolic BP + 10mmHg divided by a tissue padding coefficient (calculated from arm circumference and with a value of ≤ 0.91) was enough to achieve arterial occlusion pressure (122). More recent research assessing the methodology of FMD have chosen cuff pressures of 40-50mmHg above systolic BP (correcting for blood pressure inflation level) (123), 250mmHg (113), >200mmHg and

300mmHg (124). It has been reported that different pressures do not alter the FMD response (114, 123).

In a statement on assessment of endothelial function, recommended cuff inflations of 300mmHg for adults, 200mmHg for children or 50mmHg above the volunteer's systolic BP were given (74). However no evidence was provided for choosing these values. An inflation time of five minutes and pressure of 200mmHg has been shown to be well tolerated with low levels of discomfort when performing FMD (125).

The controversy regarding forearm or upper arm placement of the BP cuff on the arm (96) has now been resolved with distal occlusion the recommended technique (17, 100). A number of studies reported that upper arm occlusion results in a significantly greater FMD response when compared to forearm occlusion (123, 126-130). The dilatation response was completely attenuated after lower arm occlusion and infusion of L-NMMA (a NOS inhibitor) (130). When the upper arm occlusion technique was used and L-NMMA was given, complete attenuation of the vasodilator response failed to occur. This indicates that FMD from forearm occlusion is NO mediated, but some of the upper arm occlusion FMD is due to ischaemia.

The most recent guidelines state that an inflation (occlusion) time of five minutes is recommended as any longer results in a non-NO mediated response (17, 100). An early paper from Corretti's group (with the cuff placed on the upper arm) demonstrated that 1-3 minutes of occlusion was insufficient to produce significant dilatation (129). When randomly comparing occlusion times of 30 seconds, 1.5, 2.5, 3.5, 4.5 and 8 minutes, a dependency between cuff occlusion time and FMD was shown (131). The optimal

inflation time was 4.5 minutes. At this point all subjects had reached $96 \pm 6\%$ of maximum dilatation. A later paper (132) demonstrated when lower arm occlusion time was 5 minutes, L-NMMA infusion reduced the FMD response significantly indicating a NO mediated response. When 15 minutes of occlusion was used, this resulted in a prolonged hyperaemic response. Consequently, there was no reduction in FMD with L-NMMA infusion indicating this effect was NO independent.

1.4.8 When to measure post occlusion arterial dilatation.

Historically the time chosen for the measurement of arterial diameter after blood pressure cuff release has been 45-60 seconds (16). There is evidence that peak diameter does not fall within this period. After peak hyperaemic flow occurs (as assessed by Doppler ultrasound), there is a delay in the time it takes for peak FMD to occur (133). In a study assessing the magnitude of FMD over time, it was found that peak FMD occurred at 49 ± 3.1 seconds in 75% of volunteers. This means that 25% of cases had not reached peak vasodilatation within the suggested 45-60 second period (127). No recommendation was made on the optimal time for measuring peak dilatation. A recommendation was made for continuous diameter measurements for a minimum of 90 seconds after cuff release (17). Of note, none of these recommendations were referenced so the evidence was mainly anecdotal. A more recent study (123) demonstrated that with continuous automated measurement of the brachial artery for 180 seconds, it is possible to measure peak dilatation. The mean “time-to-peak” maximum dilatation was 67.8 ± 8.9 seconds, although 30% of volunteers had not reached maximum dilatation by this point. Maximum for all participants was reached by 180 seconds. Interestingly they commented that the failure to reach maximum peak dilatation in 45-60s may contribute to the variability often reported between different studies.

Another study (134) designed to establish the optimal time to measure post occlusion arterial diameter compared young physically fit (mean age 26 ± 3.3 years), “old fit” (58.9 ± 5.1 years) and “old unfit” (57.3 ± 3.9 years) subjects. Arterial diameter was continuously measured for 1 minute at baseline and then from 30 seconds before cuff inflation to 3 minutes after cuff deflation. From the data collected at baseline, arterial diameter, blood flow and shear rate were calculated. Custom designed software automatically detected time to peak diameter and peak diameter with FMD calculated as the percentage increase in diameter. The time to peak period was significantly less in the young group compared to both older groups. Forty two percent to 100% of all subjects fell outside the usually reported time bracket (50-90 seconds) for measuring peak arterial diameter. In young fit subjects, the time to peak dilatation was significantly less (50 ± 11 seconds) compared to the old fit and old unfit people (80 ± 21 and 83 ± 36 seconds respectively), however, maximum dilatation was detected in 100% of younger people within 90 seconds. No significant difference in FMD was noted between the groups when the 60 second measurement time point was used (134). This work indicates that the time to peak dilatation is quite variable and in older subjects can take greater than 120 seconds.

Continuous tracking of post occlusion arterial diameter would be necessary to calculate true FMD and edge-detection software has been developed for this purpose (135-137).

The time period to calculate FMD would not need to be as long in young subjects as older ones. Whether this relationship holds in pregnant women is not addressed by any of these studies. In conclusion, the brachial artery needs to be monitored for up to 180 seconds to detect maximum dilatation (100).

1.4.9 Factors that may influence the measurement of FMD: Cardiovascular risk factors.

Traditional cardiovascular risk factors as well as environmental factors and subjects' demographic variability have been shown to influence FMD (15). It is important to try and control for some of this variability, or at least document factors that can alter FMD as part of the methodology. Reduced FMD has been demonstrated in coronary artery disease, when risk factors for cardiovascular disease are present (16), insulin dependent diabetes (138) and in children who had a low birth weight (139). The large Framingham heart study (140) demonstrated that FMD reduced with increasing age (subjects aged between 33 and 88 years), a rise in systolic BP of 20mmHg, increasing body mass index (BMI), lipid lowering therapy and smoking within the previous six hours. In contrast, predictors for an increased FMD were being female until 70 years of age was reached, an increased heart rate of 10 beats per minute (bpm) and exercise prior to the FMD test.

1.4.10 Gestational diabetes mellitus.

Reduced FMD has been demonstrated in pregnant women at 20 weeks gestation with type 1 diabetes (141). A graded reduction in FMD was found in women in the third trimester when comparing normal pregnancy with pregnancies complicated by impaired glucose tolerance and gestational diabetes mellitus (GDM) (142). Non-pregnant women with previous GDM also had reduced FMD compared with women who had never had GDM (143).

1.4.11 Hypertension and previous gestational hypertension.

Hypertension was reported to result in reduced vascular relaxation by forearm plethysmography (10). Since then numerous studies have demonstrated reduced FMD in mixed male/female, middle age people with hypertensive disease when compared to

normotensive individuals (144-146). In young healthy normotensive women, FMD was reduced when there was a history of gestational hypertension (147). Gestational hypertension was defined as a diastolic BP >90mmHg and proteinuria <300mg/24 hours. The control group was evenly matched with the gestational hypertension group in terms of BMI, age and baby demographics from the incident pregnancy. All women abstained from alcohol, food and caffeine for eight hours and were non-smokers, had no other medical disorders and were not on vasoactive medication or the oral contraceptive pill. The study was performed on average twenty months after delivery. The FMD in the previous gestational hypertension group was 8.9 ± 1.1 versus 19.8 ± 1.3 ($P < 0.0001$) in the control group. Four and half minutes of forearm cuff occlusion at 250mmHg was used for the FMD technique and post occlusion brachial artery diameter was measured between 50-60 seconds (147).

1.4.12 Mental stress.

As mental stress has been associated with an increased risk of adverse cardiovascular incidents (148) and endothelial dysfunction is a risk factor for cardiovascular disease (149), studies have been performed to assess the effect of mental stress on FMD. A study on young healthy medical students (mean age 23.5 years) who volunteered to have a baseline FMD test performed, then underwent a stressful mental arithmetic test during the FMD retest, found enhanced vasodilatation of the brachial artery (150). In contrast, in middle aged men (mean age 50.4 years) FMD was reduced for up to four hours after mental stress was induced (151). Similar results were reported in a younger cohort (age range 20-31 years) where mental stress was found to induce prolonged (up to 45 minutes) reduced FMD (152).

Mental stress is thought to induce a transient endothelial dysfunction (151) and cortisol is a key component of the stress response (153). Thirty-six healthy non-smoking volunteers (aged 18-55 years) were randomised to a placebo group or the drug metyrapone (metyrapone inhibits cortisol synthesis and thereby decreases stress as measured by cortisol levels) to assess the effect of mental stress on FMD. There were no differences in baseline FMD between the two groups. Metyrapone was found to block cortisol production with no difference in FMD found after stress. Cortisol levels were higher in the placebo group and FMD was significantly lower than the baseline level in this group (154).

Stress can result in acute sympathetic nerve activation. Because results have differed when assessing stress, a study using four different methods to increase sympathetic nerve activation was designed (155). This work found that impaired FMD is not a generalised response to sympathetic nerve activation. As there are conflicting results regarding mental stress and its effect on FMD, it would be important to ensure volunteers are as stress free and comfortable as possible when performing the test.

1.4.13 Age.

The evidence suggests that FMD is inversely proportional to age. As subjects grow older, FMD decreases (105, 140, 156). In both men and women FMD did not decrease until around 40 years in men and until around the time of menopause in women (early 50s) (156). The relationship between FMD and age is also confounded by other variables, for example how physically fit people are (157) and artery size (16). In one study noting the inverse FMD and age relationship, people with other co-morbidities which are known to affect FMD were recruited. When comparing 35 year old women to 55 year old women, less dilatation was noted in the older group. More of the older women had pre-existing

cardiovascular disease, hypertension or were on oestrogen replacement therapy (158). For this reason a study was designed comparing FMD in young healthy women (mean age 22 ± 1 years, day 1-7 of the menstrual cycle) with older healthy women (mean age 70 ± 2 years) who had no chronic diseases (105). Neither group took any medication including the oral contraceptive pill or hormone replacement therapy. Age was still related to a 40% reduction in FMD. Endothelium independent dilatation (assessed after administration of sublingual nitro-glycerine) was also reduced in older women suggesting that the reduction in FMD may be due to a problem with the smooth muscle responding to the dilators produced after shear stress stimulus.

Interestingly another study demonstrated the age decline in FMD in women only occurred if the older women were sedentary (157). A large study (4040 individuals) examined the relationship between age, gender and baseline artery diameter (101). It was found that the best predictor of percentage change in diameter (FMD) was age. However, when adjustments were made for age and baseline diameter in both men and women, women had five percent less change in FMD than men.

In general FMD is thought to decrease more in men because they have a larger baseline brachial artery diameter compared to women. Aging also results in progressive dilatation of the brachial artery at rest (156).

1.4.14 Variations in the menstrual cycle.

Gender may affect FMD not only because of the difference in baseline arterial diameter between men and women (159) but also because of the effect of oestrogen on endothelial function (160). For this reason it is important to standardise the time in the menstrual

cycle when FMD is assessed (161, 162). In young women FMD ($11.22 \pm 0.58\%$) (mean \pm SEM) was comparable to young men ($10.6 \pm 0.75\%$) only when assessed during menstruation. Increases in FMD were noted during the follicular ($18.2 \pm 0.81\%$) and luteal phases ($17.53 \pm 0.74\%$) although no corrections were made for the significant difference in baseline arterial diameter (161). In comparison, another study demonstrated variations throughout the menstrual cycle with the lowest FMD value obtained during the early luteal (post ovulatory) phase of the menstrual cycle. Flow-mediated dilatation increased during the follicular phase, fell after ovulation and rose again during the late luteal phase (162).

The decrease in FMD during menstruation corresponded to low levels of serum oestradiol (163) and was found to be related to a decrease in arterial distensibility as measured by a distensibility/blood pressure curve (164). It has been suggested that FMD should be assessed between days 1-7 in premenopausal women (100).

1.4.15 Diet.

Diet also has an effect on FMD. In a study assessing the effect of either a single meal high in saturated fats (McDonald's Corporation breakfast McMuffins, hash browns and non-caffeinated drink), or a low fat meal of cereal, skim milk and orange juice it was found that a single high fat meal reduced FMD 2-4 hours after eating. In comparison, the low fat meal resulted in no change in FMD up to six hours after eating (165). One problem with this study is upper arm cuff occlusion was used which has been shown to not wholly represent NO mediated dilatation (130).

When comparing the consumption of meals either high or low in saturated fats, ultrasound of the brachial artery using lower arm cuff placement FMD and plethysmography were used to determine if there was a difference in FMD and forearm blood flow (FBF)

respectively. This study found that FBF and baseline artery diameter increased significantly after a fatty meal, but FMD was unchanged. The rise in FBF was thought to be mediated by changes in levels of insulin and triglycerides. It was suggested that no change in FMD meant fatty meals did not affect NO mediated endothelial function (166).

Only a diet high in saturated fats was shown to decrease FMD in a more recent study. No change in FMD was found when diets were modified to a high monounsaturated fat, high polyunsaturated or low fat high carbohydrate diet for three weeks (167). A recent review article concluded that when FMD was used, high fat food intake results in reduced endothelium dependent vasodilatation (168). Both oral and intravenous high fat loading on healthy normotensive obese ($BMI \geq 30$) subjects also resulted in reduced FMD (169). It has recently been recommended that FMD subjects abstain from food, alcohol, caffeine, drugs and medication for at least six hours before assessment (100).

1.4.16 Caffeine.

Abstinence from caffeine is also recommended when performing FMD (15, 74, 96) although no studies demonstrating the effect of caffeine on FMD were referenced when these guidelines were written. Ingestion of caffeine was shown to have no effect on FMD after two hours (170). A study in 2005 demonstrated caffeine in coffee produced an acute decrease in FMD which occurred in the first 30 minutes after ingestion and reached its lowest point at 60 minutes. By 90 minutes FMD had improved. De-caffeinated coffee had no effect (171). These two studies suggest that caffeine produces an acute reduction in FMD which resolves between 90-120 minutes. In contrast green and black tea (172), dark chocolate (173, 174), all rich in flavonoids and de-alcoholised red wine rich in polyphenols (175) have been shown to improve FMD. This is probably due to their high flavonoid

(176) and polyphenol content (175). Most studies are therefore performed after volunteers have fasted or have consumed a low fat, caffeine free meal.

1.4.17 Smoking.

Tobacco smoking was shown to result in reduced FMD in Celermajer and colleagues original study (16). Since then other studies have demonstrated impaired FMD in smokers. Flow-mediated dilatation decreases as the number and period of time cigarettes have been smoked increases, with the mean \pm SD FMD in the smoking group reported as 4.0 ± 3.9 compared with an FMD of 10.0 ± 3.3 in non-smoking controls (177). Reduced FMD has also been reported in both active and passive smokers (178, 179) and users of smokeless tobacco (180). Smoking both light and heavy nicotine content cigarettes reduced FMD in non-smokers which was of shorter duration (FMD returned to baseline in 30 minutes) when a light cigarette was smoked. The FMD did not return to baseline values after smoking the heavy nicotine cigarette until 60 minutes (181).

The same group also compared the effect of smoking the same strength cigarette in smokers and non-smokers. Unlike other studies, this research showed no difference in baseline FMD between smokers and non-smokers. Smoking caused similar decreased FMD values in both groups at 30 minutes that was prolonged for up to 60 minutes in the smokers (182).

Two studies have assessed the acute and chronic effects of cigarette smoking on FMD using a test-retest method (183, 184). Lekakis' group demonstrated no difference in baseline (day 1) FMD between smokers (n=10) and non-smokers (n=17) (both genders, 9 men, 18 women, age not reported) after an eight hour break from smoking. On day two a

reduced FMD was reported after both smokers and non-smokers smoked a cigarette. This research used a mixed study group and had small numbers (n=27) which were divided into four groups for analysis which would further reduce the power of the study (183). The second study (184) enrolled young (mean age 24.9 ± 1.9 years) apparently healthy smokers to investigate the effects of smoking a cigarette or using nicotine nasal spray on men (n=8) and women (n=8). This work also demonstrated no effect from the chronic use of cigarettes on baseline FMD, with the FMD from smokers almost twice that of other studies (FMD = 10.2 ± 4.4 (nasal spray group) and 9.4 ± 3.8 (cigarette group)). A significant reduction in FMD occurred after both treatments (184). In this study the BP cuff was placed on the upper arm reflecting ischaemia, rather than endothelial function (130). It was suggested that it was the nicotine in cigarettes that caused the reduction in FMD (184). Interestingly, no studies had been done looking at the effect of smoking in pregnancy on endothelial function using the technique of FMD so it was decided to perform such a study as part of this thesis.

Recent work has analysed the effect of smoking in young women of reproductive age throughout their menstrual cycle, comparing women who smoked (n=13) to those who did not smoke (n=12). Similar FMD in both groups at menstruation and in the mid-follicular phase was found. During the mid-luteal phase FMD and oestradiol levels were significantly reduced in the smoking group. It was suggested that nicotine use over a long period of time would decrease oestrogen levels and FMD (185). This study highlights the importance of standardising studies in terms of age, sex, time of menstrual cycle and smoking status.

1.4.18 Vasoactive medication in pregnant and non-pregnant people.

The FMD guidelines state that vasoactive medication “be withheld for at least four half-lives, if possible”. If the study is observational, data should be collected on the type of medication the volunteer is taking (96). A literature search was performed on “Pregnancy and medication and FMD” and variations thereof. This search did not reveal any studies examining the effect of vasoactive medication on FMD in pregnancy. There is some work using techniques other than FMD that have assessed the effect of vasoactive medication on the endothelium in pregnancy. Results are conflicting. One study demonstrated medication had no effect on the levels of the NO metabolites nitrate and nitrite in pre-eclampsia (186). More recently the effect of methyldopa on soluble *fms*-like tyrosine kinase (sFlt-1), soluble endoglin (sEng), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in pre-eclamptic and gestational hypertensive pregnancies was assessed (187). Both sFlt-1 and sEng serum concentrations were reduced after methyldopa treatment. There was no significant difference in sFlt-1 and sEng in the gestational hypertension women. The authors felt they could not exclude an effect from methyldopa on the endothelium.

The only literature specifically addressing FMD appears to be on non-pregnant people where the effect of vasoactive medication depends on the population studied and the type of medication given. A study performed in 1999 demonstrated an improvement in FMD after long term (6-12 months) antihypertensive treatment (188). In 2002 a single study was performed on two groups (189), in which the first group (n=73) were healthy, young volunteers, two thirds of whom were males. After a baseline FMD, they were randomised in a double blind fashion to take a placebo, felodipine, metoprolol or enalapril and FMD was performed three hours later. The second group were mostly men (78%), older, heavier, all had coronary artery disease, 47% were smokers, most had other co-morbidities

and were taking various vasoactive drugs. After withholding their vasoactive drugs for 24 hours, a baseline FMD was performed, their usual medication was given and FMD was performed three hours later. In both groups FMD was not affected by the ingestion of vasoactive drugs. The authors' conclusion was that it is probably not necessary to withhold vasoactive drugs unless an interventional trial was being held. One problem with this study (apart from the multiple confounders in the older group) was the upper arm placement of the BP cuff to induce reactive hyperaemia. (189).

Another study (190) used FMD to assess patients with coronary artery disease (n=35) on vasoactive therapy comparing the peripheral vasodilator and beta-blocker nebivolol with the beta-blocker atenolol. The subjects were randomised to either drug. Before drug therapy commenced FMD was performed and repeated four weeks later. Analysis was performed using Student t-tests which would have increased the risk of a type 1 error, as a mixed between-within analysis of variance would have been more appropriate. The results showed FMD was unchanged after atenolol therapy. Nebivolol therapy resulted in an increase in FMD compared with baseline values and atenolol therapy.

Another study (146) assessed the effect of six months therapy with four different drugs on 168 hypertensive people. This work randomized subjects to either an angiotensin converting enzyme (ACE) inhibitor (perindopril), calcium antagonists (nifedipine or amlodipine), beta blockers (atenolol or nebivolol) or an AT1-receptor antagonist (telmisartan). Only the ACE inhibitor perindopril significantly increased FMD.

In a review article (191) on hypertension and medication, ACE inhibitors were described as improving endothelial function as tested by FMD, whilst calcium antagonists improved

endothelial function as tested by markers of oxidative stress and FMD. Drugs like nifedipene were not as good as ACE inhibitors at improving endothelial function. In general beta-blockers have no effect on endothelial function except for drugs such as nebivolol because it is a NO donor. The addition of medication into the FMD picture appears to add another layer of confounding information that would need to be addressed in any studies undertaken.

1.4.19 Obesity.

Obesity is known to result in a reduction in FMD (192, 193). In healthy women of reproductive age who were classified as lean, overweight or obese based on their BMI, FMD was found to be similar in the lean and overweight groups but significantly reduced in the obese group. A negative correlation was found between FMD and BMI ($r = -0.37$, $P=0.005$) (193). A mixed male and female group of morbidly obese people also demonstrated that as size increased FMD decreased with weight being a strong negative predictor of FMD (192). It would therefore be important when assessing FMD to collect weight and height data to ensure different groups are matched.

1.4.20 Diurnal variation.

Variation in FMD can occur throughout the whole day. Some studies show decreased FMD in the morning. In young healthy Japanese males, FMD was significantly lower when measured at 08:00 and 12:00 compared to measurements performed at 17:00 and 21:00 (194). A similar finding was reported in 17 healthy young men with the lowest FMD reported at 08:00, increasing to similar values in the afternoon (between 12:00 and 14:00) (195).

In a slightly older mixed male and female cohort (mean age 41.6 ± 2.1 years), FMD was still significantly lower in the early morning (06:00, 4.4 ± 0.7 ; $m \pm SE$) compared with 11:00 (7.7 ± 1.0) and 21:00 (7.5 ± 1.0) (196). Significant diurnal variations in FMD were found in 16 healthy premenopausal women aged between 19-31 years with the lowest FMD recorded in the afternoon.

When FMD was assessed over a 24 hour period (at 08:00, 14:00, 20:00 and 02:00), the lowest value was recorded at 14:00 ($m \pm SE$), 3.1 ± 0.4 ; and then increased gradually until 02:00 (20:00, 4.4 ± 0.4 ; 02:00, 5.1 ± 0.9), dropping slightly at 08:00 (3.9 ± 0.8) ($P < 0.05$) (197). Even though this variation was significant, in the clinical situation, it is unlikely that such small differences in mean FMD would be detected.

When assessing FMD at two time points, 09:00 and 14:00, where results were reported as absolute difference and mean difference $\pm SD$ using Bland-Altman plots, no significant difference was found in FMD (198). The authors concluded that there was more inherent variability in FMD measurements due to the technique, than there is biological variability due to the time of day. Therefore, as long as other experimental cofounders are controlled for, it is not obligatory to standardise the time of day when FMD is performed.

1.4.21 Exercise.

The FMD guidelines recommend that no exercise is performed 4-6 hours before testing (15, 96) and ischaemic handgrip exercise should not be used (17). Ischaemic hand grip exercise with the addition of cuff occlusion to induce reactive hyperaemia results in an increase in blood flow velocity and FMD compared to just occlusion alone in older men (199, 200). This increase in FMD was only partially blocked by L-NMMA infusion which

suggests that adding hand grip exercise may not be wholly representative of a NO mediated dilatation (199). A later study established ischaemic hand grip exercise alone did not detect changes in FMD after a single event (high fat meal) designed to cause endothelial dysfunction and reduced FMD, whereas cuff occlusion FMD did demonstrate a difference (201). A review article (202) concluded that one of the most important changes exercise may induce is an increase in baseline artery diameter due to the increase in blood flow with a resulting decrease in FMD, although this may be age and sex dependent. Acute exercise also results in changes in sympathetic nerve activity. This means that exercise may induce changes in FMD that are not wholly NO mediated, instead reflecting a combination of “NO bioavailability and sympathetic modulation” (202).

A study (203) assessing the effect of acute exercise on healthy pre- and post-menopausal women found no significant change in baseline artery diameter (mean \pm SEM) (3.2 ± 0.16 versus 3.1 ± 0.11 , $P=0.674$) or FMD (12.1 ± 1.5 versus 14.4 ± 1.2 , $P=0.236$) in the pre-menopausal group. Exercise in post-menopausal women also resulted in no change in baseline arterial diameter (3.21 ± 0.16 versus 3.25 ± 0.17 , $P=0.007$), but FMD was doubled (5.3 ± 1.3 versus 9.9 ± 1.4 , $P=0.007$). As exercise appears to add another layer of complexity to the FMD test, it would be better if strenuous exercise was avoided, although this may not be as important in premenopausal women.

1.4.22 Racial differences.

Studies have assessed whether there are racial differences in FMD, although these have been reported in the context of black people having a higher burden of cardiovascular disease. When comparing African Americans with Caucasian Americans of both sexes aged in their mid-forties, half hypertensive and half normotensive, race was not found to

influence FMD (204). However, when young healthy black African subjects, recently migrated to Italy, were compared to Caucasian subjects, it was found that the African people had lower FMD. This was found to be related to their “total infectious burden” and inflammatory state from common viruses such as cytomegalovirus and herpes amongst others (205). In elderly African American women (mean age 78.4 ± 4.4 years for both groups), FMD was reduced when compared to elderly Caucasian American women even after adjustment for cardiovascular risk factors (206). These papers provide conflicting reports on whether race affects FMD, probably because the groups are varied in age and disease burden.

Many factors can affect FMD as the previous section demonstrated. Variations in subject demographics, risk factors and technical aspects need to be taken into account when performing this test. Physiological variables such as the volume of flow through the artery and the degree of reactive hyperaemia also need to be taken into account and these will be addressed in the next section.

1.4.23 Measuring baseline and post-occlusion volume flow and reactive hyperaemia.

Historically, baseline and post-occlusion volumetric flow (used interchangeably with the term volume flow) and reactive hyperaemia have been used and reported to assess the degree of flow stimulus (shear stress) that occurs in a FMD study (16). It was later appreciated that when comparing different groups, as long as baseline vessel diameters are similar, then volume flow can be considered an indicator of shear stress (17). Baseline volumetric flow is calculated from the formula: Baseline flow (mL/min) = VTI x baseline heart rate x $(0.5 \times \text{baseline vessel diameter})^2 \times \pi \times (1/\cos 60)$ (16, 207, 208) where VTI is velocity time integral in metres, $[(0.5 \times \text{baseline vessel diameter})^2 \times \pi]$ is the area of the

artery and $1/\cos 60$ is the Doppler angle correction, assuming that the angle between the ultrasound beam and direction of flow is 60 degrees. The VTI is the time averaged peak velocity (or area under the Doppler velocity time curve) multiplied by the time period of flow (usually one heart cycle) (132, 209). The basal VTI is averaged from a number of heart cycles when the subject has rested for at least 10 minutes (16).

Post occlusion volumetric flow uses the maximum VTI taken from one cardiac cycle and recorded by Doppler ultrasound during the reactive hyperaemia that occurs after cuff release (207, 208). Post occlusion (PO) volumetric flow is calculated from the formula:

PO flow (mL/min) = PO maximum VTI x PO heart rate x $(0.5 \times \text{PO vessel diameter})^2 \times \pi \times (1/\cos 60)$. Reactive hyperaemia or peak flow increase is calculated from the formula:

Reactive hyperaemia % = $[(\text{maximum PO flow} - \text{baseline flow})/\text{baseline flow}] \times 100$ (207, 208).

The baseline and post occlusion volumetric flow and reactive hyperaemia formulae (that is the shear stress stimulus for FMD) use the baseline and post occlusion arterial diameters in their calculation and are therefore not independent of FMD. A suggestion for dealing with this problem is to measure the baseline artery diameter at different times, first during the initial baseline collection of artery diameter (74). The second time point would be within the 10 second period after cuff deflation, before the artery dilates (101). Although as previously stated this may be a problem when dealing with different age groups as the artery dilates during cuff occlusion in younger people (106).

The term “volumetric flow” has been used as the technique results in an estimate of true volume flow (210) because a number of sources of error are encountered when calculating

volume blood flow by Doppler and two dimensional (2D) ultrasound. These errors mainly relate to the size and placement of the Doppler sample gate and the measurement of the artery diameter. Calculation of true volume flow involves encompassing the entire width of the vessel with the Doppler sample gate (115, 210-212). However, if a large sample gate is used there is the risk of inadvertently measuring another vessel at the same time (115).

In the method described originally by Celermajer (16) a small sample gate is placed in the middle of the vessel to calculate the VTI. This results in an overestimation of velocity, since maximum Doppler shift occurs at the centre of a vessel that demonstrates parabolic (laminar) flow (212). In the larger conduit arteries (for example the brachial artery) flow tends to be forward in direction and laminar with maximum flow at the centre and the slowest velocities recorded at the vessel wall (17, 212). Error can also occur in the estimation of blood flow if an incorrect Doppler angle is used as this results in incorrect velocity calculations (210). An angle of 60 degrees is the maximum recommended (80) and be used for assessing the brachial artery. This is because the angle the vessel courses in the arm makes it difficult to use a smaller angle.

The vessel diameter is an important part of volume flow calculations. The diameter is squared to calculate the area of the vessel. Any error in the vessel diameter would also be squared (115). The pulsatility of the vessel wall also means the diameter changes during the cardiac cycle; however this is compensated for by standardising the diameter measurements at end-diastole (16). An end-diastolic measurement means that the vessel diameter is at its smallest which could theoretically reduce the calculated volume flow. The area formula also assumes the vessel is circular in cross-section which cannot be

guaranteed (210). The maximum diameter of the vessel should be imaged. This can be difficult and is done by ensuring the imaging plane is perpendicular to the vessel wall (212) and the intima-media complex of the vessel walls are clearly identified (103). Even though only an estimate of volume flow is obtained, as long as a reproducible technique is used, comparisons between groups should still be possible.

1.4.24 Some evidence that FMD may not be wholly nitric oxide mediated.

As previously mentioned there are a variety of other vasoregulators apart from NO, produced by the endothelium. These vasodilators (NO, PGI₂ and EDHF) or vasoconstrictors (ET-1 and PAF) may be released in response to varying disease states as well as increased flow stimulus due to changes in occlusion time, cuff position, the addition of ischaemic limb exercise and the use of different limbs (17). The technique that produces the shear stress that results in a NO mediated FMD is very specific (17) although some evidence is available that even the technique as it stands may not result in a completely NO dependent vasodilatation. In a complicated study (124) trialling various lengths of reactive hyperaemia stimulus profiles and assessing the degree of NO dependent dilatation with the use of L-NMMA, it was reported that there was no effect of L-NMMA on FMD in any of the trials. This led the authors to conclude (at least in the radial arteries of healthy young men) that NO may not be solely responsible for radial artery FMD. Other vasodilators and/or dilatory mechanisms may play a part in the response.

The mechanisms that may contribute in the complex biology of FMD include flow rate, blood viscosity (213) and arterial diameter (107). As shear stress is the stimulus for FMD, attempts are being made to measure and quantify the shear stress stimulus and use these measurements to normalise the FMD response. This is felt to be necessary because of the

inherent variability the FMD technique displays, possibly due to the variability in shear stress between people (214). Indeed, reduced hyperaemic stimulus may be the cause of reduced FMD, rather than impaired NO release (215).

1.4.25 Variability in the FMD technique.

Three reasons for the variability in FMD measurements have been suggested. The first is due to measurement error when performing the technique, the second is due to the differences in the physiological response of the endothelium and the third is due to shear stress variability (34). The following section will concentrate on shear stress and its substitute measure shear rate, viscosity and its relationship with haematocrit and the effect of arterial diameter as a stimulus for FMD. Different methods of normalising to the shear stimulus will also be discussed.

1.4.26 Shear stress and shear rate as a stimulus for FMD and their relationship with viscosity, velocity, haematocrit and vessel size.

Shear stress is a product of the viscosity and velocity of blood divided by the vessel diameter and represents the drag or friction on the endothelial cells that results from blood flowing through the vessel (216). As such, it is a stimulus for FMD. The importance of viscosity and flow rate were demonstrated in 1989 (213), when in-vivo cat femoral arteries were used to demonstrate that changes in both blood viscosity and flow rate resulted in endothelium dependent arterial dilatation or constriction. Decreases in viscosity resulted in vasoconstriction, and an increase in viscosity caused marked vasodilatation. By comparing viscosity and flow rate by varying the haemodilution/concentration and flow dynamics the researchers ensured the variations in shear stress were matched. An increase in shear stress caused by the equal increase in viscosity and/or flow rate resulted in the same increase in

arterial diameter. The study also established that the stimulus for changes in arterial diameter is the shear stress placed upon the endothelium (213).

Viscosity is the “resistance of a liquid to flow” due to its “internal molecular friction”. The term shear rate when related to viscosity identifies the imaginary layers of fluid molecules that move over each other (shear), producing internal friction. Eventually these layers form a laminar flow profile with the difference in velocities between the layers the shear rate. Homogenous fluids that maintain a constant viscosity regardless of their flow rate are referred to as Newtonian fluids. Examples are plasma or water. Blood however is a non-Newtonian fluid because as the shear rate or velocity of blood changes the behaviour of the red cells change. The red cells either clump at low velocity or at higher velocities disaggregate, elongating and orientating in the direction of flow. This means the accurate and reproducible measurement of whole blood viscosity is very difficult (217).

In blood, increases in shear rate see a decrease in viscosity and decreases in shear rate result in increased viscosity at a set haematocrit (218). This inverse relationship is highly dependent on haematocrit. A higher haematocrit results in greater increases in viscosity even at higher shear rates (218, 219). Indeed, haematocrit is the most significant “determinant of whole blood shear-dependent viscosity” because of the ability of red blood cells to aggregate and disaggregate at different velocities (219). For this reason and due to the difficulty in measuring viscosity, haematocrit has been used as a surrogate viscosity measure in some studies (105) whilst others assume a standard viscosity for the purposes of their study (215, 220).

The ability of red blood cells to react to the surrounding conditions also means that vessel diameter is an important determinant of viscosity. This is particularly important for vessels less than 1mm. However, in medium to large size arteries viscosity can be regarded as constant (219).

1.4.27 Shear stress, shear rate and Poiseuille's formula.

The precise measuring of shear stress non-invasively is difficult not only because of the problems with measuring viscosity, but also because of the pulsatile nature of blood flow velocity. Due to the variations in velocity across the artery, measurements should be made near the vessel wall and the distance from the arterial wall to where the velocity measurement is taken should be recorded (216). Even if this measurement were possible, the concentration of red blood cells dilute near the vessel wall, so viscosity and shear stress will always be underestimated (216). Therefore a surrogate measure of shear stress, termed in the FMD literature, shear rate, is often used instead (17). Numerous formulae, all variations of Poiseuille's equation have been proposed to calculate shear rate. To understand how Poiseuille's equation is related to shear stress, a discussion on basic physics is required.

For fluid to flow through a tube, a pressure difference (ΔP) is necessary. Volumetric flow rate (Q) can be calculated by the difference in pressure (ΔP) and resistance to flow (R). This equates to the formula: $Q \text{ (mL/s)} = \Delta P \text{ (dyne/cm}^2\text{)}/R \text{ (poise)}$. As resistance to flow also depends on viscosity (η), tube length (L) and radius (r) this can be expressed as: $R = 8 \times L \text{ (cm)} \times \eta \text{ (poise)}/\pi \times r^4 \text{ (cm}^4\text{)}$. If flow resistance is inserted into flow rate and diameter D is used, this gives Poiseuille's equation: $Q = (\Delta P \times \pi \times D^4)/(128 \times L \times \eta)$ (80). This can also be expressed for laminar flow in a straight vessel as: Shear stress (τ) = $32 \times \eta \times Q/(\pi \times$

D^3) (216). Another way to express this formula, usually when viscosity can be measured (in animal or *in vitro* experiments) and referred to in some articles as the Hagen-Poiseuille formula is: Shear stress (τ) = $4\eta Q / \pi r^3$ (221) or: Shear stress (τ) = $4\eta / \pi r^3$ (219, 222). Shear stress has also been estimated by the formula: Shear stress = viscosity x velocity/diameter (17). The use of Poiseuille's formula assumes that blood behaves like a Newtonian fluid (which it does for shear rates above 100s^{-1}), that there is laminar flow with no turbulence and that the flow is not pulsatile. The cross section of the vessel should be circular with a constant diameter and the vessel wall remains rigid (219). In reality most of these assumptions are not met in vessels in the human body.

Due to the difficulties in measuring viscosity and therefore shear stress, the literature contains many variations of Poiseuille's formula adjusted to calculate shear rate, with no consensus reached on a formula for shear rate or what form of normalisation for the variation in shear rate to FMD should be used (222). The 2005 consensus statement on FMD suggested: Shear rate (γ) = velocity/diameter (17) which has the advantage of not using viscosity. According to Poiseuille's law: Shear rate (γ) = $8 \times V_m / D$ where V_m is the mean blood flow velocity and D is lumen arterial diameter at end-diastole (221). An alternative is, in the case of parabolic flow, and using only the central peak flow velocity (V_{\max}): Shear rate (γ) = $4 \times V_{\max} / D$ (221).

To provide a few examples of the uses of shear stress and shear rate, early work calculated shear rate from the basal artery diameter and volume flow, measuring maximum flow 10 seconds after cuff release. No information was provided on the shear rate formula used (197). After assuming viscosity, another variation of the formula for shear stress was calculated using mean flow velocity as: Shear stress = $8 \eta V_x / D_{BL}$ where V_x is mean flow

velocity at baseline or during reactive hyperaemia and D_{BL} is baseline artery diameter (215). The formula used in this study appears to be an amalgamation of the shear stress/shear rate formulae. Many of the scholarly articles published recently assessing the physiology of the FMD technique use one of the following: shear rate = V_{peak}/D (124), shear rate = V_{mean}/D (34, 223-225), shear rate = $4V_{peak}/D$ (105) or shear rate = $4V_{mean}/D$ (107, 134) where V_{mean} and V_{peak} are mean and peak blood velocity respectively. It would appear that researchers have chosen any combination or variation of these formulae, choosing indiscriminately whether to use peak or mean blood velocity which makes comparing results across different studies difficult (222).

The arterial diameter used in the shear rate formula has also been measured at different times. If basal shear rate is calculated then a resting diameter is used (105). As the artery does not dilate in the 10-12 seconds post cuff release (101, 105), theoretically, if simultaneous Doppler and 2D ultrasound imaging were available then the artery diameter immediately post cuff release could be used, although the limitations of changes in arterial diameter with different age groups would need to be considered (106). For post occlusion shear rate the maximum arterial diameter is not used. This is because it would not give an indication of the shear stimulus during reactive hyperaemia as the arterial dilatory response to the shear stimulus generally begins around 45 seconds post cuff release. By this time the shear stimulus is decaying (223). Instead, baseline arterial diameter has been used for the post occlusion shear rate (215) as has arterial diameter measured just before cuff release (105). More recent studies have utilised continuous artery diameter monitoring up to the point of maximum dilatation, with hundreds of automatically calculated diameters recorded, calculating shear rate as the area under the curve (AUC) (134).

1.4.28 The normalisation of shear rate to FMD.

Recent literature recommends normalising or correcting FMD to shear rate (17). Early work only reported volume flow and reactive hyperaemia (16) with, as previously discussed, volumetric flow (referred to now in current literature as volume flow) calculated from the vessel area multiplied by the velocity of the blood flow over a period of time. Volume flow does not give an indication of the frictional force that is occurring on the endothelial cells. However if volume flow and vessel size are similar, then shear rate will be similar. In contrast, if the vessel sizes differ even when volume flow is the same, then velocity changes and shear rates differ dramatically. A small vessel will experience a greater shear rate and shear stimulus than a larger vessel (17, 34).

As previously reviewed, the updated consensus article (17) stated that Shear rate = V/D and FMD should be “normalized by dividing the peak percentage change” in artery diameter (that is FMD) by the size of the reactive hyperaemia stimulus (that is FMD/stimulus). Both the peak stimulus and duration of the stimulus should be reported. For the chosen duration of the stimulus that is measured (the time frame was not specified), the AUC should be measured and either peak or AUC used for normalisation of FMD (17). The peak shear response that occurred after occlusion was one of the first methods chosen for normalisation of FMD with shear stress (105), probably because this information was available from the data collected when performing FMD according to Celermajer and colleagues (16) original technique. Parker and co-authors (105) used the formula shear rate = $4V_{\text{peak}}/D$, calculating peak velocity from the maximum VTI in the first 30 seconds post cuff release and using the arterial diameter collected immediately before cuff release. A baseline shear rate was also calculated and the normalisation of FMD involved using the difference in shear rate from baseline to peak (105).

The next form of normalisation to the shear stimulus came in the form of calculating a shear rate correction using the recorded Doppler velocity information to calculate the AUC. This was done from data that were collected from between nine seconds post cuff release (223) to within the first minute (34). This was followed by the suggestion that the AUC should be calculated until peak arterial dilatation was reached. Continuous monitoring with Doppler and 2D ultrasound is necessary to achieve this (134).

A study using repeated measures within subjects design and graded increases in shear stimulus demonstrated that the least contribution came from the peak shear rate (223). After demonstrating a strong association between FMD and shear rate AUC (measured for nine seconds) they suggested this technique as the appropriate method to normalise FMD. Another study (226) that adopted a between subjects design (by examining groups of different ages) to assess the relationship between FMD and shear rate had different findings. Shear rate was assessed as peak shear rate AUC from 0-9 seconds, AUC 0-30 seconds, AUC 0-60 seconds and shear rate AUC time to maximum dilatation for each individual data set. Only in young adults did FMD correlate with all the above parameters except peak shear rate AUC 0-9 seconds. No correlation was found from the four stimuli in children and older adults. This work demonstrated that AUC normalisation explained only 10-15% of the FMD response which is highly dependent on the groups studied and dividing FMD/stimulus multiplied any error present. They suggested more research is necessary before normalising FMD to shear rate AUC is adopted and that FMD and the shear stimulus response should be reported separately (226).

Currently it appears that normalising FMD to shear rate AUC is only appropriate when a repeated measures design is used with the same degree of shear stimulus producing the

same quantity of arterial dilatation. There should also be a “strong linear relationship between FMD and shear rate AUC” (214). It has also been suggested that normalising the shear stimulus to FMD can be done with an analysis of variance, inserting shear rate as a covariate into the analysis (227). It would appear however, from the literature that a consensus on normalising FMD to shear rate is yet to be reached. This position was recently reinforced by the latest FMD guidelines which stated it was currently “not possible to recommend a method for correcting for differences in shear” (100) stimulus.

1.4.29 Measuring the time to peak response from cuff release to maximum arterial diameter.

In healthy subjects of varying ages, time to maximum dilatation after shear stimulus has been shown to vary, with older subjects reaching maximum dilatation after a longer period of time (134). Another study (107) demonstrated this delay was related to baseline artery size, because as artery diameter increases, time to maximum dilatation increases. It was suggested that artery diameter was more important than shear rate AUC in explaining arterial response to shear stimulus. This dependence on arterial diameter may be due to differences in vessel wall structure with smaller vessel lamina possessing greater smooth muscle compared to larger vessels (107).

Measuring the time delay until maximum dilatation may offer further information about the underlying mechanisms controlling FMD. When assessing FMD on healthy males, the time course after step increases in shear stress demonstrated the brachial artery dilated initially. A second, slower dilatation occurred over time. The authors felt that two distinct mechanisms were responsible for this effect (224). Previous work has shown that a shorter stimulus (5 minutes) produces a NO dependent FMD, whereas prolonged stimulus does not

completely attenuate with L-NMMA. This suggests factors other than NO are involved with prolonged stimulus (132).

As an increased FMD response is felt to reflect NO availability, measuring the time course to peak dilatation may represent how well the endothelial and smooth muscle cells respond to NO. When comparing individuals at low and moderate risk for cardiovascular disease no difference in FMD was found. Time to peak (TTP) dilatation (in seconds) was increased in the moderate risk group. It was suggested a FMD/TTP ratio may provide more clinically relevant information as it assesses how well the vessel responds to stimulus (228).

An increased time to maximum dilatation has been reported as people age, more so in males than females. Complications in pathological states such as diabetes and hypertension have demonstrated a higher correlation with maximum arterial dilatation compared with dilatation measured to only 60 seconds (229). A study (230) measuring FMD and the different time response to maximum dilatation in normal controls and type 2 diabetics demonstrated different subgroups in the diabetics. Peak FMD was reached in a mean time of 50 seconds in the control group. In the diabetics, the mean time to peak dilatation was 120 seconds with three different types of response over time identified. One diabetic group had no dilatation at all. The group that responded early (50 seconds) had significantly reduced FMD compared with controls and the late responders (120 seconds to dilatation). The late responders had comparable FMD to the controls. It was suggested that both the early and late responders have endothelial dysfunction with reduced NO availability. The late responders may have later acting vasodilators other than NO that act as a “compensatory mechanism” when NO availability is reduced (230). It would appear

that whilst time to maximum dilatation can vary in healthy people with more than one dilatory response to shear stress over time, pathological conditions that affect the endothelium may be able to be measured not only as a percentage increase in dilatation (FMD) but also as a time course response.

In contrast, a recent paper demonstrated that TTP dilatation was not wholly NO mediated with L-NMMA infusion failing to totally blunt the response. The TTP measurement also varied widely between repeated tests (231). Therefore further work is necessary to determine how useful this measurement will be as a marker of endothelial function (100).

The FMD technique whilst difficult, time consuming and still evolving is feasible and when performed according to certain criteria represents stimulated endothelial function that is mainly NO mediated. Pre-eclampsia is hypothesised to be an endothelial disorder (11) and could be assessed by the FMD technique to ascertain if pre-eclampsia results in endothelial dysfunction in the form of reduced vasodilatation. The following section will discuss pre-eclampsia in detail.

1.5 Pre-eclampsia.

Pre-eclampsia is an enigmatic disorder of theories. Originally hypothesised to be a disease of the kidneys (232), then a hypertensive disorder (233), it is now thought of as a two-stage disorder (234). The first stage is thought to arise from reduced placental perfusion which then results in a susceptible subset of pregnant women developing the second stage of the disease. This second stage results in patho-physiological changes to the mother in the form of a multi-systemic syndrome affecting the perfusion of many organs. Consequently, changes occurring in the vascular system affect the maternal brain, heart, liver and kidneys.

This has led to the hypothesis that factors secreted by the placenta interact with the maternal endothelium resulting in endothelial activation and/or dysfunction and the clinical picture of pre-eclampsia (2, 235, 236). The following section will discuss the history and definitions of pre-eclampsia, discussing why it is a significant health issue for pregnant women. This will then be followed by a discussion on the risk factors for pre-eclampsia, its proposed aetiology and pathophysiology. Pre-eclampsia and its relationship to endothelial function/dysfunction and FMD and attempts to develop a screening test will then be discussed.

1.5.1 History.

There are very few references to pre-eclampsia in early medical literature, possibly because pregnancy and childbirth was the domain of midwives. In the first century A.D., Celsus mentioned often fatal convulsions of pregnancy (233). Nevertheless it was not until male physicians in France began practicing obstetrics in the late seventeenth to early eighteenth century that the written literature on eclampsia began. Eclampsia is derived from the Greek word for brightness or lightning, as in a flashing glance and was still being used as a generic term for epilepsy in the twentieth century. It was not until 1961 that all other definitions except the obstetric one were excluded (233).

In the 1800s the similarity between women with eclampsia and Bright's disease (nephritis) was noted which led to the discovery by Rayer, P (1839-1841) and Lever, JCW (1843) of proteinuria associated with eclampsia, cited in (237). As protein in the urine was found to predate the eclamptic convulsions, the term pre-eclampsia was coined. For many years it was thought that eclampsia was a result of renal disease (232).

The development of blood pressure techniques in the late nineteenth century led to the observation by Schedoff and Porockjakoff (1884) of the presence of hypertension in pre-eclampsia and eclampsia, cited in (237). In 1903 Cook, HW and Briggs, JC used a sphygmomanometer to measure blood pressure during pregnancy, cited in (238). This association of hypertension with pre-eclampsia meant that for many years pre-eclampsia was thought to be a hypertensive disorder and much of the research around pre-eclampsia was founded on this premise (233).

A clinical observation regarding the relationship between hydatid moles, pre-eclampsia and “relative ischemia of the gravid uterus” was published in 1939. The hypothesis was formulated that reduced placental perfusion (ischaemia) was a precursor to the clinical syndrome of pre-eclampsia/eclampsia. It was theorised that some “placental pressor substance” was produced in response to the hypo-perfusion of the larger than usual placenta. The title of the paper “The relation between hydatid moles, relative ischemia of the gravid uterus, and the placental origins of eclampsia” stated the belief that eclampsia had placental origins (239).

In 1989, Roberts and colleagues proposed that pre-eclampsia was an endothelial cell disorder with the ischaemic placenta the source of a factor or factors that caused endothelial cell injury. This led to the suggestion that pre-eclampsia was “more than pregnancy induced hypertension” (11). The term “injury” has since been replaced by the term “endothelial dysfunction”. This focus on the endothelium has led to the discovery of other risk factors for pre-eclampsia that are related to endothelial dysfunction and the recognition that some maternal factors interact with the ischaemic placenta to result in the clinical picture of pre-eclampsia (2). This hypothesis of reduced placental perfusion

causing the release of substances into the maternal circulation that affect the maternal endothelium has directed much of the research in recent years and will be dealt with in more detail in a later section.

1.5.2 Definitions.

There has been a lack of agreement when defining hypertensive disorders in pregnancy in the past, although it appears that different groups are moving towards a consensus. As early as 1961, Ian MacGillivray suggested that the “level of blood pressure attained” was an important indicator of pre-eclampsia and suggested the “critical level is about 90mmHg diastolic or 140mmHg systolic” (240). The Canadian Hypertension Society guidelines for the diagnosis of pre-eclampsia suggested that hypertension in pregnancy be diagnosed when diastolic BP was ≥ 90 mmHg (241). A systolic pressure of ≥ 140 mmHg was not considered definitive of hypertension in pregnancy. The diagnostic level of proteinuria was the same as later consensus statements.

It was proposed an increase in BP in normotensive women would identify pre-eclamptic hypertension with an incremental rise in diastolic BP of 25mmHg (242). A clinical classification of hypertension and proteinuria was suggested in 1988 containing multiple classifications and definitions of hypertensive disorders in pregnancy (243). To diagnose pre-eclampsia an absolute measurement of diastolic BP of ≥ 110 mmHg on one occasion or a diastolic BP of ≥ 90 mmHg four hours apart with proteinuria was proposed. However, it was found using a definition of a rise in diastolic BP ≥ 15 mmHg with proteinuria in normotensive women was not clinically useful (244). A rise in BP of 30/15mmHg in women whose BP was $< 140/90$ mmHg was not associated with an adverse outcome and should not be included in definitions of pre-eclampsia (245).

For some time there was a lack of consistency when defining pre-eclampsia in research papers (246, 247). High sensitivity is essential when defining pre-eclampsia for patient management, thus ensuring all women with the disease are detected. For research purposes high specificity for the disease is necessary. Although some pre-eclamptics will be missed, high specificity ensures that those who truly have the disease will be recruited (245, 248). The Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) has defined pre-eclampsia based on clinical indicators as well as clearly defined criteria for research purposes (249). This has been ratified by the publication of recent guidelines by the Society of Obstetric Medicine of Australia and New Zealand (SOMANZ) (250, 251) and endorsed as a policy directive by New South Wales Government Health (252).

1.5.3 Research criteria.

The criteria for including pre-eclamptic women in research are the development of proteinuria and hypertension after 20 weeks gestation in previously normotensive women (249, 250). The SOMANZ guidelines (250) have also sanctioned additional research criteria from the International Society for the Study of Hypertension in Pregnancy (ISSHP) definition (248). These additional criteria are newly occurring hypertension after 20 weeks gestation that returns to normal after delivery, pulmonary oedema, placental abruption and appropriately documented evidence of proteinuria (the definition of proteinuria will follow) (248). The National High Blood Pressure Education Program Working Group criteria for pre-eclampsia (253) was reviewed and endorsed by a “Research in Hypertension in Pregnancy” working party in 2003 (254) so both ASSHP and their classification agree for research purposes.

1.5.4 The diagnostic criteria for hypertension and proteinuria in pregnancy.

For both clinical and research purposes, hypertension is defined when the systolic BP is ≥ 140 mmHg at Korotkoff phase 1 (K1) and/or the diastolic BP at Korotkoff V (K5) is

≥ 90 mmHg. Korotkoff phase 1 is when the first sound is heard and K5 is when the diastolic sound disappears when recording BP with a mercury sphygmomanometer.

Proteinuria is present when a 24 hour urinary protein collection demonstrates values of ≥ 300 mg/24h or a spot urine protein/creatinine ratio of ≥ 30 (248-250, 253-255). The

American Society of Hypertension (ASH) has recently published a position article on hypertension in pregnancy. As well as the above criteria for hypertension and proteinuria they also include dipstick proteinuria of $\geq 1+$ (256).

1.5.5 The criteria for the clinical diagnosis of pre-eclampsia.

The ASSHP criteria also give a clinical diagnosis of pre-eclampsia probably because of the seriousness of the disorder and because it is a syndrome that is better over diagnosed (249).

Serious morbidity can occur in women without proteinuria but with involvement of other organ systems (257). A clinical diagnosis of pre-eclampsia is made when there is hypertension arising after 20 weeks gestation with the onset of one or more of the following problems;

1. Proteinuria as defined above; or renal disease with oliguria or serum/plasma creatinine ≥ 0.09 mmol/L.
2. raised serum transaminase and/or severe right upper quadrant/epigastric pain indicating liver disease.
3. neurological sequelae presenting as eclampsia (convulsions), hyperreflexia with clonus or severe headaches, or visual disturbances that are persistent.

4. disturbances in haematological results such as thrombocytopenia, disseminated intravascular coagulation or haemolysis.
5. intrauterine restriction in fetal growth (249).

1.5.6 Other hypertensive disorders of pregnancy.

The ASSHP and SOMANZ guidelines also address the classification of gestational hypertension, essential and secondary chronic hypertension in pregnancy and pre-eclampsia superimposed on chronic hypertension (249, 250). Gestational hypertension is defined as BP \geq 140/90mmHg that develops after 20 weeks gestation and resolves three months after delivery with no proteinuria or other multi-system signs of pre-eclampsia (249, 250). For this reason the diagnosis of gestational hypertension is often not made until after delivery (253). Chronic essential hypertension relates to women who present with BP \geq 140/90mmHg before pregnancy or during the first 20 weeks of gestation with no cause found. Secondary hypertension has the same BP criteria with a definitive aetiology (for example renal, vascular or endocrine cause or coarctation of the aorta) for the BP increase. Pre-eclampsia superimposed on chronic hypertension is diagnosed with the additional development of proteinuria as defined above (249, 250).

1.5.7 Gestational hypertension.

Gestational hypertension and pre-eclampsia are considered by some as separate disorders (249, 251). This is because the increased BP in gestational hypertension may be the only manifestation whereas pre-eclampsia results in a multisystem disorder. The alternative viewpoint is that pre-eclampsia is a progression of gestational hypertension, both sharing a common pathophysiology (236). Both pre-eclampsia and gestational hypertension share

common risk factors such as obesity, kidney or heart disease, diabetes, late maternal age and previous pre-eclampsia (258).

The most common hypertensive disorder in pregnancy is gestational hypertension.

Gestational hypertension can be divided into mild and severe disease although this division is controversial. The definition of gestational hypertension is detailed in section 1.5.6.

Severe gestational hypertension occurs when BP measures $\geq 160/110$ mmHg for at least six hours (9). Severe disease is not considered a benign condition as it can lead to serious maternal, fetal and newborn morbidity (259, 260). Gestational hypertension is more likely to progress to pre-eclampsia if it occurs prior to 30 weeks gestation (261).

Gestational hypertension and pre-eclampsia both result in long term morbidity and mortality from an increased risk of cardiovascular disease (CVD) and hypertension (262). Pre-eclampsia is hypothesised to be an endothelial cell disorder (11) resulting in reduced flow induced dilatation (263). Non-pregnant hypertensive people also have reduced vasodilatory endothelial function (10). At the time this thesis was commenced there were no studies using FMD to compare endothelial function in women with gestational hypertension compared to pre-eclampsia. A study to assess if these two groups have the same or different degree of vasodilatation may be instructive. This information may be able to help distinguish whether the pathophysiology in these disorders is similar or different.

1.5.8 Sub-classification of pre-eclampsia.

Although a consensus appears to have been reached on a basic definition for pre-eclampsia, there is still some controversy over whether pre-eclampsia should be sub-

classified to try and define severity of disease within the pre-eclamptic population. One discussion focuses on whether pre-eclampsia should be further divided into groups of differing gestational ages, for example severe disease occurring at $<34 + 0$ weeks and less severe disease at ≥ 34 weeks (264, 265). The rationale behind this sub-classification is not only that a more advanced gestational age provides a better outcome for the baby, there is less maternal morbidity and mortality when pre-eclampsia occurs later (265).

This does not mean that late pre-eclampsia is a harmless disease, especially in developing countries. A study that assessed pregnancy outcomes in women who delivered after 34 weeks gestation in a socially deprived area found around 20% of women developed eclampsia or HELLP (haemolysis, elevated liver enzymes, low platelet count) syndrome, 6.1% were admitted to intensive care units and other maternal complications such as placental abruption, severe renal disease, thrombocytopenia and pulmonary oedema still occurred. The perinatal mortality rate was 18 per 1000 births. The authors suggested late onset pre-eclampsia was more a maternal disease as greater than 90% had normal umbilical artery Doppler flow studies (266).

There is a growing consensus that early onset pre-eclampsia and late onset pre-eclampsia are differing disorders with separate pathophysiology, the late disorder arises from maternal factors, the early disorder is placental (267). The ASH position paper rates “more severe” pre-eclampsia as presenting at <35 weeks gestation (256). The recent Society of Obstetricians and Gynaecologists of Canada (SOGC) guidelines use a gestational age severity cut off of <34 weeks (255). The earlier and current Australasian consensus statements and the Royal College of Obstetricians and Gynaecologists (RCOG) guidelines do not have a definition of severity based on gestational age (248-250, 268).

A recent paper (269) suggests early (<37 weeks) and late onset (≥ 37 weeks) pre-eclampsia have distinct maternal and fetal features that differentiate them. The women who developed pre-term pre-eclampsia were significantly younger, had a lower pre-pregnancy BMI and were more often smokers. Newborn birth weight was significantly decreased in babies born from pre-term pre-eclamptic women with more pre-term babies <10th percentile. Results did not change if a 34 week gestation cut off was used. These results support the hypothesis that there are different phenotypes of pre-eclampsia presenting as a late maternal and early placental disorder (269).

The RCOG (268) have published guidelines on the management of severe pre-eclampsia/eclampsia. Both the RCOG and SOMANZ recognise a category of severe pre-eclampsia as proteinuria plus a systolic blood pressure ≥ 170 mmHg and/or diastolic BP ≥ 110 mmHg (250, 268). This cut off is still controversial with a systolic BP of 160mmHg also suggested for severe disease (250). Both ASH and SOGC use a BP of $\geq 160/110$ mmHg to define severe pre-eclampsia (255, 256).

Defining degrees of severity of pre-eclampsia does not predict which mother and fetus are at increased risk of adverse outcome and should not be used in deciding on the delivery of a pre-term fetus (270). Although agreement has been reached on definitions of pre-eclampsia and other hypertensive disorders of pregnancy, it would appear until the pathophysiology and aetiology of pre-eclampsia is unravelled, an absolute definition for pre-eclampsia will remain as suggestions and guidelines.

For the research being presented in this thesis, the ASSHP research definition of pre-eclampsia (hypertension and proteinuria after 20 weeks gestation in previously normotensive women) was chosen. The definition of gestational hypertension was onset of

hypertension after 20 weeks gestation without the proteinuria or any other multi-system signs of pre-eclampsia (249).

1.5.9 Pre-eclampsia, morbidity and mortality.

According to Australia's Mothers and Babies 2007 report on perinatal statistics (271) in women who gave birth, pregnancy induced hypertension is the second most common maternal medical condition at 45.2 per 1000. Only gestational diabetes ranked higher at 49.5 per 1000. The World Health Organization reports pre-eclampsia/eclampsia is in the top four of major complications in childbirth worldwide with an incidence of 3.2% of live births. The incidence is highest in developing countries. However the case fatality rate is the highest of all the pregnancy related complications at 1.7%. In 2000 there were 63000 maternal deaths, that is 12% of all maternal deaths were due to pre-eclampsia/eclampsia (272).

The latest report on maternal death in Australia reported a maternal death ratio of 8.4 per 100000. Hypertensive disorders of pregnancy were the third most common cause of maternal death after amniotic fluid embolism and thromboembolism (273). Pre-eclampsia/eclampsia can result in morbidity to the woman other than death including cardiac arrest, cerebro-vascular insult, respiratory distress, pulmonary oedema, renal or hepatic failure, pulmonary embolus, HELLP syndrome, disseminated intravascular coagulation and coma (274-276).

The rate of hospital admissions for pregnancy related hypertension in the United States is increasing, possibly due to the increase in age and obesity amongst pregnant women. Hospitalised women, especially those with eclampsia/pre-eclampsia are more likely to

suffer from severe obstetric complications than those hospitalised without a hypertensive disorder (276).

In Australia, the hypertensive disorders of pregnancy (especially pre-eclampsia superimposed on pre-existing hypertension) result in increased perinatal morbidity and worse outcomes compared to normotensive women. Hypertensive women were more likely to have a preterm birth, small for gestational age (SGA) (<10th percentile) infants and either be induced or deliver by caesarean section. Perinatal mortality had decreased significantly in hypertensive pregnant women when the study period of this report (1998-2001) was compared to an earlier time period (1991-1997) (277). Interestingly, for reasons unknown, babies born to mothers with pre-eclampsia at a gestation greater than 37 weeks are also more likely to be admitted to the neonatal intensive care unit compared with late preterm (34-37 weeks gestation) babies (278).

The morbidity of pre-eclampsia extends beyond the affected pregnancy. Women who have had pre-eclampsia in their first pregnancy at a gestational age of less than 34 weeks (compared to pregnant women who had pre-eclampsia greater than 34 weeks in their first pregnancy) are more likely to deliver a premature SGA baby in their next pregnancy (279). If severe pre-eclampsia occurs before 24 weeks, the risk of pre-eclampsia in a subsequent pregnancy is 50% with a greater future risk of chronic hypertension (280).

A study (281) assessing 49 women with gestational hypertension, 45 with pre-eclampsia and 45 normotensive women, found the rate of chronic hypertension was significantly increased seven years after a pregnancy complicated by either gestational hypertension or pre-eclampsia. Blood pressure was higher in the gestational hypertension group.

Compared to a control group, hypertension in a subsequent pregnancy was also significantly more likely after pre-eclampsia. Pre-eclampsia in any pregnancy was an independent risk factor for coronary artery disease with an adjusted odds ratio (AOR) of 4.8 [95% confidence interval (CI) 1.2 to 19.0]. Other risk factors that are related to endothelial dysfunction such as diabetes, smoking, obesity, hypertension and hypercholesterolemia were also more common in the coronary artery disease group compared with the control group (282).

A retrospective cohort study (283) from a 19 year period (1951-1970) of over 3000 women with pre-eclampsia/eclampsia in their first pregnancy was compared to women with gestational hypertension and normotensive pregnant women. This study found that any of the above hypertensive diseases in pregnancy increased a woman's risk of hypertension and stroke over the long term but not the risk of coronary artery disease.

Another retrospective cohort (284) study of over one million women assessed the future risk of cardiovascular disease when they combined risk factors for cardiovascular disease and maternal placental syndrome. Maternal placental syndrome was defined as pre-eclampsia, gestational hypertension, placental abruption or infarction. The presence or absence of fetal growth restriction or fetal death in-utero (FDIU) was also ascertained. The presence of maternal placental syndrome increased the risk of cardiovascular disease. If growth restriction and/or FDIU had occurred or other traditional risk factors for cardiovascular disease were present then the risk increased even more. Tragically the mean age for the first cardiovascular event in this cohort was just 38 years.

A systematic review and meta-analysis that included 25 articles (262) and assessed the risk of future disease after pre-eclampsia found that pre-eclampsia increased a woman's risk of hypertension, ischaemic heart disease, stroke, venous thromboembolism and death. Pre-eclampsia did not change the risk of cancer. Early pre-eclampsia (<37 weeks gestation) conferred a greater risk of mortality (relative risk 2.71 (95% CI 1.99 to 3.68)) compared with normotensive women. The relative risk of hypertension was 3.23 (95% CI 2.32 to 4.52) after a single pregnancy complicated by pre-eclampsia. This risk increased to 5.96 (95% CI 3.42 to 10.38) if pre-eclampsia was present in any pregnancy rather than just the first one. Pregnancy induced hypertension also increased the relative risk of future CVD 1.66 (95% CI 0.62 to 4.41) and hypertension 3.39 (95% CI 0.82 to 13.92) but to a lesser extent than pre-eclampsia.

An intergenerational (285) study of women with and without a history of pre-eclampsia and their parents, found a history of pre-eclampsia was related to a three times increased rate of metabolic syndrome. This included a greater BMI and waist measurement, increased fasting glucose levels and higher blood pressure. All these studies reinforce the necessity of an accurate diagnosis of pre-eclampsia as lifelong risk for CVD is changed and this could alter follow-up and long term management of these women.

1.6 The pathophysiology of pre-eclampsia.

The aetiology of pre-eclampsia is unknown, however it is thought to be a two-stage disorder (234). Stage 1 is proposed to begin with a normal pregnancy-specific maternal inflammatory response. This response interacts with many risk factors for pre-eclampsia which will be detailed in a following section. The maternal constitution then either modifies or interacts with the placenta or causes abnormal placentation and/or reduced

fetal/placental perfusion. Abnormal placentation may be the result of either an immune course where the mother rejects the fetus or a vascular process which involves an ischaemia-reperfusion response (234, 286).

Stage 2 occurs when factors are released into the maternal system because of the hypoxic placental problems. These are proposed to be either syncytiotrophoblast basement membrane (STBM) shedding into the maternal vascular system or the release of soluble endothelial growth receptors which bind to vascular endothelial growth factors released from the hypoxic placenta. These factors interact with the maternal/fetal endothelium resulting in endothelial activation/dysfunction and the clinical picture of pre-eclampsia. It is also suggested that the maternal constitutional status is the risk factor that affects the long term outcome in the form of recurrent pre-eclampsia or future cardiovascular disease (234). The following sections will discuss the pathophysiology of pre-eclampsia in more detail.

1.7 Stage 1: Maternal inflammation in pregnancy, risk factors for pre-eclampsia and the placenta.

1.7.1 The normal pregnancy-specific and pre-eclamptic maternal inflammatory response.

As previously stated, the up regulation of the inflammatory system may be a contributor to endothelial dysfunction (74). The inflammatory system and the endothelium are intimately related. Disease in the vascular system can promote an inflammatory response and the inflammatory system can provoke the endothelium to respond. The response to inflammation can be local or generalised and stimulated by injury or immune activation and involves immune cells such as monocytes, leukocytes and natural killer cells as well as the clotting, complement and endothelial systems (287, 288).

Communication within the inflammatory network is by cytokines. Cytokines are proteins created to act upon immune and haematogenic cells, the main source being macrophages and monocytes. Cytokines include chemokines (for example interleukin-8 (IL-8)), adipokines (secreted by adipocytes- for example, IL-6, leptin, resistin, adiponectin, PAI-1, and angiotensinogen) and angiogenic factors such as VEGF (288). Systemic inflammation produces an acute phase systemic response (which can become chronic). This leads to changes in plasma protein concentrations (the most well-known one is CRP). This process is stimulated by cytokines. The pattern of cytokine production differs according to the inflammatory condition. Fever and leucocytosis can result (288). Other metabolic responses occurring in relation to systemic inflammation include insulin resistance and hyperlipidaemia. Both these conditions are more common in obesity (a risk factor for pre-eclampsia) with the production of adipokines enhancing the inflammatory response (289).

Normal third trimester pregnancy was found to be a state of maternal systemic inflammatory response. An experiment (290) was designed to assess whether circulating leukocyte activation occurred in pre-eclampsia using whole blood flow cytometry. Four groups were studied, 21 normal pregnant women, 21 pre-eclamptic women, 21 non-pregnant women and 6 patients with systemic sepsis who acted as positive controls. Normal pregnancy had changes in peripheral blood leukocytes comparable to sepsis patients in intensive care. Pre-eclampsia was associated with a general inflammatory response which, while not large was additional to normal pregnancy.

In a review on inflammatory response in pregnancy, granulocytes and monocytes, TNF α and its soluble receptors (for example IL-6) were shown to increase in pre-eclampsia (287). The clotting (291) and complement system are also activated in pre-eclampsia (292). As

the endothelium is an integral part of the inflammatory system, the endothelial dysfunction of pre-eclampsia would be part of a more extensive intravascular inflammatory response with pre-eclampsia an exaggeration of normal pregnancy. Redman and colleagues (287) noted that common characteristics between the state of normal pregnancy and pre-eclampsia are often found and hypothesised this may preclude the search for a definitive screening test for pre-eclampsia. Interestingly they postulated that a single cause or gene for pre-eclampsia would not be found, that the fetal genotype would be an important contributor and women with risk factors that make them more susceptible to inflammatory stimulus (such as infection and metabolic disease) would be at increased risk of pre-eclampsia (287). A number of these predictions have been tested and are addressed in the following section.

1.8 Risk factors for pre-eclampsia.

1.8.1 Parity and the immune maladaptation hypothesis.

The increased risk of pre-eclampsia/eclampsia in nulliparous women was recognised in the very early medical literature. According to Chesley, LC in 1694 Mariceau a seventeenth century obstetrician, described that convulsions in pregnancy occurred more commonly in nulliparous women compared to multiparous women (232). During a ten year period, all the cases of eclampsia in Denmark were assessed. It was discovered that pre-eclampsia occurred at the extremes of reproductive age, demonstrating a U-shaped distribution. At around 15 years and 47 years the rate of eclampsia was about 8 per 1000 (293).

The incidence of pre-eclampsia was found not to be influenced by age but was rather a disorder of first pregnancy (294) with the incidence decreasing in subsequent pregnancies (240). A systematic review of risk factors for pre-eclampsia (295) found that nulliparity

results in almost three times the risk of pre-eclampsia. Women with pre-eclampsia were more than twice as likely to be nulliparous compared to women without pre-eclampsia.

One suggested reason for the increased risk of pre-eclampsia in nulliparous women is a difference in angiogenic factors (angiogenic factors and their relation to pre-eclampsia will be addressed more fully in a later section). The level of sFlt-1 was significantly increased in first pregnancy compared with second pregnancy and higher in Hispanic women compared to white women in their first pregnancy. There was no difference in PlGF (296).

Pre-eclampsia is proposed to be disease of primigravidae (264, 294). The risk of pre-eclampsia is decreased in a second or subsequent pregnancy (240), especially if the woman was normotensive in her first pregnancy (297). However, previous pre-eclampsia increases the risk of pre-eclampsia in subsequent pregnancies (298). In contrast, if a first pregnancy ended in a miscarriage or termination of pregnancy, then the risk of pre-eclampsia is reduced in the next pregnancy but if a term pregnancy is achieved the risk of pre-eclampsia is even less (294, 299).

A history of abortion in nulliparous women also reduces the risk of pre-eclampsia by half. This holds true only if the woman conceives with the same partner. If the woman conceives with a new partner after abortion, her risk is the same as being pregnant for the first time (300). Work from the Norwegian Mother and Child Cohort Study found that two or more terminations of pregnancy significantly reduced the risk of pre-eclampsia which was the same as having had a previous full-term delivery. This relationship held regardless of whether the pregnancy resulted from the same or a new partner (301).

MacGillivray suggested in 1958 that an “immunity” to pre-eclampsia “is rapidly and effectively acquired” such that the risk of pre-eclampsia in pregnancies after the first is reduced (294). This may be due to regular exposure to the father’s sperm resulting in what was termed “immunogestosis” or an immune tolerance to the partner’s antigens (302). This theory has been tested by assessing the risk of pre-eclampsia after a change in paternity in multiparous women. Three groups have found more multiparous women with new partners developed pre-eclampsia compared with multiparous women without a change in partner (303-305) although the risk was still less than a nulliparous woman (304). When there was a change in partner, an inverse relationship between pre-eclampsia, eclampsia and gestational hypertension patients and length of co-habitation was found in both primigravidae and multigravidae (306, 307).

The exposure of the vaginal mucosa during intercourse to seminal fluid has been shown to induce mucosal allo-immunisation (that is confer immunity against the partner’s antigens) in women (308). This has led to the theory called the “Immune Maladaptation Hypothesis” where the fetus is rejected by the mother because of a “maternal-fetal (paternal) immune maladaptation” (309). This atypical response of the mother to the fetus is being postulated as a cause of pre-eclampsia (310).

The increased risk of pre-eclampsia may not be due to a change in partner but to a longer inter-pregnancy interval (311, 312) although this has been a controversial finding (313). The difference in the primiparity theory and birth interval theory may be due to the different populations studied. Young primiparous women are more commonly seen in developing countries whereas the populations studied in the birth interval studies are older, more obese and from developed countries. This form of pre-eclampsia may reflect a

different disease and pathophysiology (for example women with metabolic syndrome) (314).

1.8.2 The large placenta.

Another risk factor for pre-eclampsia is a large placenta. Large placentas can occur in women with naturally conceived multiple pregnancies (294, 315-317) or multiple pregnancy due to assisted reproduction technology (318).

Twin pregnancies were found to have 2.2 times greater levels of the angiogenic factors sFlt-1 and sFlt-1/PIGF ratio compared to singleton pregnancies. The level of sFlt1 and sFlt1/PIGF ratio was found to be highly correlated with placental weight ($R^2 = 0.75$) which in twins was 1.74 times heavier than in singletons. There was no difference in sFlt-1 or PIGF mRNA expression. These results suggest that twin placentas (in women without pre-eclampsia) are not hypoxic or programmed to produce more sFlt-1. Instead, more sFlt-1 is produced affecting the maternal endothelium because there is more placenta (319). An increased level of sFlt-1 and sFlt-1/PIGF ratio was also demonstrated in normotensive multiple pregnancies compared with singletons between 22-36 weeks gestation (320).

Hydatidiform mole results in a large placenta. Pre-eclampsia can occur in 12-27% of women with hydatidiform mole and can be the cause of early onset (<20 weeks gestation) pre-eclampsia (321, 322). Elevated levels of sFlt-1 are present in women with hydatidiform mole providing more evidence of the role of sFlt-1 in the pathogenesis of pre-eclampsia (323).

1.8.3 Aneuploidy.

An association between trisomy 13 and pre-eclampsia but not the other more common aneuploidies (trisomy 21 and 18) has been reported (324, 325). A difference in angiogenic factors may explain this increased risk with a significant decrease in PlGF in trisomy 13 found. The gene for sFlt-1 is found on chromosome 13. When the ratio of sFlt-1/PlGF was assessed this was significantly increased in trisomy 13 compared with normal controls. There was no difference between trisomy 13 and trisomy 18 or 21 (326).

1.8.4 Medical conditions and maternal characteristics.

Various medical conditions and maternal characteristics such as chronic hypertension and/or a raised blood pressure early in the pregnancy, maternal obesity, renal disease and diabetes mellitus (both pre-existing and gestational) have been shown to increase the risk of pre-eclampsia (295, 327-329). A large multicentre study of 39615 pregnancies from the World Health Organization (WHO) Antenatal Care Trial (258) reported a rate of pre-eclampsia of 2.2%. Risk factors for pre-eclampsia were diabetes, renal or cardiac disease, pre-eclampsia in a previous pregnancy, urinary tract infections, chronic respiratory conditions, maternal age >40 years, twin pregnancy and obesity. Primiparity even after adjusting for maternal age <16 years was a risk factor.

1.8.5 Maternal disease.

Two review articles on chronic renal disease and pregnancy have both quoted the risk of pre-eclampsia in women with moderate to severe chronic renal disease as 40% (330, 331). With mild renal insufficiency, the risk of pre-eclampsia is 22% (330). Other maternal diseases that increase the risk of pre-eclampsia are rheumatologic diseases such as systemic lupus erythematosus, rheumatoid arthritis and rheumatologic disorders. This suggests an autoimmune link (332).

An association between thrombophilias and severe pre-eclampsia has been reported (333, 334). A recent Australian study prospectively assessed the risk of inherited thrombophilia polymorphisms in asymptomatic women with the adverse outcome variables of pre-eclampsia/intra-uterine growth restriction (IUGR)/placental abruption/stillbirth or neonatal death. They found that only women heterozygous for the prothrombin gene mutation were at increased risk of an adverse event (335).

1.8.6 Diabetes and insulin resistance.

Type 1 diabetes is also a risk factor for mild and severe pre-eclampsia (336) as is insulin resistance (337). Insulin resistance peaks in the third trimester of pregnancy which coincides with the peak of pre-eclampsia (338). There are a number of conditions that have insulin resistance as a feature, for example gestational diabetes mellitus (GDM), polycystic ovarian syndrome and obesity (339, 340). Glucose intolerance has been shown to be more common in pre-eclamptic women. Increasing insulin resistance has been touted as a possible link between pre-eclampsia and the future risk of CVD and type 2 diabetes (338).

1.8.7 Obesity.

Overweight has been classified by the WHO as a BMI (kg/m^2) ≥ 25.00 and obese a BMI ≥ 30.00 . Obesity has been further sub-classified into Obese class I (BMI 30.00-34.99), Obese class II (BMI 35.00-39.99) and Obese class III (BMI ≥ 40.00) (341). Body mass index is an imperfect measure of body fat. It may well be fat distribution (central/visceral obesity versus peripheral obesity) and percentage of fat present (342) that is the important determinant of pre-eclampsia risk because not all women who are obese develop pre-eclampsia (343, 344).

Weight gain in pregnancy includes products of conception and maternal tissue increases in the uterus, breasts, blood volume, fat and extracellular fluid. Normal weight women who gain the recommended weight in pregnancy may have a BMI up to 29.9. There is no evidence that a pre-pregnancy BMI of 19.8-26 is normal apart from the fact it encompasses the 25th to 75th percentiles (345). A systematic review demonstrated BMI was a weak predictor for pre-eclampsia with pooled estimates for a BMI \geq 25 demonstrating a sensitivity of 47% (95% CI, (33 to 61) and specificity of 73% (95% CI 64 to 83). The pooled estimate for a BMI \geq 35 was sensitivity 21% (95% CI 12 to 31) and specificity 92% (95% CI 89 to 95) (346).

Obesity occurs when the number and size of adipocytes increase. Fat functions as a storage organ as well as an endocrine organ. Increases in adipocytes result in an increase in the production of pro-inflammatory adipokines (TNF α , leptin, IL-6), a decrease in anti-inflammatory cytokines (adiponectin, interleukin-10 (IL-10)) and increases in adipose tissue macrophages which results in alterations in the immune and inflammatory response (343, 347). The inflammatory type macrophages also emit TNF α , interleukin-2 (IL-2), IL-6 and produce ROS through the activation of iNOS and are related to insulin resistance and endothelial dysfunction (347).

Complications of obesity include insulin resistance, endothelial dysfunction, hypertension, dyslipidaemia, and an increase in the pro-thrombotic state with changes in the immune and inflammatory state (343). In non-pregnant people, blood vessel structure is altered in obesity. Brachial artery vessel diameter and arterial stiffness increase with increasing obesity, occurring across all age groups from childhood to old age (348). Visceral fat as measured by abdominal ultrasound was negatively correlated with FMD in morbidly obese

(BMI 42.3 ± 4.3) people. This relationship was independent of traditional CVD risk factors (349).

In complicated and uncomplicated pregnancy, decreased microvascular dilatation occurs with increasing BMI (350-352) (this will be dealt with in more detail in the section on endothelial function in pregnancy). The risk factors for pre-eclampsia are very similar to the risk factors for CVD. It has been suggested that asymmetric dimethylarginine (ADMA), a natural inhibitor of NOS, which is also elevated in people at risk for CVD, in obesity (344) and before (353) and during (354) pre-eclampsia may be the link that results in obesity increasing the risk of pre-eclampsia (344). An alternative hypothesis is that the interaction of the ischaemic placenta along with existing maternal endothelial dysfunction from obesity/metabolic syndrome factors results in pre-eclampsia (355).

Many studies have assessed the relationship between obesity and pre-eclampsia. Obesity is a risk factor for pre-eclampsia even if women with essential hypertension are excluded from analysis (356). This risk from obesity has been demonstrated in a number of studies (357-359) with the risk of pre-eclampsia increasing in a linear fashion with increasing BMI (360). This linear relationship with increasing BMI was also demonstrated in the “super-obese” (BMI >50) with the pre-eclampsia risk quoted at 17.4% (361).

The rate of obesity is increasing in Australia (362), the developed (344) and developing world (363). An Australian study demonstrated overweight or obesity was present in 34% of the pregnant study group with an increased risk of hypertensive disorders (pre-eclampsia and gestational hypertension) in the obese (BMI ≥ 30) and morbidly obese (BMI ≥ 40), AOR 3.0 (95% CI 2.4 to 3.74) and 4.87 (95% CI 3.27 to 7.24) respectively (343). A

similar increased risk was found for pre-eclampsia when pre-pregnancy BMI was in the obese range with an AOR of 3.74 (95% CI 1.95 to 7.17) (364). Another Australian study in which 43% of the pregnant women were overweight or obese found obesity increased the risk of pre-eclampsia threefold (365).

The relationship of increasing obesity being a risk factor for pre-eclampsia holds true across different populations in the developed and developing world (328, 366-370), in black and white women (370) and in both nulliparous and multiparous women although the risk is greater for nulliparous women (328). Obesity is also a risk factor for early and late, mild and severe pre-eclampsia (328, 365, 370).

1.8.8 The metabolic syndrome.

Many of the risk factors for pre-eclampsia such as chronic hypertension, obesity and pre-existing diabetes along with the increased risk of later CVD are similar to those factors found in metabolic syndrome (371). It is not known whether the women who develop pre-eclampsia are at increased risk of CVD because of metabolic syndrome or whether pre-eclampsia acts as a positive stress test for CVD and metabolic syndrome in the future (372).

Metabolic syndrome has been defined by the National Cholesterol Education Program Expert Panel III as the presence of three or more of the following risk factors for CVD: abdominal obesity, atherogenic dyslipidaemia (high triglycerides, small low-density lipoprotein (LDL) particles, low high-density lipoprotein (HDL) cholesterol), increased blood pressure, insulin resistance with or without glucose intolerance and pro-thrombotic, pro-inflammatory states (373, 374). Normal pregnancy has a number of these factors present and demonstrates a degree of insulin resistance, hyperlipidaemia, increased

coagulation factors and increased white cell count (371). For this reason the non-pregnant criteria for metabolic syndrome as it stands cannot be used in pregnancy.

Several studies have attempted to develop a pregnancy specific metabolic scoring system to see if pre-eclampsia and the metabolic syndrome are associated. This has been done by modifying the definitions of metabolic syndrome to suit pregnancy. One group (372) developed a modified metabolic scoring system to see if there was an association with severe pre-eclampsia. Each woman was given a score of 0, 1 or ≥ 2 based on the presence or absence of obesity (BMI ≥ 30), chronic hypertension and pre or gestational diabetes. The results showed in women greater than 30 years of age, a metabolic score of ≥ 2 increased the odds of pre-eclampsia by OR 5.3 (95% CI 1.75 to 16.409). In women less than 30 years, no association between metabolic syndrome and pre-eclampsia was found.

Another study (375) combined gestational hypertension (n=41) and pre-eclampsia women (n=10) together to create a metabolic scoring system for these groups (termed pregnancy induced hypertension (PIH)) in the third trimester (34+ weeks) and compared them to 97 normotensive controls. They dichotomised a score as yes/no based on a BMI >31 , increased triglycerides, decreased high density lipoproteins (HDL) and fasting serum glucose >90 mg/dL. This study found the PIH group had higher scores for metabolic syndrome. The women were larger, had increased triglyceride and glucose levels. There was no difference in HDL levels.

When comparing the presence or absence of a modified metabolic syndrome in normal healthy pregnant women, women with pre-eclampsia and women with late onset GDM,

30% of the women with pre-eclampsia had metabolic syndrome. This compared with only 10% of GDM and none in the control group (376).

Due to the recognition of a relationship between metabolic syndrome and pre-eclampsia a review of all the articles published on maternal triglyceride levels as a risk factor for pre-eclampsia was done. This article (377) found that the risk of pre-eclampsia increased in a dose dependent fashion with increasing triglyceride levels. When the highest triglyceride levels were compared with the lowest levels, a fourfold increase in the risk of pre-eclampsia was noted with the higher readings. This increased risk of pre-eclampsia with high triglyceride levels has also been reported in more recent studies (378, 379).

Even after pregnancy, a number of risk factors for metabolic syndrome can still be found in women with a history of pre-eclampsia. Increased central obesity adds to the risk of insulin resistance in previously pre-eclamptic women but not normal controls (380). The risk of metabolic syndrome is twice as high in women with a history of early onset (<32 weeks) pre-eclampsia, IUGR, placental abruption or stillbirth (381).

1.8.9 Infection and systemic inflammation of pregnancy.

A link between maternal infection and pre-eclampsia has been hypothesised (382) with an increased risk in primiparous women with a urinary tract infection (383) and severe maternal periodontal disease (384).

As pre-eclampsia increases the risk of future CVD (262), the association between CVD, pre-eclampsia and infection has led to the hypothesis that pre-eclampsia and atherosclerosis may share a common inflammatory pathophysiology with both conditions

having similar lesions. Lesions found in both atherosclerosis and pre-eclampsia have foci of endothelial disruption and artery wall necrosis, deposition of lipoproteins and macrophages. These similarities suggest an infectious trigger that may explain why some women develop pre-eclampsia whilst others develop IUGR without hypertension (382).

As previously stated, normal pregnancy results in a condition of maternal systemic inflammation (290). It has been hypothesised that a causal pathway may exist between the inflammation resulting from infection, maternal inflammatory disease such as autoimmune disease and the inflammatory response from obesity which results in the exaggerated inflammatory response of pre-eclampsia (264).

1.8.10 Familial nature and genetic predisposition.

In 1968 Leon Chesley and colleagues reviewed the literature back to 1873 assessing the familial aspect of pre-eclampsia in female relatives (mainly mother-daughter pairs and sisters). Also performed was a review of his data between 1931-1966 of daughters, daughter-in-laws and granddaughters of women with eclampsia. The study looked at “toxemia of pregnancy” which included pre-eclampsia, essential hypertension and unrecognised renal disease. Daughters of eclamptic women had an eight fold increase in the incidence of pre-eclampsia. The incidence of “toxemia” was 26% in daughters and 8% in daughter-in-laws (385).

Studies assessing the familial nature and/or genetics of pre-eclampsia have considered the relationships of family members to try and determine the inheritance of pre-eclampsia. It is estimated the contribution of maternal genes in the development of pre-eclampsia is 30%. This estimate was based on full sisters, mother-daughters, maternal and paternal

half-sisters in over one million Swedish births (386). As well as maternal genes, paternal (and therefore fetal genes) contribute to the risk (387). A population study assessing the contribution and risk of maternal, fetal and paternal genes found that mitochondrial (maternal) genes are not solely responsible for pre-eclampsia. Whilst there is a contribution from the mother, the fetus and therefore paternal gene inheritance also plays a part (388).

Maternal susceptibility can be inherited from mother to daughter but maternal susceptibility does not pass from mother to son. Women born from a pre-eclamptic pregnancy pass the alleles for pre-eclampsia to their fetus. Men born from a pre-eclamptic pregnancy are more likely to carry pre-eclampsia susceptibility fetal alleles which increase their partner's risk. This familial association is not affected by order of birth (389).

Pre-eclampsia has been shown to cluster in families with nulliparous pre-eclamptic women more than twice as likely to have a sister with pre-eclampsia (390). Familial aggregation (grouping of a disease occurring amongst members of a family) also occurs in white western Europeans (391) and isolated populations (392). Grand-paternal systolic blood pressure is a significant determinant of maternal blood pressure in pregnancy suggesting a heritable factor (391).

A genetically isolated Netherlands population provided evidence for a recessive inheritance of pre-eclampsia when an increased number of consanguineous relationships resulted in pre-eclampsia (392). However two studies assessing consanguineous relationships found no difference in pre-eclampsia outcomes between first cousins (393) and no increase in pre-eclampsia in the 38% of women who were in a consanguineous relationship (394). Other isolated population studies have identified differing

chromosomes that may “harbor susceptibility genes for pre-eclampsia” (395) or suggest a parent-of-origin effect through the maternal line (396).

A recent review (397) concluded many studies have indicated a genetic predisposition to pre-eclampsia with a large number of candidate genes tested, but results are currently inconclusive. This may be due to previous research on the subject misclassifying gestational hypertension as pre-eclampsia. Another source of error may be the grouping of primiparous and multiparous women together when pre-eclampsia in these two groups may have different aetiologies. Almost all testing has been done on maternal samples when the evidence suggests that paternal genes may also play a part. It would appear that apart from testing performed on single families, it is most likely that susceptibility to pre-eclampsia will involve multiple genes (397) which can be transmitted by maternal or paternal inheritance.

Some authors have suggested that the paternal transmission (or lack of) of pre-eclamptic genes may result in a changed risk of pre-eclampsia. This is in opposition to the primipaternity immune maladaptation theory which suggests a first pregnancy from a new father will increase the pre-eclampsia risk (301).

If pre-eclampsia is so dangerous to the mother and fetus, it raises the question as to why the genes have not been eliminated from the gene pool. One reason may be hidden benefits as occurs with sickle cell anaemia and malaria (398). The interaction between maternal and fetal cells is a finely balanced juggling act. Changes in the gene pool may also signify pre-eclampsia as part of the evolutionary “moves and counter-moves” of primate development gone askew (399).

The variety of risk factors reviewed in the preceding section seem to support the theory that pre-eclampsia is a multi-faceted multi-system disease with multiple aetiologies that affect different populations of women in different ways. The disease pre-eclampsia may be different in nulliparous women, multiparous women with new partners, women with multiple pregnancy, women with pre-existing disease, women with a genetic predisposition and women with risk factors for metabolic syndrome.

1.9 The Placenta.

Pre-eclampsia is a disease of human pregnancy. The definitive treatment is delivery, which removes the placenta and fetus (400). The placenta appears to be the essential component. The reason a fetus is not necessary is because pre-eclampsia and eclampsia can occur when a fetus is not present or has been delivered, for example in hydatidiform molar pregnancy (323) and abdominal pregnancy after removal of the fetus (401). Case studies and a review of the literature have demonstrated persistence of postpartum eclampsia until retained products of conception (at times microscopic amounts of trophoblastic material) were removed by curettage (402). Due to the essential nature of the placenta in pre-eclampsia, much research has been conducted on the appearances of the placenta in normal and abnormal pregnancy.

1.9.1 Normal development.

A successful pregnancy is dependent upon successful placental implantation and access to the maternal blood supply, the understanding of which has evolved (and is still evolving) over many hundreds of years (403). To support a successful pregnancy the maternal uterine blood vessels need to undergo structural adaptive changes. The invading

trophoblast causes these changes to the spiral arteries to increase the maternal blood supply to the placenta and eventually the fetus (404).

In 1967 Ivo Brosens and co-workers were able to obtain and study serial sections of two normal term caesarean hysterectomy specimens with intact placenta. Their work demonstrated the spiral arteries, from the inner third of the myometrium to the distal position where they open into the intervillous space are physiologically changed, demonstrating a wide tortuous lumen and loss of elastic and smooth muscle tissue within the artery walls (405). The spiral artery walls are replaced with fibrinoid and fibrous tissue. These changes can extend as far as the terminal parts of the radial arteries (406), occur between 6-18 weeks of pregnancy (407) and are usually completed by around 20 weeks gestation (400).

The changes in the spiral arteries result in a low resistance placental bed which can accommodate the increase in maternal blood supply and render the arteries unresponsive to vasoactive agents (408). This endovascular invasion by the trophoblast demonstrates a graded invasion, not an all or none invasion (409).

1.9.2 Pre-eclampsia.

In pre-eclamptic pregnancy, the spiral arteries fail to undergo the physiological remodelling seen in normal pregnancy. The artery walls are similar in appearance to the non-pregnant state with retention of the internal elastic lamina and arterial structure and a markedly reduced artery diameter. Acute atherosclerosis which is “an acute arterio-necrosis with intramural foam cell infiltrates” and is “characterized by fibrinoid necrosis and infiltration of lipophages into the damaged vessel wall” is present in the decidual and myometrial

spiral artery sections although all these changes are mainly limited to the decidual portions of the spiral arteries in pre-eclampsia (406, 410). It is not known whether these lesions are the result of pre-eclampsia or occur before pre-eclampsia develops (411).

More recent studies have demonstrated abnormalities in the placentas of pre-eclamptic women are linked to gestational age, with more severe placental pathology occurring at an earlier gestation (412-414). Early onset pre-eclampsia may be a placental problem whereas late onset pre-eclampsia could be a maternal problem (415). This is due to the difference in placenta morphology and interaction with maternal metabolic disease (412, 413). Early onset pre-eclampsia placentas have increased disease rates with a greater number of ischaemic lesions compared to late onset pre-eclampsia placentas. The babies of early onset pre-eclampsia are also more likely to be growth restricted (413, 414).

This sub classification of pre-eclampsia as an early syndrome (occurring less than 34 weeks gestation and usually presenting with SGA/IUGR) and a late syndrome (occurring after 34 weeks gestation with a normally grown or even larger than average fetus) (416, 417) was suggested by von Dadelszen and colleagues in 2003 (265). Studies are now being performed looking at pre-eclampsia in this new light. Others argue there may be no difference in early and late pre-eclampsia, instead it is the combination of early pre-eclampsia with IUGR that makes the disorder so severe (267).

The faulty implantation and remodelling of the placental bed does not solely occur in pre-eclampsia. Failure of spiral artery remodelling has been demonstrated in SGA pregnancies without pre-eclampsia (418), in late (13-24 weeks gestation) spontaneous miscarriage

(419), preterm birth, premature rupture of membranes and in healthy normal term pregnancies (420).

As failure of spiral artery remodelling can occur in a number of pregnancy complications other than just pre-eclampsia, this leads to the question of what is the originating factor that results in pre-eclampsia and when does the failed placental implantation occur? It is possible that maternal factors are what differentiate between placental pathology evolving into pre-eclampsia and other pregnancy complications.

Pre-eclampsia is thought to develop because of abnormal implantation and/or inadequate trophoblast invasion and failed vascular remodelling. This may result from immune mediated abnormal implantation, microvascular or maternal disease, or an increase in placental size (12).

Abnormal placentation then results in reduced flow into the intervillous space and hypoxia-reoxygenation of the placenta which damages the villous trophoblast releasing micro-fragments of STBM into the maternal circulation (234). An alternative hypothesis is because spiral artery diameters are not increased in pre-eclampsia, there is increased turbulence within the intervillous space that causes a “jet hose” effect, mechanically shearing the villous syncytiotrophoblast and releasing it into the maternal circulation, interacting with the maternal endothelium and resulting in the clinical picture of pre-eclampsia (421). Faulty implantation may also occur during blastocyst implantation because of a maternal immune response against the invading trophoblast (422). In fact a number of factors control and regulate trophoblastic invasion, and an abnormality in any of these pathways could lead to impaired placental implantation (423).

The general consensus is that the placental lesions seen in pre-eclampsia are from reduced placental perfusion and chronic hypoxia (400, 424-426). The quality and extent of placental perfusion may be important with regular intermittent placental flow resulting in oxidative stress from chronic hypoxic-reoxygenation insults (424, 427). A pre-eclampsia like syndrome with hypertension, proteinuria and glomerular endotheliosis has been produced in animals by inducing placental ischaemia with unilateral uterine artery ligation (428).

Not all researchers agree that pre-eclampsia results from placental hypoxia, arguing that the vascular anomalies identified in pre-eclamptic placentas are a consequence of the disease, the same way kidney, liver and brain hypo-perfusion defects are a consequence of pre-eclampsia. They also argue that pre-eclampsia is maternal in origin if the trophoblast requires interaction with maternal risk factors (429).

Poor placental implantation could also be a major risk factor for pre-eclampsia, rather than the cause (407). However most of the histopathology on the placenta comes from women who already have pre-eclampsia. It would be almost impossible to assess exactly when the placental lesions occur as most samples are either taken before pre-eclampsia occurs (and we will never know if those pregnancies would progress to an abnormal outcome) or assessed after delivery because of pre-eclampsia.

1.10 Stage 2: The link between poor placentation, maternal risk factors and endothelial activation/dysfunction.

In 1939 it was hypothesised that some “placental pressor substance” was produced from the hypo-perfused placenta that ultimately resulted in pre-eclampsia (239). James Roberts and Christopher Redman suggested that information from the poorly perfused placenta

may be transported either “neurally or by blood borne products,” affect the endothelium and result in the clinical picture of pre-eclampsia (12). However they dismissed the former hypothesis due to work performed in the 1950s that demonstrated the “vasospasm of pre-eclampsia was not affected by ganglionic blockade” (430) when a high spinal anaesthesia had little effect on blood pressure in “toxemic patients”. This provided support for humoral products as a cause of pre-eclampsia.

1.10.1 Secretion of factors that may induce endothelial dysfunction.

A number of factors have been suggested in the search for the elusive link between poor placentation and the clinical disease of pre-eclampsia. These factors include various cytokines (431, 432) although the evidence supporting their release from the placenta in pre-eclampsia is not convincing (407), lipoproteins (433) and STBM (407).

The STBM has been detected in the circulation of normal pregnant women and is found in increased amounts in pre-eclampsia. *In vitro* endothelial cell growth is suppressed by STBM (434) and endothelial cell activity is inhibited. However there is no correlation between STBM and the severity of pre-eclampsia (435). The process by which STBM are thought to affect the maternal endothelium is by the shedding of particles into the vasculature which activates neutrophils, increases the inflammatory response and results in reduced vasodilatation and endothelial dysfunction (426). Even though cytokines, lipoproteins and STBM have been reported to induce *in vitro* endothelial dysfunction none have been shown to produce the clinical picture of pre-eclampsia (436).

It is more likely that a number of factors are released and work in concert with each other to affect the maternal endothelium. Recently, imbalances in angiogenic and antiangiogenic factors have shown promise in the search for a link between pre-eclampsia stage 1 and 2.

1.11 Angiogenesis.

There are at least three families of vascular growth factors, the vascular endothelial growth factors, the angiopoietins and the ephrins (437). Both the angiopoietin factors and their Tie receptors are important in the vascular development of the placenta (438-440). The ephrin ligands and receptors (437) are necessary in embryogenesis and placental development (441).

The VEGF members found in humans include VEGF-A,-B,-C,-D (442) and PlGF (443). The most potent angiogenic protein is VEGF-A which can induce leakage and permeability, induce mitosis and proliferation of vascular endothelial cells and promote cell survival. The VEGF family has a number of receptors including the *fms*-like tyrosine kinase vascular endothelial growth factor receptor-1 (VEGFR-1 (also known as Flt-1)) and VEGFR-2 (or KDR in humans or Flk-1 in mice). The interactions between the VEGF ligands and their receptors and the signalling pathways they arbitrate are complex (437, 442).

Vascular endothelial growth factor induces vasodilatation and plays a role in regulating blood pressure and vascular tone via the production of endothelial cell NO and PGI₂. It also preserves the integrity of the glomerular filter membrane (444).

1.11.1 The interaction between angiogenic factors, nitric oxide, prostacyclin and the endothelium.

Functional VEGF receptors are found on endothelial cells, monocytes, uterine smooth muscle cells and pancreatic epithelial cells (445). Excessive amounts of VEGF can result in an increase in vascular permeability and increased coagulability (446). Vascular endothelial growth factor is thought to play a role in endothelial function because it can induce production of NO (447, 448) and PGI₂ from endothelial cells in a dose dependent manner (447). It can also modulate growth factor signalling in endothelial cells (445).

Nitrite and nitrate levels can also be measured as an indicator of NO production and endothelial function and have been measured in pre-eclamptic women (186). The measurement of circulating levels of whole blood and plasma nitrite were compared with sFlt-1 and sEng levels in normal pregnancy, gestational hypertensive and pre-eclamptic women. Nitrite levels were significantly decreased in the hypertensive women and the soluble receptor levels were increased compared to the normotensive women. Nitrite levels and sFlt-1/sEng levels demonstrated a significant negative correlation. This suggests that these soluble antiangiogenic factors may inhibit NO formation with resulting endothelial dysfunction (449).

1.11.2 Angiogenesis in pregnancy.

While VEGF and Flt-1 are localized in the placenta, a discrepancy observed for Flt-1 suggested the presence of a soluble receptor for Flt-1, that is soluble VEGFR-1 (sVEGFR-1) also known as *fms*-like tyrosine kinase-1 or sFlt-1 (450). The placenta was found to be an abundant source of sFlt-1 which was also present in maternal serum (451). It has been shown that sFlt-1 is an antagonist to VEGF and PlGF, inhibiting and neutralizing these

factors (451). Recently, sFlt-1 has been shown to be important in the pathogenesis of pre-eclampsia.

1.11.3 An imbalance in angiogenic and antiangiogenic factors in the pathophysiology of pre-eclampsia.

In pre-eclampsia an imbalance of angiogenic factors occurs in the placenta with the expression of VEGF-A and Flt-1 decreased, PlGF unchanged and sFlt-1 increased (452). Although it was known that sFlt-1 was released into the maternal circulation (451), it was not known what occurred to the level of circulating sFlt-1 in pre-eclampsia. This led to the hypothesis that an imbalance in angiogenic factors and antagonism of VEGF factors due to excess levels of sFlt-1 in the maternal circulation may result in the proteinuria and hypertension of pre-eclampsia (453).

1.11.4 Measuring sFlt-1 levels in pregnancy and pre-eclampsia in basic science experiments.

Using a combination of human and animal experiments Maynard and colleagues (453) assessed sFlt-1 levels in pre-eclampsia. The level of total serum sFlt-1 was increased almost five times and free VEGF and PlGF were decreased in pre-eclampsia compared with normotensive pregnancy. Treatment with sFlt-1 resulted in hypertension and proteinuria with pregnant rats developing glomerular endotheliosis. These results demonstrated the effects of sFlt-1 are directed towards the maternal endothelium. Both PlGF and VEGF needed antagonism for pre-eclampsia to develop. However none of the rats demonstrated a HELLP like syndrome therefore other factors were hypothesised to be involved in pre-eclampsia (453). This was later shown to be sEng (454).

1.11.5 The level of sFlt-1 in pregnancy and pre-eclampsia.

The level of sFlt-1 was shown to be higher in pre-eclamptic women compared with normotensive women and decreased in both groups postpartum (455). Compared to normal pregnant controls increased levels of sFlt-1, and decreased free PIGF and free VEGF in pre-eclamptic women was demonstrated using stored serum from women enrolled into the Calcium for Preeclampsia Prevention trial (456). In general sFlt-1 levels increased 9-11 weeks before the onset of pre-eclampsia and rose more rapidly within 5 weeks of clinical disease. Increased levels of sFlt-1 between 21-32 weeks predicted preterm pre-eclampsia (<37 weeks gestation) with an OR 5.1 (95% CI 2.0 to 13.0). Increased sFlt-1 between 33-41 weeks predicted term disease (pre-eclampsia \geq 37 weeks) with an OR 6.0 (95% CI 2.9 to 12.5) (456).

1.11.6 Measuring VEGF levels in pregnancy and pre-eclampsia.

In pregnancy (normal and pre-eclamptic), most of the free VEGF is bound to circulating sFlt-1 (453) resulting in reduced VEGF measurements (457, 458). However measuring free VEGF concentration in maternal plasma and serum is difficult because it occurs in quantities close to the detection limit (459) or is undetectable (460) in normal pregnancy and pre-eclampsia. Low VEGF levels are not a significant predictor of pre-eclampsia (456) and are not routinely performed.

1.11.7 Measuring PIGF levels in pregnancy and pre-eclampsia.

In contrast low PIGF serum levels collected between 13-20 weeks gestation are a significant predictor of preterm pre-eclampsia (<37 weeks) with an OR 7.4 (95% CI 1.8 to 30.2). If serum was collected between 21-32 weeks, the OR for early pre-eclampsia was 7.9 (95% CI 2.9 to 21.5) (456). In normal pregnancy PIGF levels have been reported to be three times higher compared to women who develop pre-eclampsia (461). Reduced

urinary PIGF at 21-32 weeks was also a significant predictor for the development of pre-eclampsia with an OR 22.5 (95% CI 7.4 to 67.8) (462).

1.11.8 Angiogenesis and endoglin.

Endoglin (also known as CD105 or TGF- β type III receptor (T β RIII)) is a co-receptor found mainly on endothelial cell membranes. Endoglin along with TGF- β type II receptor (T β RII) and TGF- β type I receptor (also known as activin receptor-like kinase 5 (ALK5)) are receptors for the ligand TGF- β (463). Mice deficient in endoglin die around 10 days after birth and the embryos have fragile vessels, abnormal yolk sacs and heart defects. Alterations in vascular development such as embryo development, inflammatory processes and wound healing will up-regulate endoglin (463).

A soluble form of endoglin (sEng) exists which has antiangiogenic properties and interferes with TGF- β signalling on the endothelial cell membrane by binding with TGF- β and reducing its availability in the vasculature (463). The TGF- β signalling and receptor pathway along with sEng and NO pathways have been implicated in the pathogenesis of pre-eclampsia (454).

1.11.9 Soluble Endoglin.

Animals treated with sFlt-1 do not develop the HELLP syndrome form of pre-eclampsia (453). A placenta derived soluble factor sEng was discovered in maternal serum. Soluble endoglin acted in conjunction with sFlt-1, causing endothelial dysfunction and HELLP syndrome in pre-eclampsia (454).

1.11.10 The relationship between TGF- β , endoglin, the soluble receptors sFlt-1 and sEng, nitric oxide and pre-eclampsia.

In experiments (454) on rat renal microvessels TGF- β 1 and TGF- β 3 produced dose dependent vasodilatation which was antagonised by sEng. The dose effect on vasodilatation of VEGF and TGF- β 1 was cumulative but was blocked by sEng plus sFlt-1 (at levels seen in pre-eclampsia) and also L-NAME indicating a NOS dependent vasodilatation. This implies that circulating sEng plus sFlt-1 may counter the NO dependent dilatation seen in vessels and play a role in the hypertension of pre-eclampsia.

The endothelial cell receptors endoglin and Flt-1, circulating TGF- β and VEGF and antiangiogenic proteins sFlt-1 and sEng all interact to maintain vascular homeostasis in the circulatory system. Excess sEng and sFlt-1 interfere with the TGF- β and VEGF signalling to the endothelial cells which results in decreased production of NO and the subsequent endothelial dysfunction seen in pre-eclampsia (464).

1.11.12 The measurement of sEng in pregnancy and pre-eclampsia.

Stored sera were used to assess whether sEng was associated with pre-eclampsia. Serum levels of sEng were increased in women with preterm and term pre-eclampsia versus matched controls when the clinical picture of pre-eclampsia was apparent. This increase in sEng was significant at 17-20 weeks in women who later developed preterm pre-eclampsia, increasing steeply at 33-36 weeks compared to normotensive controls. Gestational hypertension was associated with increased levels of sEng at 33-36 weeks compared with controls but levels were lower than term pre-eclamptics at a comparable gestation. After onset of gestational hypertension sEng levels were similar to the term pre-eclamptic group (465).

1.11.13 A cautionary note on measuring angiogenic factors in pregnancy.

Care needs to be taken when interpreting studies on angiogenic factors.

Changes in angiogenic factor profiles have been reported in other obstetric complications such as SGA (466), unexplained fetal death in utero (467), “mirror syndrome” (maternal oedema plus or minus pre-eclampsia with a hydropic fetus) (468) and twin-twin transfusion syndrome (469).

Not all women with pre-eclampsia have increased sFlt-1 levels (470). It is still uncertain if placental hypoxia or excess sFlt-1 is the initiating pathological event. It is possible that increased sFlt-1 production during the clinical picture of pre-eclampsia is secondary to placental hypoxia (436).

Therefore even though there is strong evidence of a role of angiogenic factors in pre-eclampsia, due to the multifactorial nature of pre-eclampsia, other factors apart from angiogenic factors may affect the maternal endothelium.

1.11.14 The role of obesity, angiogenic factors and adipocytokines in the pathogenesis of pre-eclampsia.

As previously discussed obesity is a risk factor for pre-eclampsia. Various secretory proteins (adipocytokines) are produced by adipose tissue including leptin, TNF α and the hormone adiponectin. These help control energy expenditure, lipid metabolism and insulin resistance. Leptin modulates appetite and also helps in the regulation of fetal growth. Adiponectin is a cytokine that acts against atherogenesis, diabetes and inflammation. Adiponectin levels are reduced when obesity, insulin resistance and type 2 diabetes occur (471, 472).

Low adiponectin levels are associated with hypertension (473), reduced endothelial dependent dilatation (FMD) (474) and endothelial dysfunction in obese subjects (475). A number of studies have demonstrated reduced adiponectin levels in overweight (BMI >25) pre-eclamptic women and higher levels in normal weight pre-eclamptic women (471, 476).

A study was designed to assess adiponectin and angiogenic factor levels in both normal weight (BMI >18.5 and \leq 25) and overweight (BMI >25) healthy pregnant and pre-eclamptic women (471). In normal weight pre-eclamptic pregnancy adiponectin levels increase, as BMI increases in both normal weight and pre-eclamptic women adiponectin decreases. Overweight pre-eclamptic women have a less extreme imbalance in angiogenic factors and decreased adiponectin levels when compared to normal weight pre-eclamptics. The authors suggested late pre-eclampsia in overweight women is a multifactorial disease and multiple mechanisms are probably at play affecting the maternal endothelium and resulting in the clinical syndrome of pre-eclampsia (471).

Another study divided normal and overweight severe pre-eclamptic women into early (<32 weeks) and late (\geq 32 weeks) syndrome. Both pre-eclamptic groups had an angiogenic factor profile consistent with the disorder. In early pre-eclampsia there was no difference in the angiogenic imbalance or adiponectin levels between normal and overweight groups. Therefore the placenta rather than maternal factors were thought to play a greater role. Late pre-eclamptic overweight women demonstrated low adiponectin levels and a less deranged angiogenic profile compared with early overweight pre-eclamptics. These results suggest obesity is a contributor to late pre-eclampsia and implies an extra mechanism for the insult to the maternal endothelium (472).

1.12 Physiological changes in normal pregnancy and pre-eclampsia.

1.12.1 Normal Pregnancy.

Physiological changes and adaptations occur very early in human gestation with the objective of providing increased nutrients to the placenta and fetus. These changes are necessary for a healthy normal pregnancy. Increases in body weight, percentage of body fat and skin-fold thickness occur as early as the first fifteen weeks of pregnancy (477).

Haematocrit decreases continuously as pregnancy increases (478-481) with this finding noted as early as 7 weeks gestation (481). Haemoglobin concentration also falls progressively (478, 481), resulting in the “physiological anaemia of pregnancy” (482).

This decrease in haematocrit and haemoglobin concentration is a result of the increase in plasma volume (479, 481).

Cardiopulmonary adaptations occur by week seven of pregnancy. These include an increase in heart rate, minute ventilation, oxygen consumption and respiratory exchange.

Mean arterial pressure (MAP) is defined by the formula $MAP = (DBP + 1/3[SBP - DBP])$ (where DBP = diastolic blood pressure and SBP = systolic blood pressure). Mean arterial pressure as well as systolic and diastolic BP all decrease with eighty percent of the change in MAP having occurred by week seven (477). Changes in maternal heart rate have been shown to occur very early in pregnancy. By week eight, 50% of the maximum increase in heart rate has occurred (483). Heart rate continues to increase during the second trimester and then plateaus after 32 weeks gestation (484, 485).

Systolic BP, when taken in a sitting position, changes little over the course of pregnancy with the mean being 103mmHg at less than 16 weeks and 109mmHg at greater than 40 weeks gestation. Slightly higher systolic BPs are recorded with the women supine (113-

116mmHg) (486). Diastolic BP does not differ with either sitting or lying. Around the middle of the second trimester, diastolic BP reaches its lowest point (485, 486) and then increases to pre-pregnancy values by term with the mean range from less than 16 weeks to greater than 40 weeks being 56-59mmHg (486). When comparing BP taken in a sitting versus recumbent left lateral position, systolic and diastolic BP is consistently lower throughout pregnancy in the latter (487).

Cardiac output is the stroke volume in litres multiplied by heart rate (bpm) (488). Stroke volume equals the VTI in centimetres multiplied by the cross-sectional area of interest (cm^2). The area of interest may be the cross-sectional area of the aorta, pulmonary artery or mitral valve (484).

Central haemodynamic measurements in term pregnancies show cardiac output to be 43% greater than postpartum values (489). Non-invasive studies of normal pregnancy have shown cardiac output to increase by 30-50% with the maximum values occurring in the second and early third trimesters (around 26-34 weeks gestation), then decreasing near term (484, 488, 490-493). Stroke volume is also increased compared to non-pregnant values (484, 492, 493), rising steadily until 20 (484) to 31 weeks (491). These maximum values are maintained until around 38 weeks when a slight drop occurs (484, 491). The decrease in cardiac output near term is due to the decrease in stroke volume, however cardiac output is maintained because heart rate continues to increase (484).

Systemic vascular resistance (SVR) is the ratio of mean arterial pressure and cardiac output (CO): $\text{SVR (dyn.s}^{-1}.\text{cm}^{-5}) = \text{MAP(mmHg)} \times 80/\text{CO(L/min)}$ (484). This measurement gives an estimate of the peripheral impedance to cardiac output or the force needed to eject

blood into the circulation (482). Multiple studies agree that SVR decreases in pregnancy when compared to non-pregnant values (484, 488, 491-493). The changes in SVR take place before placentation occurs with minimum values obtained by the mid first trimester (490).

This decrease in SVR together with a decrease in blood pressure allows for the increase in cardiac output without an increase in blood pressure. Decreased SVR in normal pregnant women compared with non-pregnant women was demonstrated using pulsed Doppler of the brachial artery. Both peak systolic and end diastolic velocities were increased and PI decreased in pregnant women in the first and third trimester (494). Pulsed Doppler was also used to demonstrate decreasing SVR (decreasing PI in the cubital artery) in pregnant women with increasing gestation that was correlated with decreasing resistance in the uterine arteries (495). All these physiological changes are necessary for the successful maintenance and outcome of pregnancy.

1.12.2 Systemic vascular resistance and cardiac output in pre-eclampsia.

Normal pregnancy results in profound changes in the maternal cardiovascular system.

Stroke volume and cardiac output increase by 30-50% until the early third trimester then decreases near term although cardiac output is maintained because of heart rate increases. Systemic vascular resistance falls in pregnancy compared with non-pregnant women. This decrease in SVR allows for an increase in cardiac output without an increase in BP (484).

Conflicting results have been reported when measuring maternal haemodynamics prior to and when pre-eclampsia is diagnosed. A number of studies demonstrated a hyperdynamic circulatory system with increased cardiac output and decreased SVR (491, 496). Others

have reported decreased cardiac output and increased SVR in pre-eclampsia (497). Easterling and colleagues study (491) reported elevated cardiac output throughout pregnancy compared with normal controls. Systemic vascular resistance in women who developed pre-eclampsia (n=9) was decreased and similar to normotensive controls. Interestingly the pre-eclamptic women were significantly heavier term pre-eclamptics with no growth restriction (491) suggesting this group of women may represent the late onset maternal form of pre-eclampsia. Another group (496) also demonstrated increased cardiac output and decreased SVR in women destined to develop pre-eclampsia, although they demonstrated a “crossover” state when pre-eclampsia was diagnosed with a switch to lower cardiac output and a high resistance circulation. Again, the pre-eclamptic women had a significantly higher median BMI and delivered greater than 34 weeks gestation suggesting a similar form of pre-eclampsia to Easterling’s group. A later study (498) divided pre-eclamptic pregnancies into those with and without growth restriction and assessed cardiac output and SVR. All pregnancies resulted in term deliveries. Findings were similar to Easterling et al. (491) and Bosio et al. (496) when there was no growth restriction involved. However, in pregnancies complicated with growth restriction the opposite results were found, cardiac output decreased and SVR increased (498).

Combining the adverse outcomes of pre-eclampsia and growth restriction into the one group, in women who delivered <32 weeks, a decreased cardiac output and increased SVR before clinical disease (at 24 weeks gestation) compared to uncomplicated pregnancies was demonstrated (497). This suggests that early and late onset pre-eclampsia may be different diseases with different haemodynamic states.

For this reason 1168 women with uterine artery notching were recruited. Those with a normal outcome served as controls. Seventy-five and 32 women developed early and late pre-eclampsia respectively. At 24 weeks and one year postpartum maternal SVR and cardiac output were calculated. Two different haemodynamic states were identified. The early (<34 weeks) pre-eclamptic women demonstrated low cardiac output/high SVR at 24 weeks probably linked to failed placental vascular remodelling, whereas the late onset pre-eclamptic group had high cardiac output/low SVR at 24 weeks gestation which probably represents a pre-eclampsia linked to maternal risk factors such as increased BMI (499). This study explains the conflicting results of earlier studies.

1.12.3 Plasma and blood volume in pre-eclampsia.

In normal pregnancy plasma volume begins to rise in the first trimester, peaks at about 32 weeks gestation and maintains this level until delivery (479, 480, 500, 501). Plasma volume measured with Evans Blue dye is significantly decreased in women with pre-eclampsia compared with normotensive pregnant and non-pregnant women (502). In women who subsequently develop pre-eclampsia this decrease in plasma volume occurs by 14-17 weeks gestation (503). When comparing normotensive, pre-eclamptic and gestational hypertensive women, total blood volume and plasma volume were significantly less in the pre-eclamptic group compared with the normotensive and gestational hypertension groups. In pre-eclampsia, red blood cell volume (mass) was significantly decreased compared with gestational hypertension. There was no difference between the normotensive and gestational hypertension groups when measuring plasma, total blood or red blood cell volume (504).

Data collected between 1958-1978 using chromium 51 tagged red blood cells to assess total blood volume in eclamptic nulliparous women were analysed and published recently (505). Contemporary ethics approval was obtained for this (506, 507). Twenty-nine eclamptic women and 44 normotensive pregnant controls were studied. Twelve of the eclamptic women were reassessed in a subsequent normotensive pregnancy. Compared with postpartum blood volumes which were very similar in the two groups, women with a normal pregnancy demonstrated a 47% increase in blood volume but in eclamptic women blood volume expansion was only 9%. In the subsequent normotensive pregnancy, previously eclamptic women had a volume expansion that was similar to the normotensive controls. This work demonstrates that eclamptic women have severe haemoconcentration (505). These studies however do not reveal whether plasma/blood volume is reduced because of failed expansion or because of significant volume reduction due to leakage of plasma into the interstitial space (505, 508).

The decrease in intravascular volume has been hypothesised to occur because the vasoconstriction and increased BP of pre-eclampsia forces fluid out of the vascular system (509). This theory was supported by the finding that pre-eclampsia is a state of sympathetic overactivity with sympathetic activation resulting in vasoconstriction and increased SVR and BP (510). The hypothesis was contradicted when it was shown that in women destined to develop pre-eclampsia, plasma volume decreased many weeks before BP increased (511). A more recent theory, as previously discussed involves sEng and sFlt-1 which can increase vascular permeability in rodent tissue and causes severe hypertension in pregnant rats (454). This may explain to some extent the change in plasma volume that occurs in pre-eclampsia due to interstitial leakage.

1.12.4 Pre-eclampsia and its relationship with viscosity and haematocrit.

In normal pregnancy haematocrit decreases and plasma volume increases throughout pregnancy with a resulting decrease in viscosity (512). As discussed previously, viscosity measurements are difficult and unreliable, affected by shear rate, vessel size, haematocrit and the fact that blood is a non-Newtonian fluid whilst plasma is a Newtonian fluid (217, 219). Viscosity and haematocrit have been measured in women with pre-eclampsia and the aforementioned variables may be the reasons why there are conflicting results. Earlier studies also had broader definitions for pre-eclampsia which included gestational hypertension women (513). Nevertheless, haematocrit is the best determinant of whole blood shear dependent viscosity (219).

An early study (513) measured viscosity at high and low shear rates. Increased viscosity was demonstrated in hypertensive pregnant women compared with normotensive controls at both shear rates. Haematocrit was within the “normal range” for both pre-eclamptics and controls. Another study (514) demonstrated decreased viscosity (measured at a low shear rate) due to significantly lower packed cell volume (PCV) in moderate pre-eclamptics (defined as 300mg proteinuria plus BP >140/90). Severe pre-eclamptics had high blood viscosity measured at high shear rates with PCV no different to normal controls.

No difference in whole blood, plasma, serum viscosity and haematocrit was found when comparing third trimester pre-eclamptic women with normal controls (515). A subsequent study (516) showed no difference in haematocrit between pre-eclamptic ($35.4 \pm 2.9\%$) and normal control ($36.7 \pm 3.8\%$) subjects. This study however was very confusing. They had two groups in the study (pre-eclamptic and normal), defined gestational hypertension, pre-

eclampsia and normal pregnancy separately and then used the terminology gestational hypertension and pre-eclampsia interchangeably throughout the text.

The general consensus appears to be that haematocrit increases in pre-eclampsia compared to normal pregnancy (504, 517, 518) with haematocrit reported as $38.6 \pm 4.1\%$ versus $34.6 \pm 3.5\%$ ($P=0.01$) respectively (518). Variations in results are probably due to the differing techniques and definitions used and best practice would dictate measuring haematocrit on the study groups of interest.

1.13 The relationship between pregnancy and the endothelium.

The endothelium plays a major role in regulating the increased vasodilatation that occurs in normal pregnancy. A number of vasoconstrictors and vasodilators are thought to be involved in this process (8). Pre-eclampsia, hypothesised to be a two stage disorder, results in endothelial dysfunction. Imbalances in endothelial factors are thought to play a role (11). As well as the various stimulators necessary for vascular control, reactive hyperaemia and pulsatile flow can induce changes in the vascular endothelium (1, 4). This increase in flow and vasodilatation can be assessed by the ultrasound technique of FMD (16) as well as other previously mentioned techniques.

As the technique of FMD is mainly NO mediated, and the work in this thesis is based on this technique the discussion on normal pregnancy and pre-eclampsia will be limited to endothelial function in relation to vasodilatation, NO and FMD. Prior to this the following section will briefly mention some studies that have assessed risk factors for endothelial dysfunction in normal pregnancy.

1.13.1 The effect of maternal characteristics on endothelial dependent dilatation.

It has gradually been appreciated that pre-eclampsia is multifactorial and also imparts a future risk of CVD with a multitude of factors likely to interact with and affect the vascular endothelium before, during and after pregnancy. For this reason studies have been performed to look at the effects of various maternal characteristics on endothelial function.

One study (351) was specifically designed to assess the effect of maternal characteristics on endothelial function in myometrial arteries taken during elective caesarean sections. Endothelial dependent dilatation was examined using wire myography and the endothelial dependent dilator bradykinin. Decreased dilatation was noted in women with increased BMI and increased dilatation was noted in women who smoke. No explanation for the increased dilatation in smokers was given considering previous studies have demonstrated reduced endothelial dependent dilatation in non-pregnant smokers (177) and smoking reduces the risk of pre-eclampsia (519). Factors that had no effect on vasodilatation were maternal age, miscarriage history, parity and a history of complicated pregnancy (pre-eclampsia, IUGR and gestational hypertension). The median BMI of women in this study were not in the obese range although some did meet the WHO criteria (341) for obesity (previous nulliparous BMI, median [IQR]: 26 [22-37]; uncomplicated multiparous 25 [18-46]; previous pre-eclampsia 23 [23-33]; previous IUGR 24 [21-29]). All the women had previously delivered at term (median 38-39 weeks gestation for all groups) (351).

Women with a normal third trimester pregnancy were recruited and divided into lean (n=24; median BMI 22.1) and obese (n=23; median BMI 31) groups to compare biochemical parameters and endothelial function using laser Doppler imaging (350). In obese women the metabolic markers of fasting triglycerides were increased, HDL

decreased and leptin and insulin increased. The inflammatory markers IL-6 and CRP were also higher in obese women. Endothelial dependent dilatation was significantly lower in normal third trimester obese women.

Women with a BMI of <30 and ≥ 30 were recruited in the first trimester of pregnancy and studied in the first, second, third trimester and four months postpartum to assess microvascular function using laser Doppler perfusion and to evaluate maternal endothelial, inflammatory and placental function (352). Endothelial factors included ICAM-1, VCAM-1, PAI-1 and von Willebrand factor, the inflammatory markers were IL-6, IL-10, TNF α and CRP and the placental function markers were PAI-1 and PAI-2. Endothelial function was significantly increased in first trimester lean women, although both groups increased in a similar fashion throughout pregnancy. Obese women had significantly less endothelial vasodilatation in the three trimesters (-51%, -41% and -39%; $P < 0.05$) and even lower postpartum (-115%; $P < 0.001$). Even though this work provided evidence for endothelial dysfunction in normotensive obese pregnant women, none of them developed pre-eclampsia.

This endothelial dysfunction in obesity was thought to be due to an increase in inflammatory cytokines in particular IL-6 and CRP which was increased throughout pregnancy and postpartum. Placental function markers can be viewed as a ratio (PAI-1/PAI-2) with an increased ratio indicating worse function. In the first trimester, obese women had significantly higher ratios with no difference thereafter suggesting early impaired placental function (352). These two studies (350, 352) would suggest that obesity and endothelial dysfunction whilst predisposing risk factors are not a definitive risk for the development of pre-eclampsia.

1.13.2 Nitric oxide and its role as a vasodilator in normal pregnancy.

A number of studies, performed as laboratory assays have implicated NO as a vasodilator in normal pregnancy, inducing the decrease in SVR. Compared to non-pregnant women, healthy pregnant women demonstrate an increase in serum nitrite and nitrate levels (520). This increase occurs throughout pregnancy and returns to non-pregnant levels by 12 weeks postpartum (521). Other studies have found no difference in nitrite/nitrate levels in normal pregnancy (522) compared with pre-eclamptic women (523) and non-pregnant controls (524, 525). This may be due to differences in methodology and maternal diet when measuring NO metabolites (522).

An indication of the availability of NO in the body is the level of cGMP which increases when NO binds to cGMP resulting in vascular smooth muscle dilatation (26). In plasma (522) and platelets (525) cGMP is increased in normal pregnancy.

A NO-cGMP pathway has been demonstrated in the human uterus (526, 527). Studies have shown NO can modulate contractions and modify cGMP synthesis in human myometrium in a dose dependent manner (526). The level of cGMP is decreased in myometrium from term labouring women compared with preterm and term non-labouring women (526, 527). Myometrial eNOS is also upregulated in the early third trimester of pregnancy (25-34 weeks gestation) and reduced at term (528). This suggests the NO-cGMP pathway may be involved in suppressing contractions during pregnancy (527) and by decreasing at or near term may have a role to play in the initiation of labour. Decreased NO in the third trimester may be a normal physiological event.

Physiological and animal studies have implicated a role for NO and shear stress in the decreased SVR of normal pregnancy. Animal work on pregnant rats demonstrated

dilatation of mesenteric arteries was induced by flow-mediated NO release (529). When the effect of NO *in vivo* was inhibited in baboons, blood pressure and SVR increased and cardiac output and heart rate decreased (530). Using the technique of plethysmography basal blood flow to the hand was significantly increased in late third trimester pregnancy compared to first trimester pregnancy and non-pregnant women. When the three groups were infused with the NO inhibitor L-NMMA, a greater dose dependent reduction in blood flow in pregnancy was noted. This augmented response to L-NMMA suggests that the decrease in SVR occurring in normal pregnancy is due to increased production of NO (7). Similar findings were reported using the same technique with an increased response to L-NMMA demonstrating enhanced basal NO activity in third trimester pregnancy (531).

The reaction to flow induced shear stress was examined in small arteries obtained from fat biopsies at caesarean and abdominal gynaecological surgery in normotensive pregnant (n=10) and non-pregnant women (n=10) (263). The arteries from pregnant women demonstrated increased dilatation in response to flow which was absent in the non-pregnant arteries. The NOS inhibitor L-NAME reduced the response in the arteries from pregnant women. This work demonstrated arteries from normotensive pregnant women have increased sensitivity to shear stress and this is mediated by NO.

1.14 The assessment of endothelial function by flow-mediated dilatation in normal pregnancy.

The ultrasound technique of FMD has also been used to study NO mediated endothelial function, SVR and the response to shear stress in normal human pregnancy. At the time this thesis was commenced only two studies had been published specifically addressing FMD in normal pregnancy compared to non-pregnant women (207, 208). A third study assessed FMD in normal pregnancy with the intention of comparing this group to non-

pregnant and pre-eclamptic women (532). Another paper was published while the normal range study for this thesis was being completed (533).

The data from the normal pregnancy FMD studies is discussed in more detail due to their relevance and also summarised in Table 1.1. All the studies had BP occlusion times between four and five minutes and scanned the brachial or radial arteries (Table 1.1). Two studies used upper arm cuff occlusion (532, 534), the rest used forearm occlusion. One study included smokers (535). The BMI was only reported in two studies with none of these women in the obese range (535, 536). Variable BP occlusion pressures were used and post occlusion vessel diameters were measured at different times (Table 1.1). Only the work by Dorup and co-workers (207) assessed endothelial independent vasodilatation with nitro-glycerine, demonstrating the same degree of response in pregnant and non-pregnant women. The same FMD data (with the addition of extra biochemical markers each time) was presented in three papers (535, 537, 538) and the fourth paper by the same group used the same set of women with almost identical results. Differences appear so slight they may result from rounding the data or the addition of one extra subject (539) so only the first (535) publication's results are tabled and discussed.

Table 1.1: Flow-mediated dilatation in normotensive pregnant and non-pregnant women.

<i>Author (year)</i>	<i>Study type</i>	<i>Non pregnant FMD% m ± SD(n)</i>	<i>Normal pregnant FMD% m ± SD (n)</i>	<i>GA at study: weeks (range) or weeks (m ± SD)</i>	<i>Occlusion pressure (mmHg)</i>	<i>Occlusion time (minutes)</i>	<i>Time post occlusion measured(sec)</i>
Yoshida et al. (1998) (532)	Cross-sectional	11.8 ± 3.6 (20)	18.9 ± 3.4* (18)	35.8 ± 3.1	>30 above systolic	5	Maximum up to 360
Dorup et al. (1999) (207)	Cross-sectional	7.2 ± 2.8 (37)	T1: 9.1 ± 4* (13) T2: 9.1 ± 3.7* (29) T3: 10.6 ± 4.4* (29)	T1: 12 (9-14) T2: 24 (22-27) T3: 35 (31-38)	250-300	4	Up to 90 (not stated)
	Longitudinal (n=8)	7.4 ± 3.5 (5)	T1: 9.9 ± 5.0 T2: 11.1 ± 1.9 T3: 12.4 ± 5.2*				
Savidou et al. (2000) (208)	Cross-sectional	6.42 ± 2.45 (19)	8.84 ± 3.18* (157)	10-30	250-300	5	55-65
Kametas et al. (2002) (536)	Cross-sectional High altitude Sea level	6.41 ± 2.98 (11) 7.12 ± 3.1 (14)	7.5 ± 3.5 (60) 7.65 ± 3.9 (54)	6-42	300	5	55-65
Faber-Swensson et al. (2004) (533)	Longitudinal (n=12)	5.8 ± 2.1 (12)	T1: 6.2 ± 2.7 T2: 6.8 ± 2.1 T3: 11.2 ± 5.5*	T1: 12 T2: 19 T3: 32	260-280	4	60
Yamamoto et al. (2005) (534)	Cross-sectional	108.7 ± 3.9 (20)	115 ± 6.5* (20)	33 ± 3	50 above systolic	5	Up to 360, maximum at 60
Saarelainen et al. (2006) (535)	Cross-sectional	9.48 ± 4.05 (62)	T1: 7.4 ± 3.5 (13) T2: 10.9 ± 4.6 (15) T3: 11.1 ± 5.3 (22)	T1: ≤ 14 T2: 15-27 T3: ≥ 28	250	4.5	40,60,80
Sierra-Laguado et al. (2006) (540)	Cross-sectional	~15 (56)	T1: ~17* (56) T2: ~ 17* (300) T3: ~ 18* (136)	T1: 11.4 ± 1.75 T2: 21.6 ± 3.49 T3: 29.2 ± 2	300	5	Up to 60

T1, first trimester; T2, second trimester; T3, third trimester. *significant difference compared to non-pregnant controls. ~approximation made by author from graphs in original paper.

The first work using the FMD technique to assess normal human pregnancy was performed by Yoshida and colleagues (532) using upper arm cuff placement and a 30MHz transducer to ultrasound the radial artery in third trimester women. The purpose of the study was to compare normotensive pregnant, pre-eclamptic and non-pregnant controls. This work demonstrated FMD in normotensive pregnant women was almost double that of non-pregnant women (532) (Table 1.1). The normal pregnancy FMD values were almost twice as high as other studies. The use of upper arm cuff placement which reflects a degree of ischaemic response (130) and not solely NO dependent vasodilatation may explain these results. Shear stress stimulus was not reported. In summary this study reported increased FMD in normotensive pregnancy compared with non-pregnant women.

A cross-sectional study (n=71 pregnant and n=37 non-pregnant) with a small longitudinal arm (n=8) was published next with the aim of assessing the normal vascular physiology of pregnancy (207). In the cross-sectional arm FMD increased significantly in pregnancy compared to non-pregnant women with the highest value obtained in the third trimester. There was little difference between the three trimester values (Table 1.1). In the longitudinal arm FMD was measured before pregnancy (n=5) and three times during pregnancy (n=8). There was no significant difference in FMD between the three trimesters. Compared to pre-pregnancy FMD, only FMD in the third trimester was significantly greater. In the cross-sectional study, a significant inverse relationship was noted between FMD and baseline vessel size ($r = -0.35$, $P=0.0001$). Heart rate increased significantly by the first trimester then plateaued compared to non-pregnant controls. Systolic and diastolic BP was lower in the first and second trimester then increased to non-pregnant values in the third trimester. Non-pregnant and first trimester brachial artery diameters were similar in size ($3.17 \pm 0.28\text{mm}$ and $3.08 \pm 0.34\text{mm}$ respectively (mean \pm

SD)), increasing significantly but also plateauing in the second ($3.34 \pm 0.28\text{mm}$) and third trimester ($3.35 \pm 0.47\text{mm}$). Baseline volume flow was similar to controls in the first trimester then increased significantly by 56% and 83% in the middle and last trimesters. The degree of reactive hyperaemia decreased throughout pregnancy with the lowest value occurring in the last trimester. In the non-pregnant controls no mention was made regarding the time of the menstrual cycle when the FMD test was performed (207). This work seems to support the notion that endothelial function is enhanced in normotensive pregnancy although numbers were small particularly in the longitudinal arm and the time when maximum dilatation was measured was not stated.

Savvidou et al. (208) published a cross-sectional study assessing endothelial function in pregnant and non-pregnant women. The pregnant group were recruited between 10-40 weeks gestation. However the pregnant mean \pm SD FMD results reported were between 10-30 weeks gestation (Table 1.1) presumably to try and demonstrate that FMD increased in normal pregnancy. Regression analysis actually demonstrated little change in FMD ($R^2 = 0.13$, $P < 0.001$) although the authors stated that FMD increased significantly until 30 weeks (based on the mean results) and then decreased. Basal brachial artery diameter was also reported to increase with increasing gestation, although again the regression results whilst giving a significant result report a weak correlation ($R^2 = 0.21$, $P < 0.001$). Basal blood flow showed a weak but significant increase throughout gestation ($R^2 = 0.35$, $P < 0.001$) although there was no significant mean difference between controls and pregnant women. Reactive hyperaemia decreased with gestational age ($r = 0.51$, $P < 0.001$) although the Y-axis on the graph is logarithmic which demonstrates the large variance in these results. The FMD was inversely correlated with brachial artery diameter ($r = -0.42$, $P < 0.001$). The significant regression analysis results in this paper would occur because of

the large number of pregnant women analysed (n=157) (208). In conclusion, these results report increased FMD in pregnancy compared with non-pregnant women.

Flow-mediated dilatation was used to discern if there was a difference in endothelial function between pregnant women at high and low altitude (Table 1.1) because high altitude has been associated with an increased risk of pre-eclampsia. No difference in FMD was demonstrated between any of the groups (536).

A slightly larger longitudinal study (n=12 pregnant and n=12 age matched non-pregnant controls) was performed in 2004 to assess endothelial function in normal human pregnancy. This study was performed between the gestational ages of 12-32 weeks (533). Although no difference in FMD was demonstrated between controls and first and second trimester women, a significant increase in FMD occurred in the third trimester (Table 1.1). Baseline brachial artery diameter was no different between the non-pregnant, first and second trimester women (3.1mm) increasing to 3.4mm in the third trimester (significance not reported). Heart rate and blood pressure did not vary between controls and pregnant women at any gestation. Data on the shear stimulus, that is baseline and post occlusion volume flow and reactive hyperaemia were not reported (533). This study reports increased FMD in the third trimester of pregnancy only compared with non-pregnant women.

A cross-sectional study was done to assess if the action of NO is decreased in pre-eclampsia compared with normotensive pregnancy and non-pregnant controls (534). Upper arm BP cuff inflation was used. A significantly increased FMD in normotensive pregnant women compared to non-pregnant controls (Table 1.1) was found. Shear stress

stimulus was not reported. This study demonstrated increased FMD in normotensive pregnancy compared to non-pregnant controls.

Another cross-sectional study compared non-pregnant controls (n=62) to normal pregnant women (n=57) in the three trimesters with the aim of comparing FMD with serum lipids (535). Whilst there was no significant difference in FMD between controls and at any time in pregnancy (P=0.067), FMD increased significantly across the three trimesters (P=0.041) (Table 1.1). Basal brachial artery diameter did not differ between the groups. Flow-mediated dilatation was significantly and negatively correlated with brachial artery diameter ($r = -0.28$, P=0.035). Shear stimulus data was not reported (535). In summary there was no difference in FMD between non-pregnant controls and normotensive women.

Flow-mediated dilatation was compared in a large number of normotensive pregnant women (n=492) and 56 non-pregnant women cross-sectionally with the aim of evaluating the effect of pregnancy on endothelial function (540). Both FMD and basal brachial artery diameter were increased significantly in pregnancy compared with non-pregnant controls, although exact mean and standard deviation values were not given (results were presented in a graph) (Table 1.1). Flow-mediated dilatation correlated inversely with brachial artery diameter. Heart rate increased and BP decreased in pregnancy compared with controls (540). Methodology of the FMD technique was referenced to an earlier study (541) and is identical to the technique as described by Celermajer and colleagues (16). Data on the shear stimulus was not presented possibly because this was a short communication. In conclusion FMD was significantly increased in normotensive pregnancy compared with non-pregnant controls.

The above results indicate that endothelial function as assessed by FMD in normal pregnancy is enhanced or at least not reduced. Basal brachial artery diameter and basal volume flow are also either increased or remain stable in pregnancy. The increase in basal flow and basal dilatation of the brachial artery would be consistent with the decrease in SVR that occurs in normal human pregnancy. Limitations of these studies include the small numbers recruited in the longitudinal studies (207, 533), the interpretation from the statistical analysis (208) and the variations in methodology. These differences in methodology include using upper arm cuff placement (532, 534), the inclusion of smokers (535), post occlusion vessel diameter measured at different times (Table 1.1) and no reporting of shear stimulus (532-535, 540).

1.15 The assessment of endothelial function in pre-eclampsia.

Endothelial dysfunction in pre-eclampsia can be measured in a number of ways, biochemically by the assessment of various products that result from the stimulation of the endothelium and its attempts at repair (431, 542) and physiologically by any of the previously mentioned methods. Proposed stimulators of the endothelium are STBM, inflammatory proteins (for example cytokines and lipoproteins), cellular components such as platelets and leukocytes, antibodies and angiogenic factors (542). A current theory proposes a “threshold for angiogenic imbalance” exists in every pregnant woman and when this is breached pre-eclampsia occurs (543). Endothelial dysfunction then manifests itself in the characteristic signs of pre-eclampsia, namely vasospasm, microthrombus and vascular permeability (542).

Pre-eclampsia was hypothesised to be an endothelial disorder by James Roberts and colleagues in 1989 (11). This theory was based on previous work that had demonstrated in

pre-eclamptic women, leakage of fluid from intravascular spaces across the endothelial cell barrier occurs with reduction in plasma volume (544). Furthermore, the evidence suggested factors known to be released when endothelial cell injury occurs are increased in serum and plasma from pre-eclamptic women (11). Also the realisation that when women die from pre-eclampsia/eclampsia the pathological changes that result are from reduced perfusion to different organs such as the brain, liver, heart, kidneys, adrenals and placenta (12, 545).

Decreased perfusion occurs because of activation of the coagulation cascade (546, 547) and loss of fluid from the intravascular space (511) resulting in micro-thrombi occluding vessels (235). Decreased perfusion may also occur because of vasospasm or impaired vasodilatation (263). Numerous studies using different techniques have demonstrated impaired vasodilatation in women with pre-eclampsia. The impaired vasodilatation may be due to variations in NO availability.

Nitric oxide is known to have cytotoxic effects as well as being a potent vasodilator. Abnormalities in vasodilatation may occur from a direct effect when there are low levels of NO available but may also occur indirectly. The indirect effect can present as either nitrosative stress or oxidative stress through ROS or RNOS. The production of ROS results in antioxidant formation and apoptosis. The creation of RNOS occurs when NO concentrations are high for a long time and can also result in NO mediated cell death. The toxic effects of NO are mediated by conversion of NO to nitrate, nitrite, NO₂ radicals, peroxynitrite and nitrogen dioxide. Due to the short half-life of NO in biological tissue (22) most studies have measured the production of NO via its metabolites nitrate and nitrite and second messenger cGMP.

1.15.1 Nitric oxide and pre-eclampsia.

Multiple studies assessing NO levels in pre-eclampsia have been published using a variety of techniques (3). The literature is contradictory whether increased, decreased or no change in NO levels occur. This may be because of differences in diet or dietary intake as well as a lack of quality control. Commercial assays are often used because of ease and availability rather than because of reliability and validity. Nitrite and nitrate are commonly measured because they are stable metabolites of NO, found in both the blood and urine. Studies have shown nitrite in the circulation better reflects endothelium dependent NO synthesis in people and animals with rapid changes in naturally produced NO measured preferably in plasma. In urine the major metabolite of NO is nitrate and its measurement is a consistent way to assess basal NO whole body synthesis changes over time (88).

A number of studies have reported increased levels of NO in pre-eclamptic women (67, 68, 186, 520, 524, 548). Increased serum nitrate levels were reported in pre-eclamptic women compared to normal pregnant and non-pregnant women (524). In women on low nitrite/nitrate (NO_x) diets plasma production of NO_x was increased in pre-eclampsia (186). A significant increase in nitrates and nitrites was demonstrated in pre-eclampsia compared with normal pregnancy (520). Serum nitrate levels were significantly increased in pre-eclampsia compared with normal pregnant and non-pregnant women, with no difference in nitrite levels. However the total amount of nitrate and nitrite was significantly increased in pre-eclampsia (68). Plasma NO_x levels were also significantly increased in pre-eclampsia compared with normal pregnancy (67). Rowe and colleagues (548) assessed decidual endothelial cells from normotensive and pre-eclamptic women. The levels of NO_x were significantly increased in pre-eclampsia. Shear stress had no effect on NO_x production, but cGMP increased fivefold. The conclusion was impaired vasodilatation in pre-eclampsia was not due to a lack of NO (548).

Nitric oxide production has also been reported as decreased in pre-eclampsia (449, 521, 549, 550). After women fasted for 12 hours, NO_x levels were significantly decreased in pre-eclampsia when compared to gestational age matched controls (521). Only when NO_x was measured in urine was it significantly decreased in pre-eclampsia. No difference was demonstrated in plasma NO_x or cGMP (549). A recent study (449) measured plasma and whole blood nitrite, sFlt-1 and sEng in 58 normal pregnant controls, 56 gestational hypertensive and 45 pre-eclamptic women. Whole blood nitrite was decreased by 36% in gestational hypertensive and 58% in pre-eclamptic women compared with controls (P<0.05). Plasma nitrite was decreased by 36% in both hypertensive pregnant groups (P<0.05). An increase in sFlt-1 and sEng occurred in pre-eclamptic women compared with gestational hypertensive women and controls (P<0.05). A negative correlation was demonstrated between antiangiogenic factors and nitrite concentrations. The authors suggested NO formation was impaired in pre-eclampsia and gestational hypertension and sFlt-1/sEng inhibit NO formation (449). Homocysteine is involved in oxidative stress and affects the endothelium. Plasma nitrite and nitrate levels were significantly decreased and levels of plasma homocysteine and ADMA were significantly increased in both mild and severe pre-eclampsia compared with normal pregnant women. A significant negative correlation occurred between NO metabolites and homocysteine ($r = 0.87$, $P < 0.0001$) and NO metabolites and ADMA ($r = -0.895$, $P < 0.0001$). The authors reported these results suggested NO, homocysteine and ADMA may be involved in the aetiology of and used as a marker for pre-eclampsia (550).

Stable levels of NO production have also been reported (433, 522, 523, 525, 551). No significant difference in maternal serum nitrite concentration was found when comparing normal pregnant and pre-eclamptic women (551). When nitrate and nitrite was collected

from a 24 hour urine collection, levels were found to be similar between non-pregnant, normal pregnant, gestational hypertensive, pre-eclamptic and essential hypertension pregnant women (523). No significant difference was demonstrated in plasma NO_x when comparing pre-eclamptic and normal pregnant women. When urinary NO_x was measured it was significantly reduced in pre-eclampsia (433). In women on reduced NO_x diets, cGMP and endogenous NO production was measured from plasma and 24 hour urine collection. In the first trimester cGMP but not NO_x was increased. In the third trimester, urinary NO_x was decreased in pre-eclampsia. There was no difference in plasma NO_x, plasma or urinary cGMP. The conclusion was either “another signal besides NO mediates” increases in cGMP and maternal vasodilatation in pregnancy, or the measure of NO is unreliable and support for decreased NO production was not demonstrated (522). No difference in plasma NO levels between non-pregnant, normal pregnant and pre-eclamptic women were demonstrated. Intra-platelet cGMP was decreased in pre-eclampsia compared with normal pregnant women (525). Conrad et al., Rowe et al. and Teran and colleagues all hypothesised that NO may be involved with abnormal action on the endothelium rather than changes in production of NO (522, 525, 548).

A number of reasons have been proposed to explain these differences in NO in pre-eclamptic women. It has been suggested low NO levels occur because of alterations in the L-arginine NO pathway such as a deficiency in the substrate L-arginine or one of its co-factors such as BH₄ or ionic calcium. Alternatively an increase in the naturally occurring NOS inhibitor ADMA or a decrease in eNOS activity may occur (3). High or unchanged levels of NO in pre-eclampsia may occur because of increased degradation of NO resulting in either oxidative stress (superoxide formation) or nitrative stress (peroxynitrite formation) which can alter vasodilatation and the activity and function of proteins (426).

1.15.2 The study of vasodilatation in pre-eclampsia.

A variety of methods have been employed to study endothelium dependent vasodilatation in pre-eclampsia. Early work on women investigated arteries in-vitro (263, 552-555).

Biopsy specimens from subcutaneous fat taken at caesarean in normal and pre-eclamptic women demonstrated less endothelial dependent relaxation (assessed by infusion of acetylcholine after drug induced pre-contraction) in the pre-eclamptic resistance arteries.

This was not totally attenuated by the NOS inhibitor L-NMMA (552). Indomethacin which determines the contribution of prostanoids to vasodilatation caused a decrease in tension in normal but not pre-eclamptic women suggesting a significant prostanoid component in the acetylcholine mediated relaxation. The authors suggested these results may be because of increased NOS activity in pregnant women (552). However it would suggest that the relaxation observed is not totally NO mediated. Endothelial independent relaxation was no different between groups. This work provided evidence for impaired endothelial relaxation in pre-eclamptic pregnancy (552).

Assessment of *in vitro* flow-mediated dilatation in normal and pre-eclamptic pregnancy was carried out in a seminal paper (263). Arteries from subcutaneous biopsies obtained either after gynaecological surgery (non-pregnant specimens (n=10)) or caesarean (normotensive (n=10) and pre-eclamptic (n=6) pregnancy) were pre-constricted with norepinephrine. The response to flow through these arteries was then assessed. In the normotensive controls, flow stimulus resulted in significant dilatation which was absent in the pre-eclamptic and non-pregnant controls. This work established that shear stress was a significant stimulus for vasodilatation in pregnancy. In the absence of flow L-NAME decreased the arterial diameters in all groups to a similar extent. This indicates no difference in basal NO across the groups. This work provided evidence for shear stress induced dilatation in normal pregnancy that was not present in pre-eclampsia (263).

Other work using resistance arteries taken at biopsy have also demonstrated a failure of shear stress mediated dilatation in women with pre-eclampsia with basal production of NO unchanged (553). In contrast no difference in vasodilatory response to bradykinin was demonstrated in myometrial arteries dissected from non-pregnant, normal pregnant and pre-eclamptic pregnancy (554).

Myometrial samples taken at caesarean from normal pregnant women were incubated with serum taken at 22 and 24 weeks from women with bilateral uterine artery notching. In women who eventually developed pre-eclampsia incubation with their plasma resulted in reduced endothelium dependent dilatation. This demonstrates plasma from subsequent pre-eclamptic women can alter vascular reactivity prior to the onset of the disease (555). All these reports (except one (554)) have demonstrated reduced vasodilatation in pre-eclampsia in resistance vessels in-vitro. Nevertheless they do not address whether resistance vessels in-vivo or conduit vessels in-vivo would react in the same way.

Techniques used to assess skin microvascular responses *in-vivo* that involve laser Doppler imaging and delivery of acetylcholine and sodium nitroprusside via iontophoresis (the introduction of substances into tissue via an electric current) have been shown to correlate significantly with brachial artery FMD (79). A study (556) using laser Doppler fluximetry to measure drug induced (acetylcholine) vasodilatation in skin vessels found an increased vasodilatory response in pre-eclamptic women compared with normotensive controls.

There was no difference in vascular smooth muscle response. The pre-eclamptic women in this group had a BMI of 30 ± 2.0 and were late pre-eclamptics (gestational age 35.6 ± 1.0) although comparable in size and gestational age to the normotensive pregnant women

(556). This group of women may represent a later form of pre-eclampsia influenced by maternal factors.

Using a similar technique pregnant women were studied longitudinally at 22, 26, 34 weeks and six weeks postpartum (557). In subsequent normal pregnancy the vascular response to acetylcholine was not significantly different at any gestation but was enhanced compared to postpartum values. Whilst there was no difference in baseline perfusion values between normotensive pregnancy (n=54) and women who developed pre-eclampsia (n=15), in the subsequently pre-eclamptic group a progressive increase in acetylcholine dilatory response occurred from 22-34 weeks which then decreased postpartum. The response to sodium nitroprusside was also enhanced showing a greater response than normal controls. Women with underlying medical conditions were excluded with no mention made of smoking status. Pre-eclampsia was diagnosed at 34.9 weeks, range 29-39 and BMI was not reported. This study demonstrated enhanced endothelial dependent and independent vasodilatation in women destined to develop pre-eclampsia. The authors felt this increase in dilatation from the two drugs in the subsequent pre-eclamptics represents changes at the smooth muscle level or a compensatory response of the endothelium to placental ischaemia (557).

Plethysmography was used to assess venous microvascular pressure. The authors (558) hypothesised that up regulation of biochemical markers of endothelial cell and neutrophil activation would increase venous pressure in pre-eclampsia. This was confirmed through a significant correlation. Compared to normal pregnant and non-pregnant women this provided evidence of impaired microvascular function in pre-eclampsia (558).

Resting peripheral blood flow using plethysmography of calf muscle in women with pre-eclampsia, pregnant chronic hypertension and normal pregnancy was measured longitudinally throughout pregnancy (16-36 weeks) (559). Blood flow increased progressively and significantly in normal and chronic hypertensive women. In women who later developed pre-eclampsia blood flow decreased progressively and significantly beginning on average 14 weeks before pre-eclampsia occurred. This suggests impairment of tissue perfusion. This group of women were not obese (pre-eclamptic BMI 25.8 ± 0.95 , no significant difference between groups) and were late onset pre-eclamptics (mean 34.1 ± 1.8) (559).

Plethysmography of the forearm was used to demonstrate a decreased vasodilatory response to acetylcholine in pre-eclamptic pregnancy. Normal pregnant women had increased baseline flow compared with non-pregnant women. The infusion of L-NMMA did not change the response in pre-eclampsia suggesting factors other than a NO decrease are involved in pre-eclamptic vasoconstriction. None of the women were obese and the mean gestational age for pre-eclampsia was 35 ± 2.0 weeks (560).

Physiological studies have been performed using various techniques on larger vessels. Veille and colleagues (561) assessed differences in Doppler ratios (systolic/diastolic (SD) and PI) before and after reactive hyperaemia which was induced by one minute blood pressure cuff occlusion. Their results showed increased ratios in women destined to develop pre-eclampsia. Williams and Kocer (562) used a similar technique to study systolic acceleration time as measured by pulsed Doppler ultrasound before and after reactive hyperaemia in women with and without pre-eclampsia. Systolic acceleration time was shorter in pre-eclamptic women (562). Both studies reported their work demonstrated

impaired endothelial dependent vascular reactivity (561, 562). The problem with these studies is the technique reflects post occlusion reactive hyperaemia and may be an indicator of shear stress but there is no evidence that it reflects endothelial dependent vascular reactivity.

In summary, reports using various methods to assess endothelial dependent and independent dilatation in pregnancy are conflicting. This may be due to the numerous techniques used. However studies using the same technique give differing results. More likely it is due to the heterogeneous pre-eclamptic groups studied.

1.15.3 Vascular function in women with a history of pre-eclampsia.

A number of studies have assessed vascular endothelial function in women who had pre-eclampsia either a number of months to many years prior. Using the FMD technique, women with a history of pre-eclampsia were more likely to have a metabolic syndrome profile (increased BMI, family history of hypertension and increased HDL to cholesterol ratio) than women with no history of pre-eclampsia (controls) (563). Women with previous recurrent pre-eclampsia had decreased FMD ($0.9 \pm 4.1\%$) compared with a single previous episode of pre-eclampsia (2.7 ± 3.5) versus controls (4.7 ± 4.3) ($P < 0.001$). When the data were reanalysed in women who were not obese and non-smokers, the same relationship held (previous pre-eclampsia 2.5 ± 3.2 versus control 4.6 ± 4.4 ; $P = 0.03$). These results indicated endothelial dysfunction in women with a history of pre-eclampsia that was not influenced by traditional cardiovascular risk factors (563).

Using laser Doppler perfusion imaging, endothelial dysfunction in the form of reduced microvasodilatation was demonstrated in a group of former pre-eclamptic women 15-25

years after the index pregnancy (564). The response to acetylcholine was also significantly reduced in these women compared to controls. The women in this study were similar in size and not obese.

Whilst basal forearm blood flow measured by plethysmography was similar in women with and without a history of pre-eclampsia, mental stress also resulted in impaired vasodilatation in a group of former pre-eclamptic women one year after pregnancy. When BMI was controlled for, the diagnosis of pre-eclampsia was no longer a significant predictor of reduced dilatation. None of the women were obese (565).

Another study (566) used plethysmography, acetylcholine and sodium nitroprusside in women with previous pre-eclampsia found attenuated vasodilatation from the administration of both endothelium dependent (acetylcholine) and independent (sodium nitroprusside) drugs in former pre-eclamptic women. In this study there was no interaction between BMI and endothelium dependent or independent vasodilatation.

In contrast, microvascular vasodilatation was increased compared with controls in a group of women with normal BMI who previously had pre-eclampsia (567). As this study assessed the microcirculation using laser Doppler perfusion imaging, the authors felt this was a compensatory response to possible large vessel endothelial dysfunction.

At least six months after pregnancy, endothelial function was assessed by changes in arterial forearm blood flow after arterial occlusion (568). No difference was demonstrated between former pre-eclamptic women and former normotensive pregnant controls. Endothelial function correlated negatively with BMI and biochemical indicators of

metabolic syndrome and positively with arterial compliance. This suggests metabolic syndrome impacts on endothelial function. The mean BMI in both groups were not in the obese range (former pre-eclamptic median [IQR]; 24[21.7-31.0] vs. controls 23.4 [21.7-24.1]).

When assessing non-obese women 11-27 months after delivery, decreased FMD and serum nitrites were reported when a history of severe pre-eclampsia or recurrent pregnancy loss was present (569). There was no significant difference in VEGF or sFlt-1 levels between the three groups.

A comparison of women with a history of severe pre-eclampsia (diagnosed at 26-34 weeks gestation with significantly smaller newborn weight) and former normal pregnant controls was done 15 ± 3 months postpartum to assess FMD, ambulatory blood pressure and plasma metabolic factors (570). Neither group was obese during pregnancy or postpartum (BMI ≤25, both groups, both times). A significant decrease in FMD was demonstrated in the former pre-eclamptic women. In women with a history of pre-eclampsia no correlation between FMD and plasma markers of endothelial function was found. These women had higher ambulatory blood pressure and insulin levels.

In a study performed two to three years after pregnancy, former pre-eclamptic women had increased arterial stiffness (measured by augmentation index) and decreased FMD compared with controls (never pregnant or normotensive pregnancies) (571). In this non-obese group no significant correlation was found between augmentation index and FMD in terms of age, BMI, blood pressure, heart rate or metabolic parameters.

Recent work has demonstrated reduced FMD only in women with a history of early onset pre-eclampsia (FMD mean \pm SEM) (3.25 ± 0.7) or those who had delivered a growth restricted baby (2.14 ± 0.44) ($P < 0.0001$). This is compared to late onset pre-eclampsia and normal controls. A history of late onset pre-eclampsia resulted in a FMD measure similar to normal controls (7.93 ± 1.33 vs. 9.14 ± 0.9). Interestingly women with a BMI > 30 were excluded (572).

In summary, some but not all studies support the notion that a history of pre-eclampsia results in reduced vasodilatation many months to years after the event and this may be one reason why these women are at increased risk of cardiovascular disease in later life.

1.16 The assessment of endothelial function by flow-mediated dilatation in pre-eclampsia.

Hypertensive disorders of pregnancy have been assessed by the ultrasound technique of FMD both when the syndrome is present and to try and predict pre-eclampsia. Presence of the disorder (pre-eclampsia) and prediction will be dealt with separately. Table 1.2 summarises the studies which were performed when the diagnosis of pre-eclampsia or other hypertensive disorders of pregnancy was made. At the time this thesis was commenced only three studies had been published assessing FMD in pre-eclampsia (532, 573, 574) and none of these compared FMD in pre-eclamptic women with gestational hypertensive women.

Table 1.2: Flow-mediated dilatation (FMD) in pre-eclampsia and other hypertensive disorders of pregnancy ($m \pm SD$) or m [IQR] (n).

Author (year)	Pre-eclampsia FMD%	HT of pregnancy FMD%	Normotensive pregnancy FMD%	GA at study: weeks (range),	Body mass index (kg/m^2)	Pre-eclampsia definition
Yoshida et al. (1998) (532)	$7.2 \pm 3^*$ (22)	N/A	18.9 ± 3.4 (18)	PE: 36 ± 3.3 NP: 35.8 ± 3	N/R	ACOG 1996
Yoshida et al. (2000) (573)	$7.9 \pm 3^{*\dagger}$ (22)	13.9 ± 2.2 (15) (chronic HT)	17.4 ± 4.2 (58)	PE: 35.5 ± 3.7 HT: 27.1 ± 7.6 NP: 26.1 ± 8	N/R	ACOG 1996
Takata et al. (2002) (574)	$2.3 \pm 3.7^*$ (25) (mild) $1.6 \pm 3.8^*$ (27)(severe)	N/A	5.0 ± 3.2 (32)	Mild: 32.6 ± 4.1 Severe: 32.3 ± 2.7 NP: 32.2 ± 3.9	All ≤ 25.9	Mild: BP $>140/90$ mmHg & 1+ protein Severe: BP $>160/110$ mmHg or protein $>5g/24$ hrs
Kuscu et al. (2003) (575)	$4.26 \pm 0.69^*$ (15)	N/A	12.18 ± 1.97 (11)	PE: 33.9 ± 1.4 NP: 36.3 ± 0.6	N/R	BP $140/90$ mmHg & 2+ protein
Yamamoto et al. (2005) (534)	$106 \pm 2.7^*$ (15)	N/A	115 ± 6.5 (20)	PE: 34 ± 3 NP: 33 ± 3	N/R	NHBPWG 1990
Brodzki et al. (2008) (576)	$9.5 \pm 5.3^*$ (20) (Ut art notches) 11.6 ± 5.4 (14) (No notch)	N/A	13.4 ± 4.0 (23)	PE: 27-38 NP: 25-27	N/R, no significant differences between groups	DBP >90 mmHg x2 hrs apart or \uparrow DBP >20 mmHg & protein >300 mg/l
Filho et al. (2010) (577)	13.6 [4.4-17.1] (14)	6.0 [1.9-10.3] (13) SPE	N/A	PE: 35.4 ± 3.3 SPE: 32.0 ± 4.4	PE: 34.4 SPE: 31.4	NHBPWG 2000
Yamamoto et al. (2010) (53)	$109.3 \pm 5.7^*$ (20)	N/A	114.8 ± 6.3 (20)	PE: 33 ± 3 NP: 34 ± 3	N/R	BP $\geq 140/90$ mmHg & protein >300 mg/24hrs
Mori et al. (2010) (578)	$3.8 \pm 2.0^*$ (15)	N/A	10.6 ± 6.4 (17)	PE: 29.3 ± 1.0 NP: 30.5 ± 1.1	PE: 23.3 ± 2.5 NP: 22.3 ± 2.3	BP >140 mmHg or >90 mmHg & protein ≥ 30 mg/100ml
Adali et al (2011) (579)	$8^* \S$ (35)	N/A	$15 \S$ (35)	PE : 33 ± 0.5 NP: 33.5 ± 0.5	PE: 27.5 ± 0.5 NP: 26.7 ± 0.4	ACOG 2002

HT, hypertension; PE, pre-eclampsia; NP, normotensive pregnancy; SPE, superimposed pre-eclampsia; BP, blood pressure; DBP, diastolic blood pressure; N/A, not applicable; N/R, not reported; ACOG, American College of Obstetrics & Gynecology; NHBPWG, National high blood pressure working group; ut art, uterine artery. *significantly decreased compared to normotensive controls. †significantly decreased compared to chronic hypertension. § SD not reported.

There are many variations in the FMD technique reported and different definitions of pre-eclampsia used. Not all the studies used a technique that solely reflects NO mediated endothelial function. It is recommended the brachial or radial artery is imaged (17) and all studies met these criteria. A variety of blood pressures were used to occlude arterial flow and induce reactive hyperaemia. As they were all well above systolic pressure (and similar to those documented in Table 1.1) this would not present a problem. Different blood pressure occlusion times were employed. Research on this variable has demonstrated an occlusion time of less than 5 minutes is insufficient to produce significant dilatation (129). The brachial artery was measured post occlusion at different time points, with some researchers measuring at fixed times. Research has shown measuring at a fixed time may not represent maximum dilatation (100) and under measuring of FMD may occur. Upper arm cuff occlusion was the technique of choice in seven studies (53, 532, 534, 573, 575, 576, 579), the forearm in two (574, 578) and not stated in one (577). As only forearm occlusion represents a solely NO mediated technique (130), upper arm occlusion presents a problem with these studies.

Smoking is known to affect endothelial function (as detailed in an earlier section) although its effect on FMD in pregnant women had not been described at the time this thesis was commenced. The majority of studies excluded smokers. One study made no mention of smoking status (575) and one reported enrolling smokers which was controlled for as a covariate in the analysis (576).

The effect of vasoactive medication on endothelial function is varied (as discussed earlier) although its effect on FMD assessment of endothelial function in pregnancy is not known. All studies except one (577) excluded women on medication and it was not stated in three

(532, 575, 576). Women with medical conditions were also excluded in most studies (no mention was made in two) (532, 575). One study (573) recruited women with chronic hypertension. Body mass index was rarely reported (Table 1.2) which is disappointing considering obesity is considered a risk factor for pre-eclampsia.

A study that talks about measuring FMD but is actually measuring the time taken to obtain a 50% reduction in shear stimulus as a measure of endothelial function (a technique that is not referenced or validated as such a measure) (580) will not be discussed.

Endothelial function in pre-eclampsia was first assessed with the technique of FMD by Yoshida and colleagues (532) in a letter to the editor. Their aim was to assess FMD non-invasively in pregnant women using a 30MHz transducer with the radial artery and upper arm occlusion pressure of 30mmHg above systolic. Blood pressure occlusion time was five minutes and post occlusion maximum diameter was measured within a six minutes monitoring period. They demonstrated that FMD was significantly reduced in women with pre-eclampsia compared with normotensive pregnant controls ($p < 0.001$) (Table 1.2). The American College of Obstetricians and Gynecologists (ACOG) hypertension in pregnancy technical bulletin (581) was referenced for the pre-eclampsia criteria. Mild pre-eclampsia was defined by a BP of 140/90mmHg and proteinuria ≥ 300 mg/24 hours. Severe disease was systolic BP > 160 -180mmHg, diastolic BP > 110 mmHg, proteinuria > 5 g/24 hours, end organ involvement or IUGR. Gestational age at time of study was well matched with this group representing the late form of pre-eclampsia (> 34 weeks) (Table 1.2). No information was presented on the shear stress stimulus (532). In summary, FMD was reduced in a group of late onset pre-eclamptic women compared with normotensive controls.

Flow-mediated dilatation was compared between pre-eclamptic, pregnant chronic hypertensive and normotensive pregnant women with the intention of assessing the relationship between vasodilatation and plasma fibronectin, a marker of endothelial injury (573). Upper arm five minute occlusion with pressures 30mmHg above systolic and scanning of the radial artery with maximum post occlusion diameter after six minutes of monitoring were used to assess FMD. The ACOG 1996 criteria (581) for pre-eclampsia was used. Radial artery dilatation was decreased in the pre-eclamptic women compared to the normotensive ($P<0.001$) and chronic hypertensive women ($P<0.001$). Flow-mediated dilatation was also significantly reduced in the chronic hypertensive women compared to normotensive women ($P<0.001$) (Table 1.2). A significant negative correlation was seen between FMD and plasma fibronectin in all groups although the strongest correlation was between FMD and pre-eclampsia ($r = 0.77$, $P<0.001$). The authors (573) concluded this provided evidence of endothelial dysfunction in pre-eclampsia. No significant difference in baseline artery diameters was found between the groups. Shear stress stimulus was not reported. There was a significant difference in gestational age between the three groups (Table 1.2) (573). As FMD is either unchanged or increased in normal pregnancy (see previous section) and ideally comparisons in terms of early and late pre-eclampsia would be made, this would be a limitation of this study. In conclusion this study demonstrated decreased FMD in late onset pre-eclampsia compared with chronic hypertensive pregnant women and normotensive pregnant women who were assessed around 10 weeks earlier than the pre-eclamptic women.

A cross-sectional study was performed to compare endothelial function in mild and severe pre-eclamptics and assess blood flow through the uterine and ophthalmic arteries with pulsed Doppler (574). The brachial artery was scanned with only two minutes of forearm

occlusion at 200-250mmHg and maximum post occlusion diameter was measured at maximum dilatation within the three minutes of post occlusion monitoring. Rather than reporting volume flow and reactive hyperaemia as a measure of shear stress, brachial artery pulsatility index was used. This would give an indication of downstream resistance rather than the shear stimulus. The authors (574) concluded that FMD was significantly decreased in mild and severe pre-eclampsia compared to normotensive controls (Table 1.2). Ultrasound of the uterine, ophthalmic arteries and FMD helped to differentiate between degrees of pre-eclampsia severity. This paper reports very low FMD values which may be a result of the short occlusion time. In both pre-eclamptic groups the standard deviations were greater than the mean (Table 1.2) suggesting the data were not normally distributed and non-parametric tests for multiple comparisons would have been more appropriate than the reported analysis of variance (ANOVA). Pre-eclampsia diagnostic criteria are detailed in Table 1.2. Mild pre-eclampsia included BP >140/90mmHg. The mean diastolic BP in the mild group was 85 ± 10 suggesting some women in this group did not meet the criteria for hypertension. The gestational age at time of study was well matched in all groups (around 32 weeks) consistent with early onset pre-eclampsia. The BMI was not in the obese range (Table 1.2) suggesting less impact from maternal constitutional factors. This study reports decreased FMD in early onset pre-eclampsia in women who were not obese, suggesting limited impact from maternal metabolic factors.

In a small number of women endothelial function was assessed in pre-eclamptic and gestational age matched normotensive controls (Table 1.2) (575). The pre-eclampsia definition is described in Table 1.2. The brachial artery was scanned with upper arm occlusion at pressures of 250-300mmHg for three minutes. Post occlusion artery diameter

was measured at 15, 30, 60, 120 and 180 seconds and the mean calculated, rather than use maximum diameter. Artery diameters were measured using colour Doppler (an unusual method). Shear stress stimulus was not reported. Gestational ages were not different, tending towards late onset pre-eclampsia. The pre-eclamptic women only were re-examined at two and six weeks postpartum. Pregnancy FMD was significantly decreased in pre-eclampsia compared to normal pregnant controls ($P=0.003$) (Table 1.2) and increased by six weeks postpartum. Statistical analysis on the pre-eclampsia group used ANOVA when a repeated measures analysis would have been more appropriate. In summary this work demonstrated decreased FMD in late onset pre-eclamptic women compared to normotensive pregnancy.

Flow-mediated dilatation and cGMP were measured to assess if both were decreased in pre-eclampsia (534). The radial artery was scanned with upper arm occlusion set at a pressure of 50mmHg above systolic with five minutes of occlusion. Maximum arterial diameter was reported to occur at 60 seconds although data was collected for six minutes. The National High Blood Pressure Working Group (NHBPG) (1990) report (582) was used for pre-eclampsia diagnosis. This is hypertension of $>140/90$ mmHg and proteinuria 1+ on dipstick or ≥ 300 mg/24 hours after 20 weeks that regresses after delivery. The FMD study (534) demonstrated significantly reduced vasodilatation in pre-eclamptic women compared with normotensive pregnant women ($P=0.049$) (Table 1.2). There was no significant difference between pre-eclamptics and non-pregnant women ($P=0.75$). Platelet cGMP was significantly increased in normal pregnant compared with non-pregnant women with no difference between normal pregnant and pre-eclamptics. Sodium nitroprusside increased cGMP in all samples. The increase in cGMP after sodium nitroprusside was higher in normal pregnancy compared with non-pregnant and no different between pre-

eclamptics and normal pregnant women. In normal pregnancy this study demonstrates that cGMP and smooth muscle cell dilatation is increased significantly and the dilatation is probably because of increased cGMP and its action on smooth muscle. Therefore the reduced FMD in pre-eclampsia was due to reduced action of NO rather than reduced production of NO (534). In conclusion this study demonstrates reduced FMD in late onset pre-eclampsia compared with normotensive pregnant women.

Women with pre-eclampsia were recruited at the time of diagnosis (mean 33 weeks) for a study on FMD, assessment of uterine artery flow and vascular mechanical properties (arterial stiffness) in the maternal aorta, common carotid and popliteal arteries (576). Histology of the placenta was evaluated after delivery. Normotensive pregnant women were recruited at their 18-20 week fetal anomaly scan and then tested between 25-27 weeks as above. Pre-eclampsia was defined as a diastolic BP >90mmHg with 1+ of protein, each measured twice four hours or more apart with proteinuria >300mg/L in two specimens or an increase in diastolic BP >20mmHg. For the FMD test, the brachial artery was scanned using upper arm occlusion for four minutes at occlusion pressures between 200-250mmHg. Post occlusion artery diameter was measured at 60 and 120 seconds. The 60 second data are presented (Table 1.2) as these did not differ significantly from the 120 second data. Pre-eclampsia data on FMD was presented as women with and without bilateral uterine artery notching. Unfortunately subject demographics and outcome data were only presented as either pre-eclamptic or normal pregnancy results. This limits interpretation of the data when trying to evaluate the differences between the three groups. This study demonstrated that bilateral uterine artery notching with pre-eclampsia was associated with significantly decreased FMD compared to normotensive controls (P=0.01) (Table 1.2). Adjustment for MAP and basal vessel diameter strengthened the association.

Shear stress stimulus and actual BMI were not reported although it was stated there was no difference in BMI between groups. There was no difference in arterial stiffness between women with pre-eclampsia and controls. Significantly more placental ischaemic lesions were seen in women with pre-eclampsia and bilateral uterine artery notches (13/15) than in pre-eclampsia without notching (5/13) ($P=0.02$). Women with pre-eclampsia and no notching also had a trend towards higher FMD than the notched group (not significant). Flow-mediated dilatation in the no notching pre-eclampsia group was not significantly different to the normotensive controls (576) (Table 1.2). Whilst the pre-eclampsia definition used may include some women without pre-eclampsia, the difference in FMD and placental histology in the two pre-eclampsia groups suggests different forms of pre-eclampsia (placental ischaemia induced or maternal metabolic syndrome related disorder) and may explain the difference in FMD in the two pre-eclamptic groups. In summary this study demonstrated reduced FMD in women with pre-eclampsia, bilateral uterine artery notching and placental ischaemic lesions compared with pre-eclamptic women without notching and normotensive women.

Another study compared FMD in women with pre-eclampsia to women with pre-eclampsia superimposed on chronic hypertension (SPE) (577). The aim was to assess if FMD could differentiate between the two groups. The NHBPWG 2000 pre-eclampsia criteria were used which is BP $>140/90$ mmHg after 20 weeks gestation and proteinuria >300 mg/24 hours or 1+ on dipstick (253). The brachial artery was scanned with the BP cuff applied to the arm (position not stated, although the Celermajer et al. paper (16) is referenced for the technique so it is probably forearm occlusion) for five minutes at pressures 20-30mmHg above systolic. Post occlusion brachial artery diameter was measured at 60 ± 30 seconds at end-diastole. No information on the shear stimulus was provided. Patient medication was

not withdrawn and no information was provided on the number medicated. No significant difference in FMD was found between the two groups ($P=0.08$) probably due to small numbers, although FMD in the pre-eclamptic women was higher than the SPE women (Table 1.2). The groups were not matched for maternal age, number of prior pregnancies, gestational age at time of study, maternal weight, and previous history of pre-eclampsia, placental weight at birth and umbilical artery vascular resistance. The pre-eclamptics were younger (24.5 ± 4.7 vs. 29.3 ± 6.0), heavier and presented at a later gestational age (mean >34 weeks) (Table 1.2). Calculation of BMI (Table 1.2) (only height and weight were presented) put both groups in the obese class I range according to the WHO criteria for BMI classification (341). As obesity is a risk factor for pre-eclampsia, this suggests these women may be presenting with the late onset maternal disease type of pre-eclampsia (577). The pre-eclampsia FMD results are also similar to those obtained by Brodzki and colleagues (576), that is a higher FMD in the group that may be presenting as late onset maternal disorder influenced pre-eclampsia. In conclusion this study demonstrates FMD cannot be used to differentiate between women with pre-eclampsia and SPE.

Flow-mediated dilatation and prostacyclin (a vasodilator) concentrations were measured in non-pregnant, normotensive pregnant and pre-eclamptic women (53). Flow-mediated dilatation was assessed as per the previously mentioned Yamamoto and colleagues paper (534). Shear stress stimulus and BMI were not reported. Pre-eclampsia was defined according to the Japan Society of Obstetrics and Gynecology, that is blood pressure $>140/90$ mmHg and proteinuria >300 mg/24 hours (53). The women were matched in terms of age, gestational age at time of study and parity. The level of prostacyclin in plasma was reduced in pre-eclamptic women possibly due to decreased production. In summary, FMD at 33-34 weeks gestation was significantly reduced in pre-eclamptic women compared to

normotensive pregnant women ($P < 0.05$) (Table 1.2) with no correlation between FMD and prostacyclin ($R^2 = 0.071$, $P = 0.085$).

Due to the association between pre-eclampsia, obesity and metabolic syndrome a study was performed to assess the relationship between adipocytokines, metabolic syndrome related parameters and endothelial function (578). Pre-eclampsia criteria are detailed in Table 1.2. After 10 minutes of rest and using a 10MHz transducer, the brachial artery was scanned. A forearm positioned blood pressure cuff was inflated to 250mmHg for five minutes and post occlusion arterial diameter was measured at 60 seconds from the mean of four measurements. The women were non-smokers with no medical conditions and did not take alcohol, caffeine or medication that affected lipoprotein metabolism. The normal pregnant women were matched with the pre-eclamptics by maternal and gestational age, BMI and weight gain in pregnancy. There was no difference in basal brachial artery diameter and basal blood flow between the two groups. Flow-mediated dilatation was significantly decreased in pre-eclampsia compared to normotensive women ($P < 0.001$) (Table 1.2). Adiponectin was decreased and other cytokines (leptin, PAI-1, IL-6, VCAM-1, E-selectin and CRP) were increased in the pre-eclamptic women. This was reported as a similar profile to that seen in metabolic syndrome. Plasma adiponectin correlated negatively with BMI ($r = -0.5$, $P < 0.05$) and body weight gain in pregnancy ($r = 0.63$, $P < 0.01$) and positively with FMD ($r = 0.5$, $P < 0.05$). In this sample of women weight gain was associated with decreased adiponectin and endothelial dysfunction. The women however were not overweight (Table 1.2) and on average did not gain excessive weight (5.5 ± 3.9 kg weight gain in normal pregnancy and 8.1 ± 3.6 kg in pre-eclampsia). They were also an early onset pre-eclampsia group (Table 1.2). It is interesting how these researchers are studying obesity in pregnancy in Japan when their women are consistently

among the slimmest. This study would be very interesting if it was replicated in obese women. In conclusion this study demonstrates reduced FMD in early onset pre-eclamptic women who were not obese, although a relationship between FMD and a metabolic syndrome profile was demonstrated even in this early onset group.

A cross-sectional study was performed to assess the relationship between CRP and FMD in pre-eclampsia compared with normotensive pregnant women (579). The brachial artery was assessed with five minutes of upper arm occlusion and 250-300mmHg of cuff pressure. Maximum post occlusion arterial diameter was measured at 60 seconds. The ACOG 2002 definition for pre-eclampsia was used. This is BP \geq 140/90 after 20 weeks gestation in previously normotensive women with proteinuria of \geq 300mg/24 hours or 1+ protein (583). The groups were well matched for gestational age at time of study and BMI with none in the obese range (Table 1.2). The women had no pre-existing medical conditions, were non-smokers and had no medical conditions. This work demonstrated FMD was significantly decreased in pre-eclamptic women compared with the controls ($P<0.01$) (Table 1.2) although SD was not reported. Flow-mediated dilatation was inversely correlated with CRP ($r = -0.436$, $P<0.01$). Around a third of the women with pre-eclampsia had uterine artery notching (11/35). When the pre-eclamptic women were grouped according to normal or abnormal uterine artery Dopplers, FMD was significantly reduced in the abnormal group (FMD of 2 versus 10, $P<0.01$; SD not reported). Shear stress stimulus was not reported. In summary this work showed decreased FMD in non-obese pre-eclamptic women compared to normotensive pregnant women which was significantly correlated with CRP (579).

The above reviews demonstrate that not only are there variations in the FMD methodology and pre-eclampsia criteria used to recruit pre-eclamptic women, but there may also be differences in the sample populations. Differences in the FMD technique make exact comparisons between studies difficult. Variations in pre-eclampsia criteria means high specificity in the pre-eclamptic groups (245, 248) may not be obtained with some women without the disease recruited into the study population. Some studies have recruited pre-eclamptics at an early, late or mixed gestational age or not stated exactly the time frame. In other groups maternal constitutional factors such as obesity and metabolic syndrome may be involved although BMI was rarely reported. Pre-eclampsia is a multifactorial syndrome and endothelial dysfunction in different populations of pre-eclamptic women may present in a variety of ways.

The technique of FMD has also been used to attempt to predict pre-eclampsia. The following section will deal with those studies.

1.17 Flow-mediated dilatation as a predictive or screening test for pre-eclampsia.

A screening test should be simple and inexpensive, have high specificity and sensitivity with a high positive predictive value (false negatives and false positive should be kept to a minimum). The test should also be convenient, painless and not result in unacceptable morbidity. Testing should be able to be applied to a large number of asymptomatic people, with the disease detectable in the pre-clinical phase. The disease should have a high prevalence in the population of interest and by performing the screening test morbidity and mortality from the disease should be reduced (584). To be able to predict pre-eclampsia a screening test should be able to classify asymptomatic women as either likely to develop or not develop the disorder at a later time (585).

The search for a screening test for pre-eclampsia has been the subject of research over many years and was the subject of a recent systematic review (586). In general screening tests for pre-eclampsia have poor results and although some have high specificity, the sensitivity is low. The only tests with reasonable specificity were BMI \geq 34 with a pooled estimates of sensitivity of 18% (95% CI, 15 to 21) and specificity of 93% (95% CI, 87 to 97), Doppler ultrasound demonstrating bilateral notching of the uterine arteries (sensitivity 48% (95% CI, 34 to 62) and specificity (92% (95% CI, 87 to 95)) and maternal serum α -fetoprotein (sensitivity 9% (95% CI, 5 to 16) and specificity 96% (95% CI, 94 to 98)) (586). Another systematic review assessing uterine artery Doppler found using diastolic notching with an increased PI at >16 weeks gestation was the best predictor for pre-eclampsia and IUGR in low risk patients with a likelihood ratio (LR) for pre-eclampsia of 7.5 (95% CI, 5.4 to 10.2) and for IUGR LR 9.1 (95% CI, 5 to 16.7). The LR improved in high risk populations (587).

Six studies have been published using the technique of FMD to try and assess if the endothelial dysfunction of pre-eclampsia occurs before the clinical disease (353, 588-592) and are described and summarised in Table 1.3. Similar to the work where FMD was assessed when the clinical disease was present, these six papers have used variations of the FMD technique, a variety of pre-eclampsia diagnostic criteria and a mixed group of women for recruitment.

Table 1.3: Flow-mediated dilatation as a predictive test for pre-eclampsia.

<i>Author (year)</i>	<i>Study type</i>	<i>Pre-eclampsia FMD% (n)</i>	<i>HT of pregnancy FMD% (n)</i>	<i>Normotensive pregnancy FMD% (n)</i>	<i>GA at study: weeks -range, (m ± SD), median [IQR]</i>	<i>Body mass index (kg/m²)</i>	<i>Pre-eclampsia definition</i>
Savvidou et al. (2003) (353)	Cross-sectional (prospective)	3.58 ± 2.76† (10) (Ut art notches)	N/A	8.15 ± 4.32 (19) (Ut art notch) 8.59 ± 2.76 (43) (No notch)	PE: 24 [24-25] NP: 24 [24-25] (Ut art notches) NP: 23 [23-25] (No notch)	N/R	ISSHP 1988
Takase et al. (2003) (588)	Cross-sectional (prospective)	1.6 ± 1.0* (9)	N/A	11 ± 4.5 (34)	PE: 29 ± 3 NP: 29.3 ± 3	N/R	NHBPWG 2000
Garcia et al. (2007) (589)	Cross-sectional (prospective)	13.4 ± 4.3* (14)	16.3 ± 5.7 (18) (GH)	18.2 ± 7.2 (64)	PE: 21.4 ± 5.8 GH: 21.9 ± 6.7 NP: 21.5 ± 5.8	PE: 25.8 ± 4.5 GH: 25.5 ± 4.6 NP: 25.2 ± 4.3	NHBPWG 2000
Savvidou et al. (2008) (590)	Cross-sectional (retrospective)	2.6 ± 2.3* (5) (PE + SGA) 3.8 ± 3.2* (7) (PE, no SGA)	N/A	8.4 ± 2.8 (40)	23-25	PE: 28 ± 4.5 NP: 28 ± 5.6	ISSHP 1988
Kamat et al. (2009) (591)	Longitudinal (prospective) (assessed x2)		FMD 1: 6.72 ± 3.24* (25) FMD 2: 6.04 ± 4.37* (25)	FMD 1: 20 ± 9 (56) FMD 2: 25.12 ± 10.9	FMD 1: 18-24 19.6 ± 1.42 FMD 2: 28-34 29.2 ± 1.24	N/R	N/R
Noori et al. (2010) (592)	Longitudinal (prospective) (no. assessed median [IQR]) 5 [2-6]	8.84 ± 1.52‡ (11) (term PE) 8.48 ± 1.42‡ (10) (Preterm PE) (10-17 weeks)	12.93 ± 0.98‡ (10) (GH) (10-17 weeks)	“Intermediate between GH & PE” ~11‡ (128) (10-17 weeks)	10-17 18-25 26-33 34-40	Term PE: 25.8 ± 2.1 Preterm PE: 28.5 ± 1.9 GH: 28.1 ± 1.5 NP: 24.6 ± 0.4	NHBPWG 2000

HT, hypertension; PE, pre-eclampsia; NP, normotensive pregnancy; GH, gestational hypertension; SPE, superimposed pre-eclampsia; N/A, not applicable; NR, not reported; max, maximum; ut art, uterine artery; NHBPWG, National high blood pressure working group; ISSHP, International society for the study of hypertension in pregnancy; *significantly decreased compared to normotensive controls; †significantly decreased compared to normotensive controls with and without uterine artery notch. ‡significant difference between groups. ~approximation made by author from graph in original paper.

When using the FMD technique for prediction purposes, forearm occlusion was used in all studies except one where it was not stated (591). The brachial artery was scanned and blood pressure occlusion was well above systolic in all cases. Five minute occlusion time was used in all except one study (588) where one minute occlusion time was reported. Only one study (592) reported measuring maximum brachial artery dilatation post reactive hyperaemia, the other studies measured between 60-65 seconds.

All studies except two (589, 591) included women who smoked. Women on medication were not recruited, although this was not reported in one study (592). Healthy women with no medical conditions or history of cardiovascular disease were recruited in two studies (353, 590). Two reported including women with a family history of pre-eclampsia or hypertension (588, 589) plus a history of other medical disorders (588). Women with pre-existing medical conditions were recruited to increase the pre-eclampsia rate (592).

The pre-eclampsia definitions were less varied in this group of studies using either the ISSHP 1988 criteria (243) or the NHBPWG 2000 (253) criteria. One study did not state the pre-eclampsia definition used (591).

One of the first papers on the subject of FMD and prediction was a cross-sectional paper (353) whose aim was to investigate if endothelial dysfunction (decreased FMD) and increased ADMA (an endogenous NOS inhibitor) preceded the clinical syndrome of pre-eclampsia. The brachial artery was scanned using five minutes of forearm occlusion at 300mmHg with post occlusion diameter measured at 45-65 seconds. The pre-eclampsia criteria used was the 1988 ISSHP (243). The criteria are diastolic BP ≥ 110 mmHg or diastolic BP ≥ 90 mmHg measured twice four hours apart and proteinuria ≥ 300 mg/24 hours

or 2+ protein. Forty-three women with bilateral uterine artery notching and 43 controls with no notching were recruited and scanned only once between 23-25 weeks gestation (Table 1.3). The women were then divided into four groups, no notch-normal outcome, notch-normal outcome, notch with IUGR and notch with pre-eclampsia. Flow-mediated dilatation in the IUGR group was decreased (6.17 ± 2.82) compared to the no notch-normal group (Table 1.3). The pregnant control group was matched by maternal age, ethnicity and smoking status. Body mass index was not reported. In the pre-eclamptic group FMD was significantly decreased compared to normal controls with and without notches ($P < 0.001$) (Table 1.3). Plasma ADMA was significantly increased in the pre-eclamptic women with an inverse correlation between FMD and ADMA ($r = -0.8, P = 0.005$). This suggests endothelial dysfunction in women destined to develop pre-eclampsia. There was a greater percentage of smokers in the pre-eclampsia group ($n=3$ (30%)) compared with the no notch-normal ($n=7$ (16%)) and notch-normal ($n=2$ (11%)) outcome groups although this was not significant. The authors report that smoking was a significant predictor of FMD (along with baseline vessel size and pregnancy outcome) with adjustment for smoking status made. The significant difference in FMD remained. The r-values for smoking and FMD were not reported so the correlation and relationship of the variables cannot be assessed. As smoking is known to effect endothelial function (177) this information would have been of interest. There was no significant difference in baseline artery diameter, blood flow or reactive hyperaemia across the groups. There are a number of limitations in this study (353), one is the smoking issue. The number of pre-eclampsics assessed was small and the SD of the pre-eclampsia FMD was large. There was also a broad scatter in the raw scores for all four groups with overlap of the data which would limit the suggested use of FMD as a screening tool for pre-eclampsia. Outcome data showed that the pre-eclamptic group were neither an early or late group in terms of gestational age at delivery

(mean 34.8 ± 3.5 weeks) compared to the other three groups who delivered at or near term. The babies born to mothers with pre-eclampsia were significantly smaller (2074 ± 723 g) than the no notch-normal outcome group (3326 ± 460). Corrected birth weight percentiles were not reported. This study demonstrates FMD was significantly reduced in women destined to develop pre-eclampsia compared to normotensive pregnant women with and without uterine artery notching.

Another study was performed to investigate whether FMD and measures of reactive hyperaemia could predict pre-eclampsia (588). A forearm blood pressure cuff was inflated to 30mmHg above systolic pressure for only one minute and the brachial artery was scanned with post occlusion diameter measured at 60 seconds after cuff release. The NHBPWG 2000 criteria were used for the diagnosis of pre-eclampsia (253). Forty-three women were screened at around 29 weeks after being recruited with risk factors for pre-eclampsia. These risk factors included primigravida, a past or family history of pre-eclampsia, diabetes and other cardiovascular, autoimmune or renal diseases, smoking, ≥ 35 years of age and multiple pregnancy although all were purported to be healthy at recruitment. Smoking is an interesting “risk factor” as multiple work has demonstrated smoking reduces the risk of pre-eclampsia (519). Of the 43 women, nine developed pre-eclampsia and the remaining were normotensive controls (588) (Table 1.3). Fourteen non-pregnant controls were also studied during menstruation. Flow-mediated dilatation was calculated as the percentage increase in diameter, blood flow was calculated with pulsed Doppler using the PI and resistance index (RI) ($RI = \frac{\text{peak systolic velocity} - \text{peak diastolic velocity}}{\text{peak diastolic velocity}}$) (588). There was a significant reduction in FMD in the women who subsequently developed pre-eclampsia compared with the normotensive women ($P=0.05$) (Table 1.3). This study (588) unlike the previous one discussed (353)

found no difference in ADMA (or endothelin-1) levels between the pregnant groups. There was no significant difference in baseline artery diameter, basal or post occlusion PI or RI. The authors calculated $\geq 3.0\%$ FMD value as a cut off for normality, so values less than this would give a positive predictive value (PPV) of 90% and a negative predictive value (NPV) of 100% (588). One limitation of this interpretation is this may only hold true in a population with risk factors for pre-eclampsia and not the general pregnant population. Other limitations are the one minute BP cuff occlusion time and the small number of pre-eclamptics (n=9). This group of women would also represent the late pre-eclampsia with a normally grown fetus. Both pre-eclamptic and normotensive women delivered at term (normotensive: 39 ± 2 , pre-eclamptic: 38 ± 1 weeks; not significant) and had similar birth weights (normotensive: 3281 ± 224 , pre-eclamptic: 3290 ± 190 grams; not significant). The maternal BMI was not reported. In summary this study demonstrates women with pre-existing risk factors, destined to develop late onset pre-eclampsia have reduced FMD.

Subsequent normotensive, pre-eclamptic and gestational hypertensive women were compared at an earlier gestation (Table 1.3) than the preceding studies. The purpose was to assess if decreased FMD preceded pre-eclampsia in these groups and if this was related to an inflammatory process as measured by CRP and leukocyte count (589). Diagnosis of pre-eclampsia was made according to the NHBPWG 2000 report (253). The brachial artery with five minutes of forearm occlusion set at a pressure of 300mmHg was used with post occlusion diameter measured at 60 seconds. From the 506 normotensive women enrolled, two controls with normal outcomes for every subsequently hypertensive woman were selected for analysis (Table 1.3), matched by BMI, gestational age and maternal age. No significant difference was demonstrated in FMD, CRP and leukocyte count between the normotensive and subsequent gestational hypertensive women at enrolment (589).

There was also no difference in basal blood flow and reactive hyperaemia in women who later developed pre-eclampsia although they had significantly decreased FMD (Table 1.3) and increased concentrations of CRP and leukocytes compared with the control group. The authors felt this supported the theory that pre-eclampsia is an inflammatory condition. No correlation between FMD and inflammatory markers were found. More of the women who developed pre-eclampsia had a history of prior pre-eclampsia or family history of hypertension. The women were not obese (Table 1.3) and most had no biochemical indicators suggestive of metabolic syndrome (589). The interval between screening and development of pre-eclampsia was 15 ± 4.3 weeks. All groups delivered at or near term although women with pre-eclampsia delivered significantly earlier (controls 39.1 ± 1.2 ; gestational hypertension 38.8 ± 1.6 ; pre-eclampsia 37.9 ± 2.0) and had babies of similar size (controls 3305 ± 457 ; gestational hypertension 3110 ± 410 ; pre-eclampsia 3094 ± 626). In conclusion this study demonstrated reduced FMD in women who had pre-existing risk factors without the metabolic syndrome profile who eventually developed pre-eclampsia at a late gestation.

A retrospective study was published with the intention of investigating if alterations in angiogenic factors precede pre-eclampsia and if this is related to endothelial dysfunction as measured by FMD (590). The technique of FMD and the criteria for pre-eclampsia were identical to the previously discussed work (353). The women were divided into two groups, the first group (A) had normal uterine artery Dopplers and normally grown fetuses, the second group (B) had bilateral uterine artery notching and normally grown fetuses. From these groups FMD was performed on a subset of each group (A, n=37; B, n=36). Pre-eclampsia developed in twelve women from group B, group A women all had normal outcomes. The FMD results were reported in terms of pre-eclampsia plus SGA and pre-

eclampsia without SGA. Flow-mediated dilatation was significantly reduced in both pre-eclampsia groups compared with group A ($P < 0.05$) (Table 1.3). Group A had an angiogenic profile consistent with a normal outcome (higher PIGF and lower sEng) and both pre-eclampsia groups had a typical angiogenic profile for pre-eclampsia (lower PIGF and higher sEng). No correlation between FMD and any angiogenic factors was found. The authors suggested “no direct causal relationship” was evident between conduit vessel endothelial dysfunction and angiogenic factors and that derangement of angiogenic factors may not be “directly responsible” for the endothelial dysfunction seen in pre-eclampsia (590). This study had very small numbers. Shear stimulus data was not reported. The women were not obese (Table 1.3) and were recruited on the basis of abnormal uterine artery Doppler studies. They all delivered at term and may therefore represent a late onset pre-eclampsia influenced by placental disease rather than maternal metabolic disease (590). This study reported women destined to develop pre-eclampsia with and without a SGA baby have reduced FMD compared to women with normal pregnancy outcomes.

A longitudinal predictive study where FMD was assessed twice in pregnancy was published (591). This study assessed FMD in women in the late second and early third trimester (Table 1.3) who had at least one risk factor for pre-eclampsia. These risk factors included nulliparity, age > 35 years, BMI > 30 , chronic hypertension not on therapy and a previous history of pre-eclampsia/eclampsia. Excluded were women with hypertension on medication, smokers or a family history of cardiovascular disease. The brachial artery was scanned with a blood pressure cuff inflated to 200mmHg for five minutes (position on arm not stated) and post occlusion diameter was measured at 60 seconds. No definitions for pre-eclampsia or gestational hypertension were given. Women with pre-eclampsia, pre-eclampsia superimposed on essential hypertension and gestational hypertension were all

combined into a group called PIH presumably because no significant difference was found between the three groups, although this was not stated. Flow-mediated dilatation was significantly reduced in the PIH groups at both study times compared to normotensive controls ($P=0.001$) (Table 1.3). When analysing this study, paired t-tests and Mann-Whitney-U tests were used to compare repeated measures on FMD and compare differences between PIH and normotensive respectively. Shear stress stimulus and BMI were not reported. The data and methodology in this study have significant deficiencies. Although the authors concluded FMD is a “promising predictor of pre-eclampsia” their data does not support this as the hypertensive disorders of pregnancy could not be separated and their study population were women with risk factors. Therefore the results may not be able to be extrapolated to a low risk population. In summary this study demonstrated FMD could not differentiate between women destined to develop hypertensive disorders of pregnancy.

The most recent study published on FMD as a predictive test was performed with the intention of assessing the relationship between angiogenic factors and FMD longitudinally throughout pregnancy (592). The right brachial artery was scanned with five minutes of forearm occlusion set at 300mmHg. Maximum post occlusion arterial diameter was measured after up to five minutes of monitoring (593). The NHBPWG criteria (253) were used to diagnose pre-eclampsia. One hundred and fifty-nine women were assessed a median of five times (inter quartile range 2-6) for FMD in pregnancy. Forty-five of these women had risk factors for pre-eclampsia (pre-existing hypertension, previous pre-eclampsia, diabetes, thrombophilias, previous IUGR and polycystic ovarian syndrome). Only the mean FMD values for the first visit at 10-17 weeks were reported (Table 1.3). In women who developed gestational hypertension, FMD was 4.5% (95% CI, 1.64 to 6.46)

higher between 10-40 weeks gestation compared to pre-eclamptics ($P=0.001$). The women who had a normal outcome had an intermediate FMD between gestational hypertension and pre-eclampsia (mean not reported). The FMD values were lower in women who delivered with both term and pre-term pre-eclampsia compared with those who did not develop pre-eclampsia (FMD: -1.7% (95% CI, -3.3 to -0.13 ; $P=0.03$). Pre-term pre-eclampsia women had decreased PIGF followed by increased sEng and sFlt-1 compared with other groups. Pre-term pre-eclamptics also had increased uterine artery PI compared with other groups. No correlation was found between FMD and any angiogenic factors at any time in pregnancy (592). One limitation is the inclusion of women with pre-existing medical conditions as the results may not be applicable to the general population. None of the women were obese (Table 1.3) so it is possible that metabolic factors played a limited role in this group of women. This study demonstrated prior to the development of disease, increased FMD in gestational hypertension and reduced FMD in pre-eclamptics.

The above studies using FMD to screen for or predict pre-eclampsia before clinical disease was apparent also demonstrated variations in FMD methodology although this was less than the studies that were performed when pre-eclampsia was present. The criteria used to diagnose pre-eclampsia were more homogenous, however the number of pre-eclamptics in each study was small (Table 1.3). The groups studied tended to have pre-existing disease or risk factors, be women with metabolic syndrome risk factors or were recruited because of uterine artery notching so the results may not be applicable to the general pregnant population. They also varied in time of delivery with some groups representing early, late or a mixed form of pre-eclampsia. Although only half the studies reported BMI, none were in the obese range. Similar to findings when assessing other biochemical markers (for example nitrates, nitrites and cGMP) associated with pre-eclampsia, these studies also

had variable results. A significant correlation between FMD and ADMA (353) was not repeated in the study by Takase and colleagues (588). The two groups that studied FMD and angiogenic factors found no relationship (590, 592). All studies attempting to predict pre-eclampsia demonstrated decreased FMD prior to clinical disease compared to their control groups (353, 588-590, 592) except Kamat et al. (591) who could not differentiate between any hypertensive disorder. Decreased FMD before the clinical syndrome of pre-eclampsia may allow this test to be used as a screening test.

The evaluation of FMD as a screening test requires that the principles of screening methods be considered. Flow-mediated dilatation may be a convenient screening test as it could be performed at the same time as a routine obstetric ultrasound adding only an extra 20-30 minutes to the appointment. The women would need to be motivated to undertake preparation by either fasting and/or refraining from cigarettes, caffeine, high fat meals and exercise (100). The test causes some discomfort, but does not cause unacceptable morbidity. The FMD method is difficult to learn and not simple to perform requiring highly trained operators and expensive high quality ultrasound equipment (96) with manual measurement of vessel diameters tedious and time consuming. The test also has a large variance with considerable overlap between screen positive and screen negative groups (353). Therefore applying FMD to a large asymptomatic population may not have the desired sensitivity and specificity and would be difficult and expensive. Only one FMD study reported PPV and NPV. Whilst these were good (PPV 90% and NPV 100%) they are limited in interpretation as there were only nine pre-eclamptics recruited from and compared with a high risk population (588).

To be more cost effective FMD could be applied to a high risk population only as most of the aforementioned studies have done. The incidence of pre-eclampsia is about 5-8% (594, 595) which is relatively low, and up to 11% in first pregnancies (594). The study by Garcia and colleagues (589) recruited a large number of women (n=506) with only 6.3% of women developing a hypertensive disorder of pregnancy and only 2.8% developing pre-eclampsia, small numbers for a lot of work. Performing FMD at 21-22 weeks gestation identified pre-eclamptic women who mostly had previous pre-eclampsia or a family history of hypertension and developed term pre-eclampsia (589).

A good screening test would identify early onset pre-eclampsia where, ideally increased surveillance, early intervention and the possibility of extending the pregnancy by even a few weeks could make a difference to the mother's and baby's outcome. A problem also arises in defining and identifying high risk women as a significant number of cases would likely be missed depending on the definition. As pre-eclampsia results in increased perinatal and maternal morbidity and mortality (595) it would be an ideal disease to evaluate for screening and prediction. At this time however, there is no treatment for pre-eclampsia apart from delivery (596, 597).

In conclusion, from the above studies it appears that FMD may be able to identify some women who may develop pre-eclampsia but seems to be limited to groups who already have pre-existing risk factors. Numbers recruited were small probably because the FMD test is difficult, expensive and time consuming but also because of the low incidence of pre-eclampsia in pregnancy.

1.18 Cigarette smoking, pregnancy, pre-eclampsia, intra-uterine growth restriction and the endothelium.

No study had used the ultrasound technique of FMD to assess endothelial function in pregnant women who smoke when this thesis was commenced. This may be because of the difficulties in obtaining ethical approval as numerous works have demonstrated the deleterious effect of smoking in pregnancy.

In the twenty first century smoking remains a significant health issue despite the known adverse effects. Although the proportion of women of reproductive age who smoke has reduced in recent years, the latest Australian perinatal statistics still quote around 25% as current smokers (579). Of these 17.3% of women continue to smoke in pregnancy. Most states of Australia dichotomise questions on smoking as a yes/no answer with quantity data reported as \leq or $>$ 10 cigarettes per day. This cut off of 10 cigarettes per day is often used as criteria for light or heavy smoking (598, 599).

Studies have shown that pregnant women can report a reduction in the number of cigarettes smoked without reducing tobacco consumption. This is achieved by changing smoking habits by drawing in smoke more deeply or smoking more of the cigarette (600). Research has also shown if a pregnant smoker consumes $<$ 10 cigarettes per day, around 71% of them will cease smoking by the second trimester (598). This information would be important when designing a study assessing the effects of smoking on endothelial function. To give women time to quit smoking before recruitment, the study could be conducted at the beginning of the third trimester. Heavy smokers ($>$ 10 cigarettes per day) are less likely to cease smoking. If a study was designed to assess the acute and chronic effects of smoking with women abstaining from cigarettes for a period of time and then smoking one

cigarette, recruiting women who smoke >10 cigarettes per day would result in women being less likely to be doing what is outside normal practise for them. Women would also need to be given the choice on whether to smoke or not after the period of abstention.

Smoking tobacco cigarettes provides a protective effect against pre-eclampsia while at the same time increasing the complications of pregnancy (601). This paradox occurs even though cigarette smoking is known to cause endothelial dysfunction in non-pregnant people (177) and pre-eclampsia is thought to be an endothelial cell disorder (2). This raises the question whether smoking in pregnancy improves vascular endothelial function as measured by the ultrasound technique of FMD. The following section will review the relevant literature on smoking in pregnancy.

1.18.1 The effect of smoking in pregnancy on the incidence of pre-eclampsia and gestational hypertension.

Numerous studies have shown that smoking in pregnancy reduces the risk of both mild and severe pre-eclampsia. In 1967 a study of over 48000 women, comparing smokers with non-smokers, found the incidence of pre-eclampsia decreased as the number of cigarettes smoked daily increased (602). These findings were confirmed in a study on primigravid women the following year (603). Since then others have shown that smoking in pregnancy significantly reduces the risk of pre-eclampsia in nulliparous women (601). Even when confounding factors such as age, socioeconomic status, body mass index and race were controlled for, both past and current smoking in pregnancy was associated with a reduced risk of gestational hypertension and pre-eclampsia (604).

Nonetheless, women who quit smoking before pregnancy do not have a reduced risk of pre-eclampsia or gestational hypertension (605). The reduced risk from smoking was

confirmed by measuring lower urinary cotinine levels (as an indicator of tobacco exposure) in nulliparous women with pre-eclampsia in a case-control study where the controls were normotensive pregnant women (606). A later study on African-American women observed the protective effect of smoking occurred only at high levels (>200ng/ml) as measured by salivary cotinine. After adjustment for medical risk factors this was no longer significant (607).

A population based retrospective study demonstrated the odds ratio (OR) for pre-eclampsia in women who smoked was 0.64 (95% CI, 0.59 to 0.7) and increasing the number of cigarettes smoked per day by five decreased the incidence of pre-eclampsia by 18% (608). Smoking reduced the risk of pregnancy induced hypertension and eclampsia in primiparous and multiparous women. The more daily cigarettes consumed the lower the risk for eclampsia (609).

In contrast a multicentre cohort study found that whilst smoking in pregnancy decreased the risk of pre-eclampsia, the risk of eclampsia was dramatically increased (OR 4.9, 95% CI 1.4 to 16.6) (610). As well as pre-eclampsia and gestational hypertension risk being reduced by 34%, smoking also reduces the risk of developing HELLP syndrome by 81% (611). In a systematic review of the literature, smoking was shown to reduce the risk of pre-eclampsia by 32% with the risk decreasing as the number of cigarettes smoked daily increased (519). Another systematic review found a similar dose dependent relationship with smoking with a 50% decrease in the risk of pre-eclampsia (612).

A recent large population based study also reported a reduced risk of pre-eclampsia in women who smoked (AOR 0.83 (95% CI 0.74 to 0.94) (613). The reduced risk of pre-

eclampsia and gestational hypertension from smoking was found to occur only when the newborn was growth restricted (<1st percentile) (614). No protection from smoking was observed when babies in heavy smokers were $\geq 20^{\text{th}}$ percentile, $\geq 60^{\text{th}}$ percentile in moderate smokers and $\geq 80^{\text{th}}$ percentile in light smokers.

Swedish “snuff” which contains nicotine but none of the combustion products of cigarette smoking such as carbon monoxide was found not to reduce the risk of pre-eclampsia whilst smoking cigarettes did in a dose dependent fashion. Women who smoked at the beginning of pregnancy and continued at 30-32 weeks gestation had a reduced risk. This protective effect disappeared if they smoked at the first visit and ceased at 30-32 weeks. The risk decreased if the women did not smoke at the first visit but smoked at 30-32 weeks. The authors concluded that cigarette combustion products (possibly carbon monoxide) rather than nicotine are the most likely protective ingredients. As a change in smoking habits in pregnancy altered the risk, later smoking is more protective of pre-eclampsia than earlier smoking (615).

A population based cohort study of pregnant women was carried out to assess the mutual effects of smoking and obesity on the risk of pre-eclampsia from data obtained from Missouri birth certificates (616). Data collected in this manner is known to introduce misclassification errors and was a criticism of this study by a later paper (617). Regardless of BMI, the risk of pre-eclampsia was consistently decreased in smokers compared with non-smokers. This population of women delivered between 2000-2001 with at least 45% overweight and obese (616).

In comparison, data from a study (617) where women were prospectively enrolled between 1959-1965 was used to assess the combined effects of smoking and maternal weight on the development of pre-eclampsia. Self-reported pre-pregnancy underweight or overweight affected approximately 10% of these women. Pre-eclampsia was less common and SGA more common in smokers compared with non-smokers. In the overweight women, pre-eclampsia occurred more frequently and SGA less frequently. Light and heavy smoking and being underweight decreased the risk of pre-eclampsia. When women were overweight and smoked there was no decrease in the pre-eclampsia risk.

Another study (618) assessed whether smoking, maternal weight and pregnancy hypertension were associated with differences in newborn weight. Baby birth weight was lower in women who smoked and were underweight although baby weight increased with increasing maternal BMI. The same pattern was seen in non-smokers but their babies were larger overall. The lowest mean birth weight and highest prevalence of SGA was in underweight smoking hypertensive women. Regardless of weight, smoking and hypertension resulted in more SGA babies.

Although for over 40 years researchers have consistently recorded an inverse dose dependent relationship between hypertensive disorders of pregnancy and smoking, it is becoming apparent that this is a complicated relationship. In general the risk of pre-eclampsia may be reduced from smoking but smoking has other deleterious effects on the mother and fetus.

1.18.2 The effect of smoking on pregnancy outcomes.

Smoking affects all stages of the reproductive cycle. Multiple studies have demonstrated smoking reduces fertility, increases pregnancy loss (619-624), results in smaller and/or growth restricted babies (601-603, 608, 610, 613, 614, 625-630), increases the incidence of placental abruption (601, 613), placenta praevia (602, 631), perinatal mortality (601, 603, 610, 613) and the incidence of pre-term delivery (384, 608, 610, 613). These adverse outcomes are dose dependent, increasing significantly with increasing number of cigarettes smoked (608) and length of exposure (632).

When assessing the number of cigarettes smoked and/or tar, nicotine or carbon monoxide yields from cigarettes, a threshold effect of 13 cigarettes per day and 15mg of carbon monoxide was estimated to affect fetal growth (633). A dose response effect was found, with the greater the number of cigarettes smoked, the greater the reduction in birth weight. This study suggested that smoking earlier in pregnancy (<28 weeks) has a greater effect on fetal growth than smoking later in pregnancy and carbon monoxide had the strongest association with decreased fetal growth. However, reducing or ceasing smoking increases newborn weight and is the most effective modifiable strategy for improving pregnancy outcomes (598, 610, 634).

1.18.3 Smoking plus pre-eclampsia equates to a worse outcome.

If a pregnant woman smokes and develops pre-eclampsia, perinatal outcome is worse (601, 603, 610, 613, 631). In a smoking/pre-eclampsia group, the perinatal mortality rate was 21.7% compared to 8.5% in the non-smoking/pre-eclamptic group (603). A large study of over 317000 nulliparous women demonstrated that in women who smoked heavily and developed severe pre-eclampsia, there was a dose-dependent increase in the risk of

perinatal death (from 24 to 36 per 1000), being born small for gestational age (from 28% to 68%) and placental abruption (from 31 to 67 per 1000) (601).

A retrospective South Australian study from a perinatal database demonstrated a twofold increased risk of delivering a small for gestational age baby in hypertensive women who smoked, although the smokers had a decreased risk of caesarean or operative vaginal delivery and decreased length of stay in hospital (635). In an observational multicentre trial (610) in women at high risk of pre-eclampsia, both maternal and baby outcomes were worse if the mother smoked and developed pre-eclampsia, although overall smoking did reduce the risk of pre-eclampsia in this population. Smokers had increased arterial stiffness (as measured by pulse pressure) and were five times more likely to develop eclampsia compared to women who never smoked. Almost 66% of current smokers had an adverse outcome which included preterm delivery (<34 weeks), birth weight <3rd percentile, admission to special newborn care units and perinatal death.

Smoking in pregnancy and pre-eclampsia are independent risk factors for an increase in adverse outcomes. Pregnant women who smoke (without pre-eclampsia) and non-smoking pre-eclamptic women have a similar increased risk for similar adverse outcomes (SGA, pre-term birth, abruption, low apgars, stillbirth and admission to special care nurseries) (613). When pre-eclamptic women who smoked were compared to non-smoking normotensive pregnant women the risk of adverse outcomes doubled.

1.18.4 Mechanisms that may be the cause of adverse outcomes in pregnancy.

Alterations in maternal haemodynamics and/or physiology and the effect of smoking on the endothelium have been proposed as a cause of worse outcomes in pregnancy. The

adverse effects on fetal growth and the complications in pregnancy from smoking are also thought to result from decreased placental perfusion and placental injury. These increase the rate of vascular complications in pregnancy (620). Due to the inconsistency of smoking reducing pre-eclampsia risk but at the same time worsening outcomes it would be interesting to know if chronic or acute smoking in pregnancy affects endothelial function in pregnancy and if that is the mechanism for the different outcomes.

1.18.5 Altered maternal vascular function, smoking and growth restriction.

The association between altered maternal vascular haemodynamics and growth restriction has long been appreciated in pregnant smokers and non-smokers with failure of normal vascular remodelling proposed as grounds for the fetal growth restriction. This association also extends to altered haemodynamics in hypertensive disorders of pregnancy.

Insufficient maternal plasma volume expansion is one maternal haemodynamic change associated with fetal growth restriction in both normotensive pregnancies of smoking women (636) and hypertensive pregnancies (511). This changed volume adaptation has been noted very early in pregnancies destined to develop growth restriction where “relative hypovolemia” and a failure to increase cardiac output have been noted (637). Decreased cardiac output and plasma volume and increased SVR have also been recorded in third trimester pregnancies complicated by growth restriction (638). Therefore failure of the normal changes that occur in pregnancy may predispose or result in adverse outcomes in pregnancy.

1.18.6 The effect of cigarette smoking on maternal and fetal physiology.

As early as 1935, concern was raised regarding the effect of maternal cigarette smoking on the fetus when the fetal heart rate (measured by stethoscope and stopwatch) was noted to increase after pregnant women smoked a cigarette (639). Numerous studies have since been published demonstrating the deleterious effects of cigarette smoking on the maternal physiological status.

Smoking has been shown to affect maternal physiology and feto-placental function.

Maternal blood pressure increases and maternal and fetal heart rate increase acutely after smoking. In a survey of BP in pregnancy, systolic but not diastolic BP was found to be higher in pregnant women who smoked (486). A later study (640) assessing maternal and fetal physiological variables, found maternal heart rate and BP increased after smoking one cigarette containing nicotine, but not after smoking a cigarette that contained no nicotine. Fetal heart rate also increased after exposure to a cigarette containing nicotine and baseline variability decreased.

Smoking also affects utero-placental blood flow. Maternal BP and heart rate increased and intervillous blood flow (IBF) decreased significantly after smoking a single cigarette in a study (641) where the radioisotope ^{133}Xe was injected intra-venously into pregnant women. Blood flow then returned to pre-smoking levels within 15 minutes. In a subsequent study (642) IBF was shown to increase in mildly hypertensive pregnant women after a cigarette. Intervillous blood flow pre-cigarette was already significantly less in these hypertensive women (n=11) compared to normotensive women (n=12). Remarkably, 23 women were studied, 21 of whom were non-smokers and all of whom had a cigarette.

The effect of smoking on fetal and maternal heart rates as well as umbilical and uterine artery Doppler waveforms is an acute response. No difference between smokers and non-smokers in any of the aforementioned variables in pregnant women who had not had a cigarette for greater than 30 minutes (643) was demonstrated. A significant increase in maternal heart rate, systolic BP and fetal heart rate within five minutes of smoking a cigarette was demonstrated. Maternal heart rate and BP returned to pre-smoking levels within 30 minutes, fetal heart rate remained elevated at 45 minutes when the study ended (644). Others have shown similar findings on maternal heart rate and BP (645).

Smoking also affected umbilical artery systolic/diastolic (S/D) ratios (a measure of placental vascular resistance) but not maternal uterine artery S/D ratios. The increase in umbilical artery ratio remained for 15 minutes, normalising by 30 minutes (644). A prospective study (646) that compared smokers with non-smokers, assessing fetal biometry, umbilical and uterine artery Dopplers at 20 weeks gestation, found smokers had higher umbilical artery Doppler RI values which were more likely to remain abnormal after adjustment for confounders. Apart from femur length which was significantly smaller in smokers, there was no difference in fetal biometry. Amongst babies born SGA, SGA babies of smokers were more likely to have abnormal umbilical artery Doppler study. There was no difference in fetal biometry between SGA babies of smokers and SGA babies of non-smokers (646).

1.18.7 Smoking in pregnancy and its effect on nitric oxide production and the endothelium.

Studies that have assessed levels of NOS in pregnancy in umbilical vessels and placentas have overall demonstrated reduced NOS levels when pregnant women smoke or have babies that are growth restricted. No difference between smokers and non-smokers in

calcium-dependent or calcium-independent NOS in first trimester placental villi was found (647). By the second trimester a significant decrease in calcium-independent NOS in smokers was demonstrated. This suggests cigarette smoke may affect the NO metabolism of the placenta as pregnancy advances.

The level of eNOS was measured in endothelial cells taken from umbilical veins at delivery in smokers and non-smokers. The significantly smaller babies of smokers had lower levels of eNOS activity and concentration which correlated positively with the number of cigarettes smoked and baby weight (648). In a later study, the same group (649) measured eNOS levels in umbilical veins and chorionic vessels from healthy pregnancies in 182 non-smokers, 43 current smokers and 41 women who had ceased smoking. In women who smoked, baby weight, head circumference and femur length were significantly smaller compared to non- and ex-smokers. The activity and concentration of eNOS was also significantly decreased in smokers compared with the other two groups in both umbilical and chorionic vessels with 25% of the reduction in weight explained by reduced eNOS levels. Both studies suggested maternal smoking reduces eNOS activity which may reduce fetal vessel dilatation and partially explain the reduction in fetal size (648, 649).

Serum nitrate and nitrite levels were measured to assess NO production in pregnant smokers between 16-22 weeks gestation. The concentration of NO was decreased in smokers (345) which could possibly result in vasoconstriction, similar to that reported in non-pregnant people (177).

Markers of endothelial cell activation were assessed in pregnant smokers and non-smokers by measuring VCAM-1 and ICAM-1. Smokers had significantly higher levels of ICAM-1

and a non-significant increase in VCAM-1. This is a similar pattern seen in CVD and in non-pregnant smokers (650).

Smoking also results in endothelial cell injury. Normal endothelial cells are spindle shaped and arranged longitudinally in a regular pattern. In ultrastructural studies (651) of endothelial cells from human umbilical arteries of mothers who smoked, pathological change in the endothelial intima was demonstrated. The endothelial cells were swollen and enlarged with a “cobblestone” appearance compared with normal endothelial cells. It was suggested this may be due to hypoxia from chronic carbon monoxide exposure.

Pregnant rats were exposed to second hand smoke to determine its effect on endothelial function in newborn pups (652). Pups born after smoke exposure had a higher birth mortality rate and were smaller at four weeks of age. Aortic rings from surviving pups were examined in-vitro. Exposure to cigarette smoke in-utero resulted in reduced endothelial dependent acetylcholine mediated relaxation in the pups indicating endothelial dysfunction from smoke exposure.

From the above studies there is a suggestion that reduced NO activity may occur in smokers and growth restriction and that smoking results in endothelial cell injury, although the studies do not address why this occurs and which factors from cigarette consumption may cause this.

1.18.8 The effect of smoking on the placenta.

The adverse effects of smoking are very evident in the placenta with poorer outcomes possibly due to the effect of cigarette by-products. In a case-control histological study

(653) of placentas from smokers and non-smokers whose pregnancies were complicated by abruption, placental lesions consistent with chronic hypoxia were found more often in smokers. Smokers' placentas had increased rates of intervillous thrombus compared with non-smokers (20.0% versus 3.0%; OR 17.5, 95% CI 3.1 to 99.4) and a non-significant trend for increased rates of villous fibrosis (55.0% versus 11.8%).

Smoking in pregnancy damages the placenta and results in abnormal placentation which may then affect the fetus' growth potential. Placental weight in term placentas has been reported to be increased in heavy smokers, probably from chronic hypoxic insult with resultant hypertrophy of the placenta (654, 655). In another study, term placentas weighed less in heavy smokers compared with non-smokers (656).

Placental abnormalities characteristic of under perfusion in the placenta (654, 655) have been demonstrated on microscopic placental examination in smokers. In a study assessing whether the placenta compensated for the chronic hypoxia affecting the fetus in women who smoked (657) no difference in oxygen diffusion across the placenta between smokers and non-smokers was seen. The authors suggested that as these fetuses are afflicted by hypoxia, this lack of compensation does not allow an increase in oxygen to the fetus which may contribute to a decrease in birth weight.

Increased apoptosis in the placentas of women who smoke has also been reported to be associated with IUGR. Using immunostaining with the monoclonal antibody M30 which is used to identify apoptotic epithelial cells, programmed cell death in fetal villous placental tissue was increased in women who smoked and had IUGR fetuses (658). From

these studies hypoxia and/or cell death appears to play a significant part in the adverse effects on the placenta from cigarette smoking.

1.18.9 Products of cigarette smoking.

Tobacco contains more than 4000 toxins and/or chemicals such as nicotine, carbon monoxide, nitrogen oxide, thiocyanate, benzene, toluene and phenol to name a few which are released as combustion products from a burning cigarette (75, 659). Various components of cigarette smoking may affect the mother, fetus, placenta and endothelium differently. Nicotine and carbon monoxide appear to be the most studied substances. They are emerging as promising candidates in the search for substances that may affect the endothelium in pregnancy resulting in the reported adverse outcomes or as a contender for the decreased risk of pre-eclampsia (615).

1.18.10 The adverse effects of nicotine on physiological status.

Nicotine, the addictive component of smoking, is the main alkaloid in tobacco that affects the cardiovascular system via stimulation of acetylcholine receptors found in the adrenal medulla, neuromuscular junctions and autonomic ganglions. Nicotine is metabolised by the liver and excreted by the kidneys with an average half-life of 1-2 hours. The main metabolite of nicotine is cotinine which correlates well and is used as a biomarker of nicotine after tobacco exposure (660, 661). Interestingly the metabolic clearance rate of nicotine and cotinine is 60% and 140% greater respectively in pregnancy compared with postpartum values. The half-life of cotinine is also 50% less in pregnancy resulting in reported plasma levels of cotinine being much less although cigarette intake was similar in pregnancy and postpartum (662).

It has been demonstrated that in non-pregnant humans it is the nicotine in cigarettes that increases heart rate and BP. Heart rate increases the most after the first few cigarettes of the day and then plateaus as tolerance develops (663). Twenty-four hours of abstinence is enough to increase heart rate by an average of 13.6 ± 6.5 beats per minute when smoking recommences (664). In pregnancy nicotine but not carbon monoxide was shown to increase maternal heart rate and blood pressure after 12 hours of abstinence in women who regularly smoked an average of 15 cigarettes per day. Fetal heart rate and volume blood flow in the fetal descending aorta and umbilical vein increased and the PI of the fetal aorta and umbilical artery decreased (665).

1.18.11 The effect of nicotine on the endothelium.

Nicotine has been shown to have detrimental effects on the vascular endothelium in some but not all studies. Early ultrastructural studies on aortic endothelium in mice, demonstrated chronic nicotine exposure (five weeks in drinking water) increased mitotic activity and cell loss contributing to endothelial cell injury (666) This induced changes in intercellular cleft morphology which would increase the vascular permeability of endothelial cells (667).

Studies assessing acute nicotine exposure on hamster cheek pouch arterioles demonstrated decreased vasodilator response to acetylcholine (668, 669). Low dose nicotine infusion resulted in decreased acetylcholine mediated vasodilatation; high dose nicotine infusion resulted in vasoconstriction after acetylcholine infusion, both indicating inhibition of NO. Nicotine did not change the response to nitro-glycerine indicating no effect on endothelial independent pathways (668).

Decreased vasodilatation from nicotine was also demonstrated in-vitro in human skin vessels (670). In an experiment (671) non-smokers were infused with bradykinin to assess endothelium dependent hand vein response. Relaxation in the veins was noted. When nicotine was added, the dilatory response to bradykinin was blunted indicating nicotine affects NO mediated dilatation. Smooth muscle dilatation was not affected by nicotine.

Other effects on the endothelium from nicotine include induction of a pro-thrombotic state (672). An *in vitro* study on pregnant ewe uterine arteries assessed the acute and chronic effects of nicotine exposure. Only chronic nicotine exposure increased contractions in the uterine arteries which was mediated via inhibition of the eNOS relaxation pathway (673). This demonstrates how nicotine may affect the vascular endothelium in pregnancy.

Nicotine has previously been shown to modulate eNOS interactions via interference with NO pathway cofactors (not receptors) (674).

In comparison lipid soluble particles from cigarette smoke but not nicotine induced decreased vasodilatation in rat and human arteries in-vitro (675). Chronic two week exposure to nicotine in rats via an infusion pump did not alter vascular reactivity of endothelial cells or vascular smooth muscle cells (676).

1.18.12 The effect of nicotine on pregnancy.

Nicotine also affects the stillbirth rate, sex ratio and birth weight. These variables were assessed in women who chewed tobacco rather than smoked it. This has the advantage of assessing the effect of nicotine intake in pregnancy (677) with the added confounder of inhaled carbon monoxide from burning tobacco removed. Women who chewed tobacco

had an increase in the stillbirth rate, a decrease in the number of boys born and a decrease in birth weight.

Growth restriction in the fetus of smoking mothers may be caused by nicotine toxicity and/or reduced blood flow to the placenta from vasoconstriction (678). Studies performed on placental tissue obtained after first trimester terminations have demonstrated the harmful effects of cigarette smoking and nicotine. Both smoking and nicotine were shown to inhibit the outgrowth and invasion of 6-10 week gestation cytotrophoblast (679).

In contrast by studying the effects of in-vitro nicotine and smoking on molecules that control the cellular response to oxygen tension in the cytotrophoblast, nicotine was found to upregulate the production of VEGF-A from trophoblast cells (680). A recent study (681) also demonstrated sFlt-1 and sEng down regulated endothelial angiogenesis and nicotine restored these functions (angiogenesis was measured by endothelial migration activity and morphogenic activity of endothelial cells). The authors hypothesised this was by stimulating growth factors as PlGF levels were increased although VEGF was undetectable and TGF- β 1 was unchanged. This positive effect from nicotine may be due to its pro-angiogenic properties (682).

Interestingly due to the vast number of chemicals in tobacco, some of the adverse effects attributed to nicotine may in fact be due to other chemicals or combinations of different chemicals with nicotine (75). Others have shown that nicotine does not increase sFlt-1 production in placental tissue (sFlt-1 was instead partly regulated by an inflammatory process) (261) and cigarette smoke which contains nicotine suppresses sFlt-1 production (683) potentially removing an anti-angiogenic effect.

Smoking has many adverse effects. However nicotine may not be solely responsible for the decreased risk of pre-eclampsia and some other substance produced from cigarette smoke may be a contender (615). One possibility may be NO, another is carbon monoxide (612).

1.18.13 Carbon monoxide, angiogenic factors and pregnancy.

Carbon monoxide is produced naturally in the body as a by-product/degradation product when the enzyme heme oxygenase (HO) catalyses heme. Carbon monoxide has both beneficial effects (in low concentrations) and toxic effects when the concentration is high. The other by-products of the HO pathway are biliverdin which breaks down to bilirubin, and iron which converts to ferritin (149). Heme oxygenase prevents programmed cell death, has anti-inflammatory properties, helps control vascular tone and decreases oxidative stress. It exists in three isoforms HO-1, HO-2 and HO-3. Of these HO-1 is inducible in response to stress injury from ischaemic-reperfusion injury, HO-2 is constitutive, involved in heme metabolism and found throughout the body. HO-3 is also constitutive but its function is not well understood (29, 149, 583, 684). For HO to produce carbon monoxide, the cofactors NADPH-cytochrome P450 and NADPH are necessary. Like NO, carbon monoxide also activates guanylate cyclase to produce cGMP to induce smooth muscle relaxation. Carbon monoxide has similar properties to NO in that it inhibits activation of platelets, suppresses thrombosis, curbs apoptosis and causes vasodilatation. Both may also work together in highlighting these protective effects (29).

After cigarette smoke inhalation, carbon monoxide is absorbed into the blood where it binds more efficiently to haemoglobin than oxygen forming carboxyhaemoglobin (659,

661). This has the effect of reducing the oxygen carrying capacity of blood to the mother and fetus (649).

The HO-carbon monoxide system is proposed to have a vasodilatory role in pregnancy, is involved in trophoblast invasion and spiral artery remodelling and exerts an anti-oxidant effect (583, 685). Women destined to develop pre-eclampsia have been shown to have decreased HO-1 gene expression in the first trimester (11 weeks chorionic villus sample) indicating early involvement in that pathway. The expression of Flt-1, Eng, VEGF-A and TGF- β 1 were increased and PlGF decreased in the five women in whom pre-eclampsia developed (73). Whilst end-tidal carbon monoxide levels do not change throughout normal pregnancy, in pre-eclamptic women there is reduced exhaled carbon monoxide (74).

Carbon monoxide from cigarette smoke has been proposed as the substance responsible for reducing the risk of pre-eclampsia (543). Cigarette smoke which contains both nicotine and carbon monoxide down-regulates the production of sFlt-1 in the placenta. As sFlt-1 is up-regulated in pre-eclampsia, this suggests exposure to cigarette smoke decreases the risk of pre-eclampsia by providing a pro-angiogenic placental environment (683). In women with normal pregnancies, cigarette smoke was associated with lower levels of sFlt-1 whilst women with pre-eclampsia also had lower sFlt-1 levels although this was not significant (445).

Heme-oxygenase-1 acts to down regulate the release of sFlt-1 and sEng in endothelial cells and placental tissue providing a pro-angiogenic environment with the authors suggesting changes in the HO-carbon monoxide pathway may be involved in the development of pre-

eclampsia (57). This pathway could provide an explanation for smoking reducing the risk of pre-eclampsia. Currently it is not known whether the decreased risk of pre-eclampsia is due to carbon monoxide and/or nicotine (or indeed some other product from cigarette smoke) and their effects on the placenta and angiogenic factors (30). Whereas normal pregnancy results in profound changes in the maternal cardiovascular system to accommodate the developing fetus, women who smoke introduce further changes into their cardiovascular system and affect their pregnancy.

1.19 Aims of this work.

The endothelium plays a significant role in regulating the cardiovascular changes that occur in normal pregnancy with dysfunction of the endothelium proposed as the final step in the complex pathophysiology of pre-eclampsia. The work in this thesis sought to assess endothelial function longitudinally in normal pregnancy. As smoking is known to result in endothelial dysfunction in non-pregnant people but paradoxically reduces the risk of pre-eclampsia, a second study was planned to assess endothelial function in pregnant smokers. This was to ascertain if smoking provided a protective effect on the vascular endothelium, thereby reducing the risk of pre-eclampsia. As both pre-eclampsia and gestational hypertension may result in endothelial dysfunction, a cross-sectional study was designed to compare these hypertensive disorders of pregnancy. The ease and precision of the FMD test was determined. All studies were performed using the ultrasound technique of FMD which is a marker of stimulated NO mediated endothelial function.

1.19.1 The studies performed.

The first study involved developing a normal range of endothelial function in uncomplicated pregnancy. The purpose of this study was to determine if endothelial

function changed throughout gestation in pregnancy and postpartum. Second was to ascertain if endothelial function differed between pregnant and non-pregnant women (Chapter 2). This was a longitudinal study that assessed healthy pregnant women five times throughout their pregnancy beginning in the first trimester (11-14 weeks gestation) and continuing until 36+ weeks. The women were then invited to return at six weeks postpartum. The pregnant women were compared to healthy non-pregnant women.

The second study was designed to determine if endothelial function in otherwise uncomplicated pregnancy was altered by smoking tobacco cigarettes when compared to healthy pregnant non-smoking women. This study used a test-retest design to assess the acute and chronic effects of smoking in pregnancy on endothelial function (Chapter 3).

The third study assessed endothelial function in women with pre-eclampsia and gestational hypertension. Pre-eclampsia is thought to result in endothelial dysfunction in the form of reduced vasodilatation. Gestational hypertension is considered either a separate disease to pre-eclampsia or a precursor to pre-eclampsia. For this reason it would be informative to assess if endothelial function differed between the two groups. The ultrasound technique of FMD has evolved over the period it has taken to complete this work, so a secondary aim was to ascertain if the FMD results in this group of women varied with the addition of revised techniques (Chapter 4). Using the normal range study, differences between hypertension in pregnancy and normotension were determined. Women with hypertension who smoked were recruited but were excluded from analysis due to the significant effect smoking had on endothelial function in pregnancy.

This work was done at Nepean Hospital which lies within the Nepean and Blue Mountains Area Health Region (formally Sydney West Area Health Service and Wentworth Area Health Service). Nepean Hospital is a tertiary level referral and teaching hospital for the University of Sydney.

Chapter 2

A longitudinal study using the ultrasound technique of flow-mediated dilatation to assess endothelial function in normal human pregnancy.

2.1 Introduction.

Profound physiological changes occur in the maternal cardiovascular system to allow for the growth and development of a normal pregnancy. The vascular endothelium is thought to play a major role in these changes including regulating the decrease in SVR and blood pressure to maintain vascular tone (484).

This regulation is achieved by the endothelium constitutively producing NO along with a number of other vasodilators and constrictors (1). The ultrasound technique of FMD has been shown to be mainly NO mediated and was developed to assess endothelial function and dysfunction in different physiological and pathological conditions. Under normal conditions and with an intact endothelium pulsatile flow or hyperaemia will result in vasodilatation. Different pathological conditions that affect the endothelium may present as vasoconstriction or reduced dilatation (16).

The endothelium is designed to react to its immediate environment. As previously reviewed, changes in diet, exercise, drug intake, time of the menstrual cycle and a variety of cardiovascular risk factors may alter the response of the endothelium. As pregnancy results in so many changes to the mother to accommodate the developing fetus, it would be important to try and control and/or document as many of these variables as possible. It would also be important to note if the changes that occur with increasing gestation in normal pregnancy affect endothelial function. Therefore, the aim of this work was first to assess endothelial function in normal human pregnancy and postpartum using the

ultrasound technique of FMD. Second was to compare endothelial function in normal pregnancy with non-pregnant women. This work will form the basis of future studies. These other studies are to evaluate if pregnant women who smoke have endothelial dysfunction and if endothelial function differs between women with pre-eclampsia and gestational hypertension.

2.2 Methodology.

2.2.1 Longitudinal normal range study.

This was a longitudinal study of pregnant women with singleton pregnancies assessed five times throughout their pregnancy and once postpartum. All routine obstetric ultrasounds were performed immediately before the study by the author (AEQ) as required.

Gestational ages in weeks were 11-14, 18-20, 22-24, 28-32 and 36+. Postpartum the women returned at 6 weeks. Gestational age was calculated from a first trimester ultrasound. Non-pregnant women were recruited and assessed once at day 1-5 of their menstrual cycle.

Inclusion criteria for all participants were non-smoking, no medical conditions, no family history of premature cardiovascular disease or pre-eclampsia and not taking any medication. Additionally, for non-pregnant women, the use of oral contraception was an exclusion criterion. For the pregnant women, development of pre-eclampsia, gestational hypertension, gestational diabetes or pre-term delivery resulted in exclusion. All women were asked to have a low fat meal before the study and refrain from caffeine products. Before the study began height and weight were measured and blood pressure was recorded manually from the left arm (the opposite arm to the FMD study) using a mercury sphygmomanometer. A detailed medical, family and obstetric history was taken (Figure

2.1). Approval for this project was given from the Area Ethics Committee and written informed consent was obtained at enrolment.

Figure 2.1: Brachial Artery Study History Sheet.

Date: ___/___/___

Study Type: control / normal / pre-eclamptic

Name: _____ MRN: _____ DOB: ___/___/___

Age: _____ Phone: _____ Referral: _____

Ht: _____ cm Wt: _____ kg BMI: _____

Current Pregnancy: LMP: ___/___/___ Sure: Y / N _____ Days/cycle

1st Ultrasound date ___/___/___; Gestation: _____ EDC: ___/___/___ (dates / U/S)

(Controls: LMP: ___/___/___ Days/cycle: _____ Day _____)

Smokes: Y / N _____ day Passive smoker: Y / N

Alcohol: No / rare / social / frequent _____ units/day

Medication: Y / N: Details _____

Other drugs: Y / N: Details _____

Previous Pregnancies

Year	Gest age	Outcome	Complications (PE, HT, diabetes, IUGR etc)

Medical History

Family history of heart disease or stroke: Y / N Details _____

(parent or sibling before age 55 years)

Mother or sister with preeclampsia: Y / N Details _____

Medical conditions:

Last meal: time _____ Content: _____ Last cig: y / n Time: _____

Pregnancy Outcome

Date of birth: ___/___/___ Gestational age: _____ Sex: Male / Female

Birth weight: _____ g Apgars: ___/___ Outcome: A&W / PND

Placental weight: _____ g

Comment: _____

2.2.2 The ultrasound technique of FMD.

Endothelial function was assessed by the mainly NO dependent non-invasive ultrasound technique of FMD as previously described (16). The women were rested in a semi-supine position for 10 minutes at 23° Celsius in a temperature controlled room before baseline measurements were taken. Continuous maternal ECG monitoring was performed. The right arm was extended comfortably on a pillow at heart level in between the author and ultrasound machine (Figure 2.2). Acoustic gel was applied to the upper arm.



Figure 2.2: Author scanning pregnant volunteer. Note the position of arm, BP cuff and ultrasound transducer. Coloured ECG leads attach to the ultrasound machine. Automatic BP machine control and compressor is between ultrasound machine and computer screen. The video recorder is controlled through the ultrasound consol.

The right brachial artery was visualised in a transverse section superior to the cubital fossa using 2D ultrasound with a linear array L12-5MHz transducer and a Philips Medical System HDI 5000 ultrasound machine (Philips Ultrasound, Bothell, WA, USA). Colour Doppler was used as necessary to identify vessels. Light pressure was applied to the arm with the ultrasound transducer to identify the artery. Veins will collapse with pressure, while arteries retain their size and shape. If necessary pulsed Doppler was used to further identify the artery as pulsatile flow will be displayed while veins demonstrate monophasic flow (80). The use of pulsed Doppler was sometimes necessary in large women. The transducer was then rotated 90 degrees 1-10cm above the cubital fossa to display a longitudinal section of brachial artery. The distance in centimetres from the cubital fossa and a still image demonstrating recognisable anatomical landmarks such as another vessel or fascia plane was recorded (Figure 2.3). These were then used to identify and measure the same section of artery for each visit. The arm was marked with an indelible ink pen to mark the distal border of the transducer so the same section of artery was scanned throughout the examination. The image was zoomed and optimised by decreasing the gain in the area of the brachial artery. This would minimise acoustic noise.



Figure 2.3: The 2D ultrasound images demonstrating brachial artery under basal conditions and post cuff release. Note the increase in size of brachial artery from 2.7mm to 3.2mm which is used to calculate FMD. The ECG tracing used to measure brachial artery diameter incident with the R-wave can be seen at the base of the images.

A single focal zone was set to give optimal resolution. SonoCT® (Philips Ultrasound, Bothell, WA, USA) which is real-time compound imaging was activated to improve resolution. Ultrasound settings, especially focal zone, gain and dynamic range were not changed throughout the examination, a practice that was verified by a later study (104). The entire study was recorded on video tape.

A basal arterial diameter was recorded for one minute using 2D ultrasound. Basal arterial flow velocity (VTI) was recorded using pulsed Doppler ultrasound and a centrally placed 1.5mm range gate at a 60 degree angle with a minimum of six cardiac cycles obtained. The High Q function (the blue tracing on the Doppler waveform) which allows automatic reporting of VTI and heart rate and is displayed in the bottom left corner of the ultrasound image (Figure 2.4) was activated. A BP cuff inflation pressure of 200mmHg was chosen for normotensive pregnant and non-pregnant women. This was based on the Doppler stethoscope work (120, 121) and the work demonstrating subject tolerance at pressures of 200mmHg (125). An automated BP cuff (Hokanson E20 Rapid Cuff Inflator, D.E. Hokanson, Inc. Bellevue, WA, USA) was placed on the right forearm and inflated for five minutes at 200 mmHg (Figure 2.2) to occlude flow to the arm (Figure 2.5). This was released to induce reactive hyperaemia (Figure 2.6). Just before cuff release, Doppler imaging was activated and flow recorded until maximum VTI was reached (Figure 2.7) and the hyperaemic response began to decay. This occurred within 30 seconds of cuff release. Peak PO VTI was documented. 2D imaging was re-activated and PO arterial diameter was recorded for up to 90 seconds from the time of cuff release (Figure 2.3). Ultrasound imaging using 2D technology has been shown to be a safe imaging modality. The highest ultrasound dose is derived from pulsed Doppler (80). In this work pulsed Doppler was used for the minimum amount of time necessary.

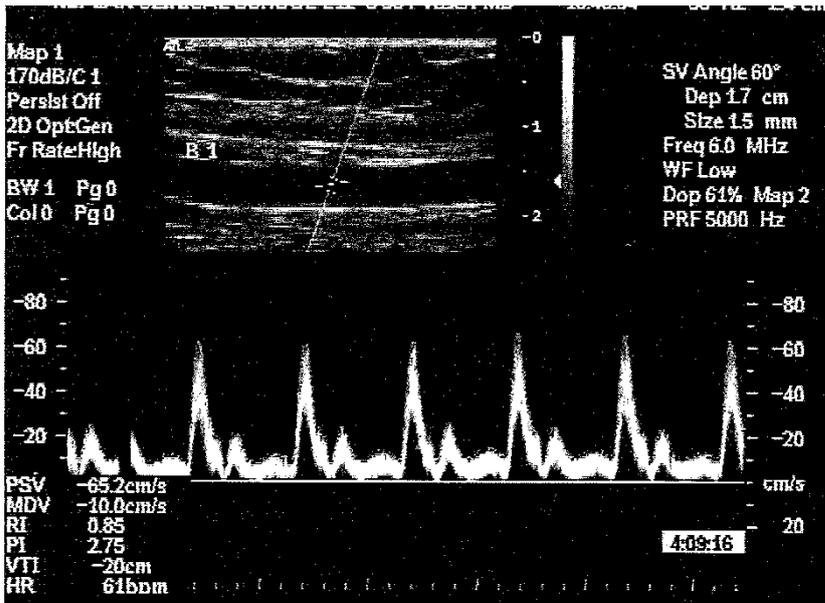


Figure 2.4: Typical Doppler waveform image demonstrating brachial artery basal VTI and heart rate. Sample gate placement can be seen in 2D part of image superiorly.

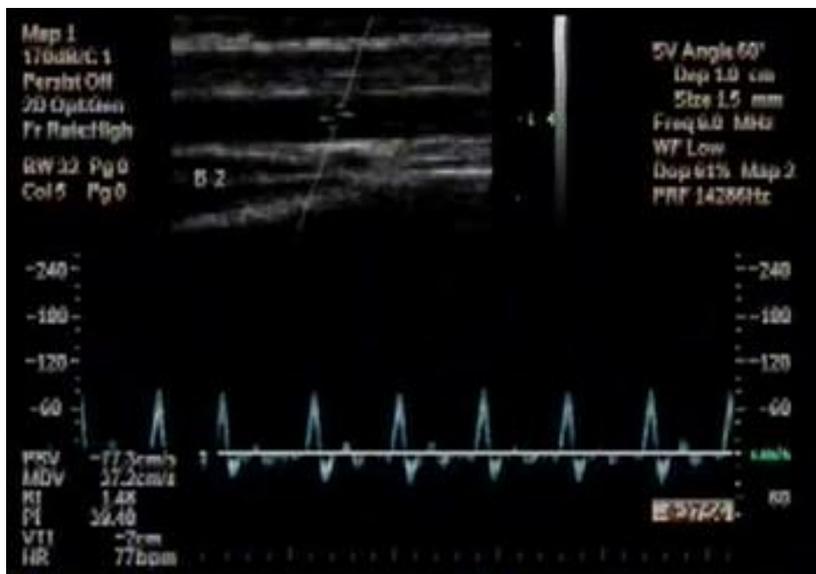


Figure 2.5: Doppler waveform demonstrating almost absent flow in brachial artery during occlusion by cuff.

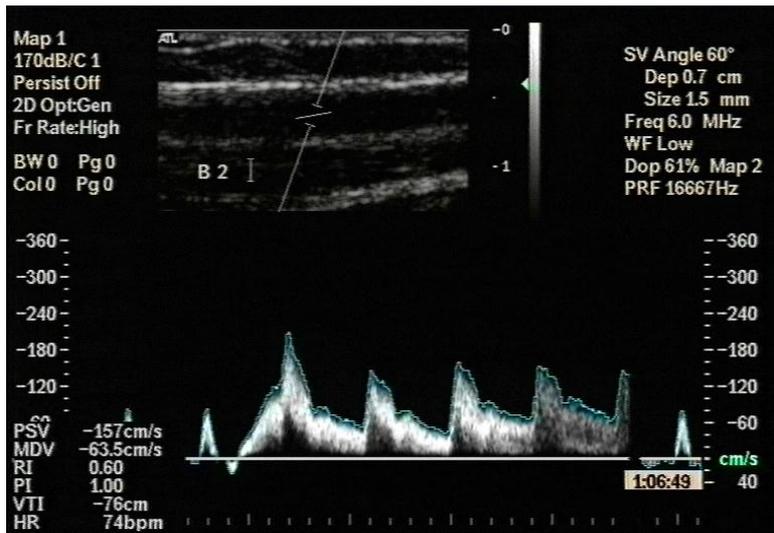


Figure 2.6: Doppler waveform image at time of cuff release. Note reduced flow at left of picture and immediate increase in flow that occurs at time of cuff release.

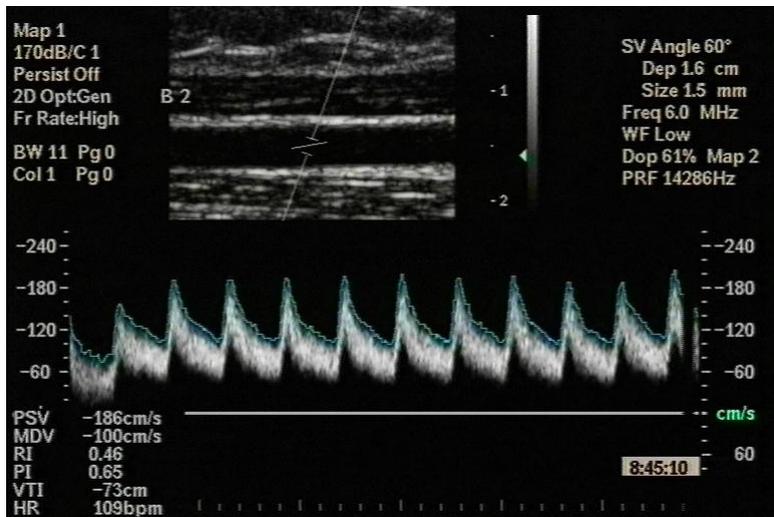


Figure 2.7: Doppler waveform image taken during reactive hyperaemia. Note increase in flow compared to figures 2.4 and 2.5.

Basal and PO vessel diameters were each measured manually with the ultrasound machine calipers, offline, from the videotape. This requires downloading of the image from the video recorder onto the ultrasound machine and recalibration of the image at the beginning of each study. Measurements were taken four times at end diastole (incident with the R-wave on the ECG) from a fixed anatomical landmark and the mean calculated (Figure 2.3). Measurements of PO artery diameters were done between 45-60 seconds post cuff release. The lumen diameter was measured by placing calipers from the anterior and posterior arterial wall 'm' line, or the intima-media complex (110), whichever was the best resolved. The same criterion for measuring was consistent throughout each individual's study. Mean heart rate was calculated at baseline and during reactive hyperaemia from six cardiac cycles from the Doppler waveform. All studies and measurements were done by the author (AEQ).

Flow-mediated dilatation was calculated from the formula:

$$\text{FMD\%} = [(\text{PO arterial diameter} - \text{basal arterial diameter}) / \text{basal arterial diameter}] \times 100 \quad (16).$$

Basal volume flow (mL/min) was calculated from the formula:

$$\text{Basal flow} = \text{VTI} \times \text{basal heart rate} \times (0.5 \times \text{basal vessel diameter})^2 \times \pi \times (1/\cos 60)$$

where VTI was calculated from the mean of six cardiac cycles (207, 208).

Post occlusion volume flow (mL/min) was calculated from the formula:

$$\text{PO flow} = \text{PO peak VTI} \times \text{PO heart rate} \times (0.5 \times \text{PO vessel diameter})^2 \times \pi \times (1/\cos 60)$$

where PO peak VTI was the maximum VTI reading obtained after cuff release (207, 208).

Reactive hyperaemia (RH) or peak flow increase was calculated from the formula: **RH% = [(PO peak flow – basal flow)/basal flow] x 100** (207, 208).

2.2.3 Comments on the FMD technique.

The technique of FMD is difficult to learn and requires experienced trained personnel to perform it. For the purpose of this work a number of visits were made to the laboratory of Professor David Celermajer to learn the technique. At least 30 volunteers (work colleagues, friends and family) were scanned to perfect the technique prior to the commencement of this work. A number of limitations have been identified when performing the FMD test. Arm and transducer movement are a problem. Slight movement of either can result in different sections of artery being measured. In recent times transducer clamps and arm cushions have been designed to try and overcome this although these were not available for this work. The brachial artery narrows as it travels distally (Figure 2.8). If different sections of the artery are inadvertently measured this could result in erroneous FMD and volume flow measures.

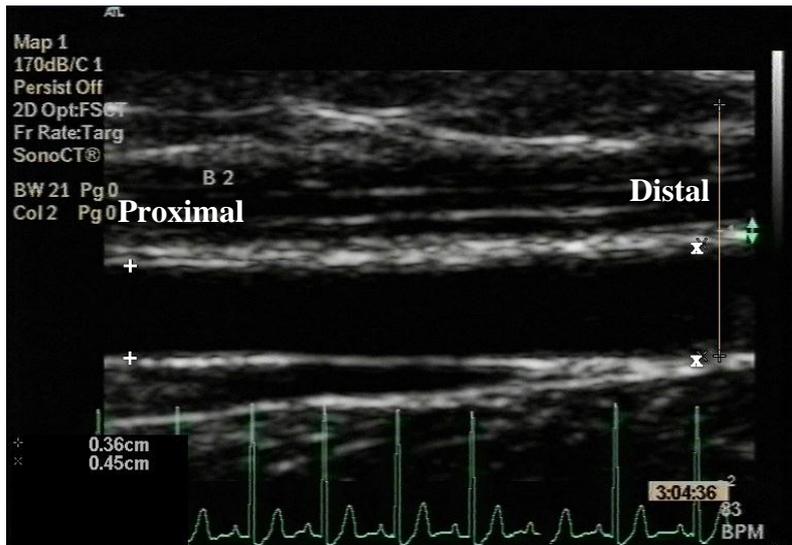


Figure 2.8: The brachial artery narrows as it travels distally, note the difference in artery diameter proximally (4.5mm) and distally (3.6mm). This demonstrates how measuring different sections of artery may result in incorrect measurements.

Even though a high resolution ultrasound transducer was used, the walls of the brachial artery can be hard to resolve. In thin women the anterior wall 'm' line is well seen, however the posterior wall 'm' line may not be resolved (Figure 2.9). In some women both walls of the artery are difficult to resolve (Figure 2.10). Some of this problem is due to the depth of the artery in larger women and attenuation of the ultrasound beam. Another cause is slice thickness artifact. The ultrasound beam is three-dimensional with axial and lateral resolution occurring in the scanning plane. The third plane is the transducer beam slice thickness. The ultrasound image is two dimensional and when an anechoic structure is scanned that is smaller than the slice or section thickness of the ultrasound beam artificial filling of the structure occurs. This artifact results in echoes outside of the actual structure appearing as echoes within the structure (80). The brachial artery appears as an anechoic tubular structure. Figures 2.11 and 2.12 demonstrate difficulty in clearly

resolving the anterior artery wall due to slice thickness artifact. Multiple slice thickness artifact echoes within the artery lumen can also be seen (Figure 2.12).

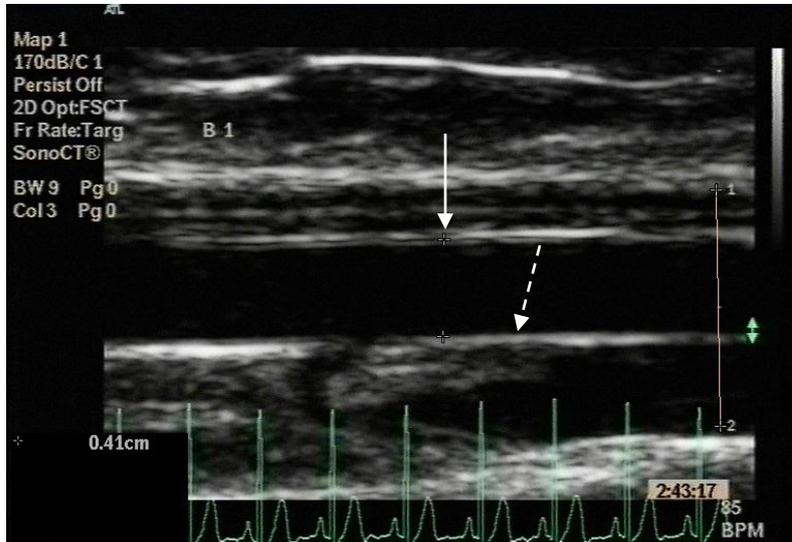


Figure 2.9: The 'm' line is resolved at the artery wall nearest to the transducer (arrow), however it cannot be seen at the far wall (dashed arrow).

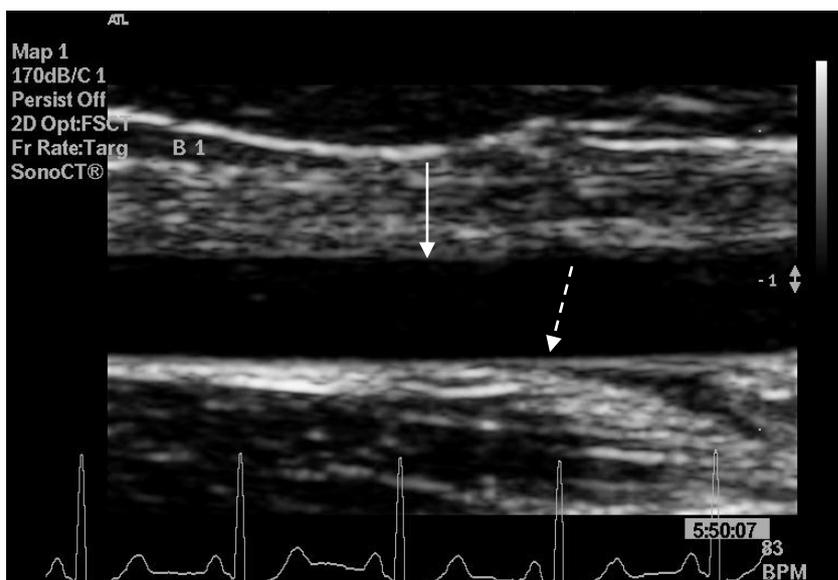


Figure 2.10: The 'm' line is not resolved in either the near (arrow) or far (dashed arrow) artery wall.

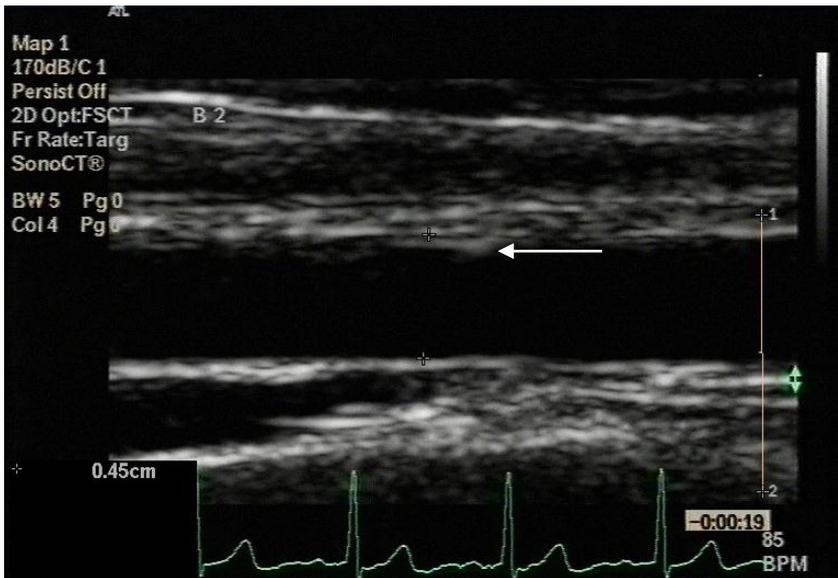


Figure 2.11: Slice thickness artifact makes the near artery wall difficult to resolve due to the irregular interface (arrow).

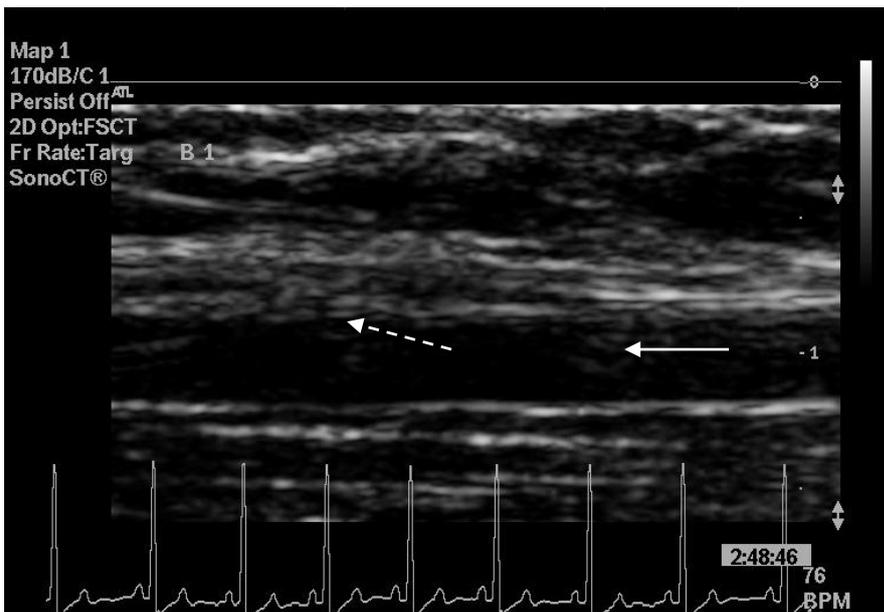


Figure 2.12: Slice thickness artifact results in echoes within the anechoic artery (arrow) and irregular, difficult to resolve artery borders (dashed arrow).

2.2.4 Statistical analysis.

Statistics were performed using the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL). For continuous normally distributed data, parametric analysis included independent t-tests, repeated measures analysis of variance (RMANOV) with a post hoc contrast polynomial trend analysis for linear and non-linear trends, or pairwise comparisons, or analysis of variance where appropriate. For data that were not normally distributed, non-parametric tests of Mann-Whitney U and Friedman matched sample were used. Correlation analysis was performed using Pearson Correlation test. Data is reported as mean \pm SD where the data is normally distributed and mean and SEM where data is not normally distributed. A Bonferroni correction was used for post hoc comparisons of repeated measures with a P-value of 0.01 used. For all other tests significance was assumed when $P \leq 0.05$.

2.3 Results.

More than 300 studies were performed to obtain a normal range. Eighteen non-pregnant women and 53 pregnant women were enrolled. Three women who delivered normal term babies but missed one study have been included in the analysis. Six pregnant women met the exclusion criteria resulting in 47 women in the statistical analysis. Women were excluded for preterm delivery (n=3), severe early onset pre-eclampsia/HELLP syndrome delivered at 28+4 weeks (n=1), gestational hypertension at term (n=1) and gestational diabetes (n=1). Seventy percent (33/47) of women returned for the postpartum visit. Table 2.1 shows the exact gestational ages at which the studies were performed.

There was no significant difference at enrolment between the pregnant and non-pregnant women in age (28.4 ± 4.8 vs. 32.1 ± 8.7 years; pregnant range 20-40 years, non-pregnant range 15-45years), height (165 ± 6.9 vs. 166 ± 6.4 centimetres), weight ($70.4 (15.7)$ vs.

68.4 (18.9) kilograms) or body mass index (25.9 (5.8) vs. 24.8 (5.9)). Gestational age at delivery in weeks was 40 ± 1.2 and birth weight in grams was 3484 ± 395 .

Table 2.1: Study data for non-pregnant, pregnant and postpartum women. Values are reported as $m \pm SD$ or mean (SEM).

	<i>Non-pregnant</i>	<i>Gestation (weeks)</i>					<i>Postpartum</i>
	<i>(n=18)</i>	<i>11-14 (n=46)</i>	<i>18-20 (n=47)</i>	<i>22-24 (n=47)</i>	<i>28-32 (n=47)</i>	<i>36+ (n=45)</i>	<i>(n=33)</i>
Actual gestation (weeks)		12.8 ± 0.9	18.8 ± 0.5	23.1 ± 0.6	30 ± 0.9	36.4 ± 0.4	6.6 ± 0.7
Basal artery diameter (mm)	3.4 ± 0.4	3.2 ± 0.3	3.2 ± 0.4	3.3 ± 0.4	3.4 ± 0.4	3.5 ± 0.4	3.3 ± 0.4
Post occlusion artery diameter (mm)	3.7 ± 0.4	3.4 ± 0.4	3.5 ± 0.4	3.6 ± 0.3	3.7 ± 0.4	3.8 ± 0.4	3.6 ± 0.4
FMD (%)	8.5 ± 3.6	8.0 ± 4.6	9.4 ± 5.3	8.9 ± 5.4	9.3 ± 5.0	6.7 ± 5.2	8.2 ± 3.9
Basal volume flow (mL/min)	170 (26)	135 (15)	182 (22)	256 (27)	342 (31)	425 (32)	259 (19)
Post occlusion volume flow (mL/min)	1138 (91)	1139 (52)	1156 (45)	1321 (46)	1529 (66)	1520 (58)	1261 (54)
Reactive hyperaemia (%)	913 (160)	1436 (259)	1121 (163)	857 (140)	650 (141)	400 (79)	464 (47)
Systolic BP (mmHg)	115 ± 8	110 ± 8	111 ± 8	114 ± 8	114 ± 8	117 ± 8	113 ± 8
Diastolic BP (mmHg)	75 ± 7	72 ± 8	69 ± 8	71 ± 8	73 ± 6	78 ± 8	77 ± 8
Basal heart rate (bpm)	63 ± 10	75 ± 10	78 ± 8	82 ± 10	88 ± 11	89 ± 11	75 ± 10

Basal heart rate increased significantly throughout pregnancy (RMANOV) ($P < 0.001$). Whilst there was a significant difference between heart rate in the first trimester and the other gestations, there was no significant difference when comparing heart rate at 28-32 weeks with 36+ weeks (Table 2.1). Systolic and diastolic blood pressures were within the normotensive range for uncomplicated pregnancy. Systolic BP increased linearly over time (RMANOV) ($p < 0.001$). Diastolic BP showed a significant quadratic decrease between 11 and 20 weeks gestation ($p = 0.004$), then increased linearly over the remainder of the pregnancy ($p < 0.001$) (RMANOV) (Table 2.1).

Basal brachial artery diameter showed a significant increase over the study period (RMANOV) ($P < 0.001$) (Figure 2.13). Post hoc pairwise comparison demonstrated basal arterial diameter increased significantly from the first two visits (11-20 weeks) compared with the last two visits (28-36+ weeks) (Table 2.1). The trend was similar for the PO arterial diameter (RMANOV) ($P < 0.001$) (Table 2.1) (Figure 2.13). Brachial artery diameter was inversely correlated with FMD ($r = -0.447$, $P < 0.0001$) (Figure 2.14).

Figure 2.13: Basal and post occlusion artery diameters (mean \pm SEM) in non-pregnant, pregnant and postpartum women.

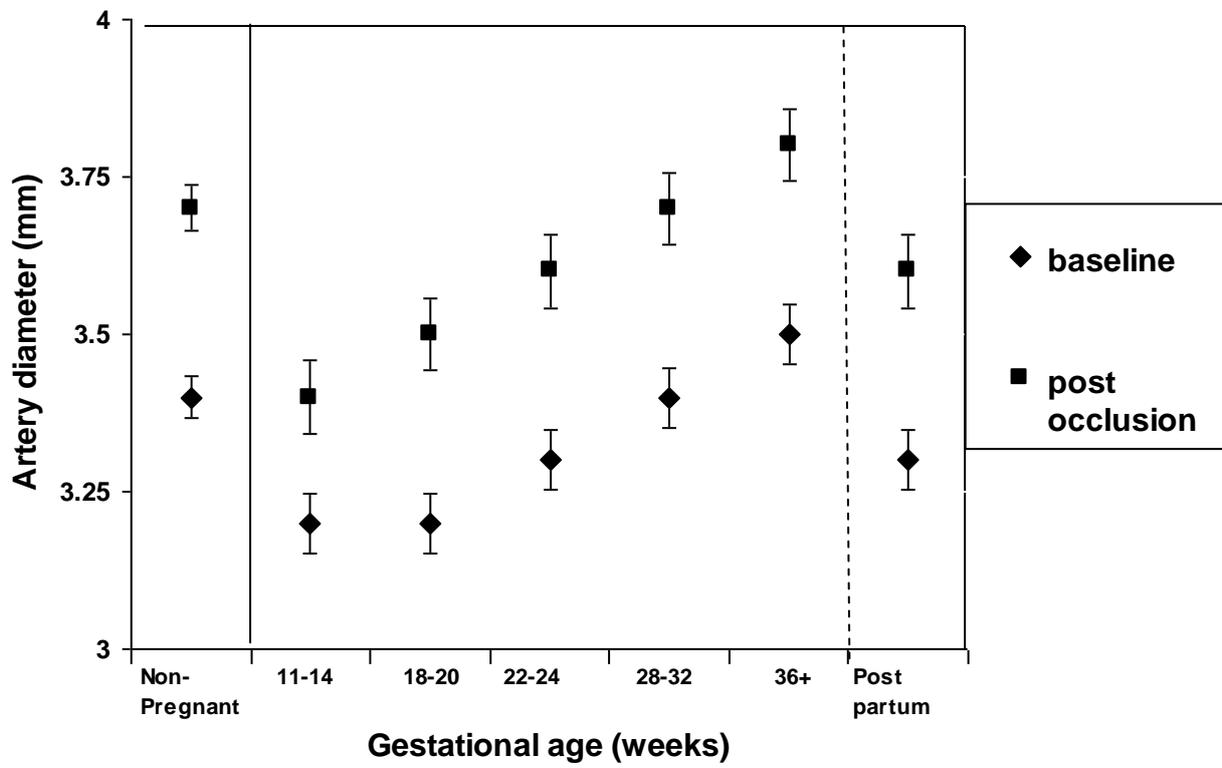
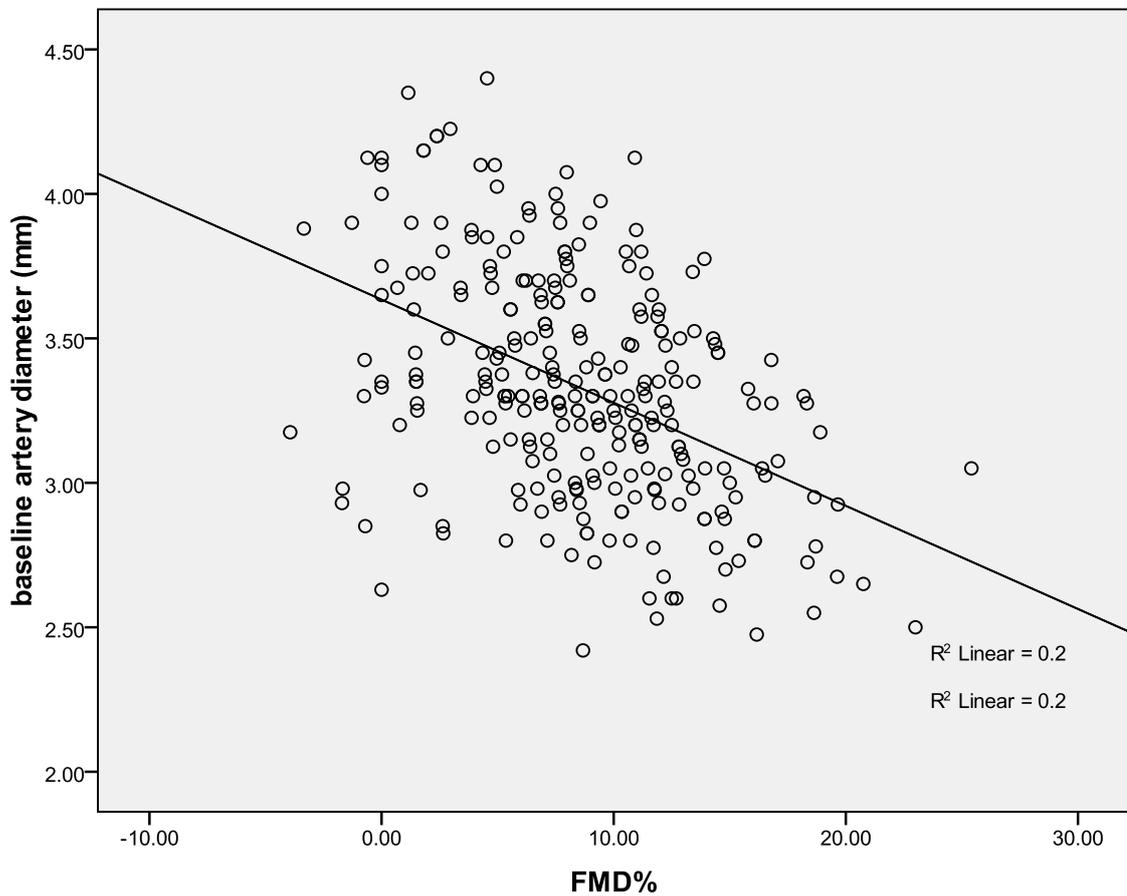


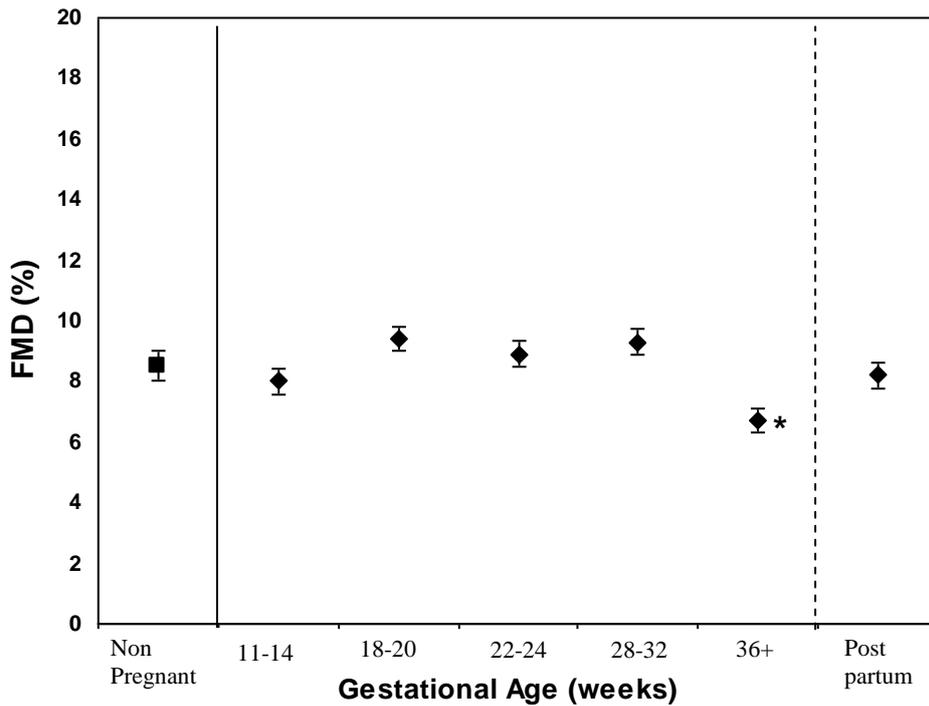
Figure 2.14: Graph demonstrating the relationship between FMD and brachial artery diameter in normal pregnant women ($r = -0.447$, $P < 0.0001$).



Repeated measures analysis of variance of FMD in the pregnant women demonstrated a significant change throughout the study period ($P=0.02$). Post hoc contrast polynomial trend analysis showed a non-significant linear component in FMD from 11-32 weeks gestation ($P=0.2$) and a significant quadratic component from 36+ weeks gestation ($P=0.007$). Analysis of variance demonstrated no significant difference in FMD between non-pregnant women and the pregnant women at any gestation or postpartum ($P=0.13$). A repeated measures sub-analysis of FMD comparing pregnant women throughout the study

and postpartum showed no significant difference ($P=0.094$) although the numbers were smaller ($n=33$) (Figure 2.15) (Table 2.1).

Figure 2.15: Flow-mediated dilatation (mean \pm SEM) in non-pregnant, pregnant and postpartum women. (*denotes significant value for FMD at 36+ weeks gestation, $P=0.007$).



Basal volume flow increased linearly with increasing gestation ($P<0.001$) (Figure 2.16) as did PO volume flow ($P<0.001$) (Table 2.1), whilst the degree of reactive hyperaemia decreased with increasing gestation ($P<0.001$) (Friedman matched samples) (Table 2.1) (Figure 2.17). The postpartum women had significantly increased basal volume flow compared with the non-pregnant women ($P=0.009$) (Table 2.1), although there was no significant difference in PO volume flow between the two groups ($P=0.8$) (Table 2.1) (Mann-Whitney U).

Figure 2.16: Basal volume flow (mean \pm SEM) in non-pregnant, pregnant and postpartum women.

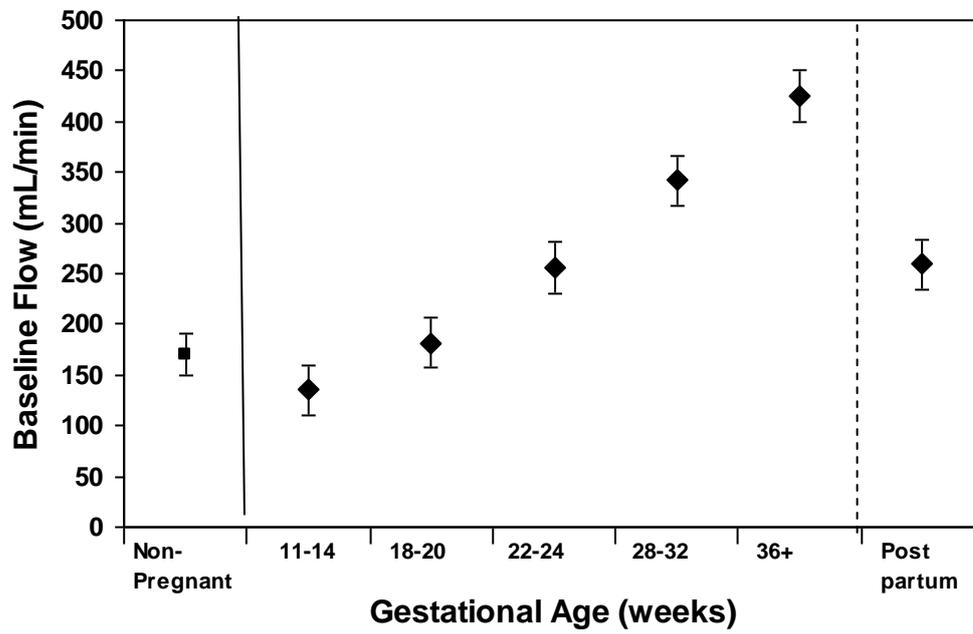
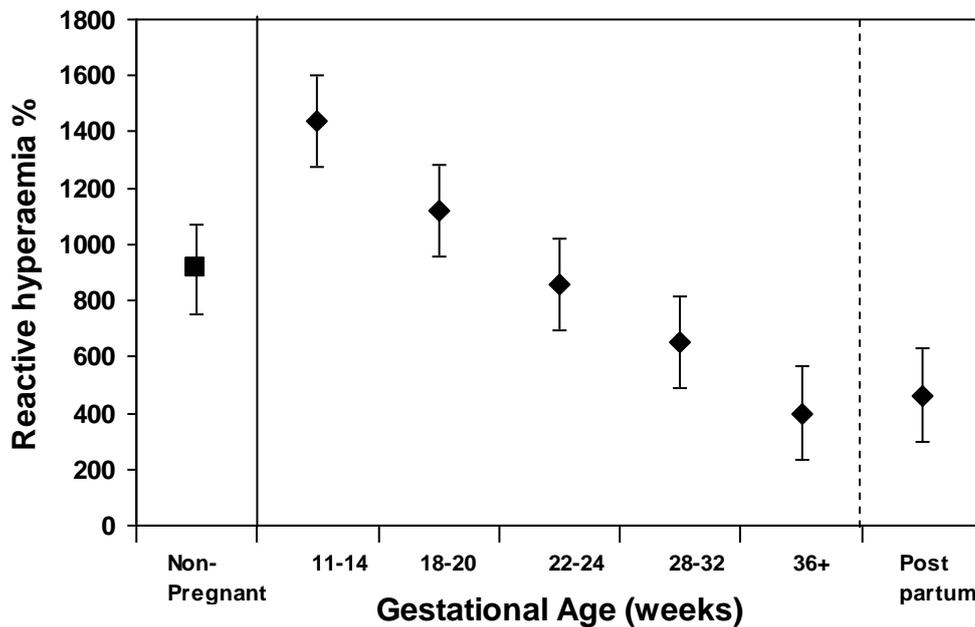


Figure 2.17: Reactive hyperaemia (mean \pm SEM) in non-pregnant, pregnant and postpartum women.



2.4 Discussion.

This study demonstrates that FMD remained stable in normal human pregnancy from 11-14 weeks up to 32 weeks gestation. Flow-mediated dilatation decreased significantly at 36+ weeks gestation. This decrease has not been previously reported. Flow-mediated dilatation then returned to the non-pregnant value by six weeks postpartum. There was no significant difference in FMD in pregnancy compared with the non-pregnant group. Basal brachial artery diameter and basal volume flow increased significantly in a linear fashion throughout pregnancy whilst reactive hyperaemia decreased.

Flow-mediated dilatation is a technique that is modulated by NO. Nitric oxide is synthesised from the essential amino acid L-arginine via an oxidation process to form NO and L-citrulline using the enzyme NOS. Nitric oxide then diffuses into the smooth muscle

cell membrane, binds to guanylate cyclase activating cGMP and resulting in vasodilatation. Nitric oxide is produced constitutively to maintain vascular tone and blood pressure (26).

Induced reactive hyperaemia stimulates vasodilatation with FMD being an indicator of stimulated endothelial function (16). The lack of difference in FMD demonstrated in our study between non-pregnant and pregnant women may be because basically the brachial artery is already dilated. This could occur because in normal pregnancy there is increased sensitivity to shear stress which is mediated by NO (263).

Endothelial dysfunction is reported when FMD is reduced (16). In our study FMD decreased when approaching term. This suggests when assessing FMD in the third trimester, analysis should be divided into early third trimester (28-32 weeks) and late third trimester (36+ weeks) or gestational age should be entered into the analysis as a confounder. This would be important when assessing FMD in pathological conditions such as pre-eclampsia. We have established reduced FMD is part of the normal physiology of late pregnancy. This decrease is also consistent with normal pregnancy NO system activity. Human studies have demonstrated NOS and cGMP levels are at their highest during pregnancy, are down-regulated at term and in labouring women (526-528). This would equate with uterine quiescence during pregnancy and the initiation of labour at term.

Alternatively the decrease in FMD may indicate the NO system is less responsive to shear stress in late pregnancy due either to the increase in vessel size or the brachial artery reaching maximum dilatation. The decrease in FMD with large arteries shown by us (Figure 2.14) and others (16, 107, 207, 208, 535, 540) by the negative correlation between

FMD and artery diameter occurs because of differences in shear stress, not because of endothelial dysfunction (108). This would make the reporting of the shear stress stimulus (usually by reporting volume flow) an important factor. This was not done in a number of studies assessing FMD in normal pregnancy (532-535, 540).

Consideration of artery size should be made when interpreting the results as it impacts on FMD. As obese people have larger arteries (348) and obesity correlates negatively with FMD in non-pregnant people (192, 193) and pregnant women (350-352) the subjects' size could also be a confounder. In our study the women were not in the obese range.

The decrease in peripheral vascular resistance in pregnancy is thought to be mediated by NO. The measurement of NO levels in pregnancy has produced conflicting results with no change (522-525) or increases (520, 521) in nitrate and nitrite levels reported. This may be due to variability in technique and maternal diet when measuring metabolites (522). Our study showed a progressive increase in basal volume flow throughout normal pregnancy which would result in increased shear stress on the endothelial cells and would augment basal NO release. Similar results were demonstrated in two other studies (207, 208).

Using the technique of plethysmography basal blood flow to the hand was significantly greater in the third trimester of pregnancy compared to first trimester and non-pregnant women. This was thought to be due to NO release because infusion of the NOS inhibitor L-NMMA resulted in a greater reduction in flow in pregnancy (7). This work was later replicated by others (531). Normal pregnancy results in profound changes in the cardiovascular system (484). The increase in heart rate and alterations in blood pressure demonstrated in our study, especially the nadir in diastolic BP in the early second trimester (Table 2.1) are consistent with these changes.

Other studies assessing FMD in normotensive pregnancy have demonstrated either no difference between pregnant and non-pregnant women (535, 536) similar to our results or an increase in FMD in pregnancy (208, 532-534, 540). Most studies have been cross-sectional with only two using a longitudinal design (Table 1.1). The two longitudinal studies were small in number, with one study combining the whole of the third trimester as one group (207). The other work recruited until 32 weeks gestation (533). Thus neither study appreciated the fall in FMD in late pregnancy. Savvidou et al. (208) in a cross-sectional study reported similar results to our own with FMD increasing until 30 weeks and then decreasing although they only reported mean FMD values up to 30 weeks in their analysis.

Cross-sectional studies assessing FMD over the three trimesters also had varied results with two demonstrating an increase compared to non-pregnant women (207, 540) and one showing no difference (535). Other cross-sectional studies assessed FMD at only one point in time in pregnancy (532, 534, 536).

As previously reviewed differences in the results may be due to variations in methodology such as cuff placement (532, 534), recruitment of smokers (535) and PO vessel diameter measured at different times (Table 1.1). At the time most of these studies were performed and designed (including our own) the recommended time for measuring PO diameter was between 45-60 seconds (16, 96).

A potential limitation of this study is the small number of non-pregnant women recruited (n=18) as many young women are either using contraceptives or pregnant. As not all the women returned for the postpartum visit this limited the statistical analysis and may have

contributed to the non-significant result when comparing FMD during pregnancy and postpartum. We also did not use an endothelial independent dilator such as sodium nitroprusside or nitro-glycerine to assess differences in endothelial dependent and independent dilatation or a NOS inhibitor such as L-NMMA. We did not think this appropriate due to the multiple studies performed on each pregnant woman. In pregnancy endothelial dependent FMD and smooth muscle vasodilatation were demonstrated to be independent of each other using nitro-glycerine (207).

True volume flow is difficult to measure and can present a number of sources of error. To calculate volume flow a constant angle of 60 degrees and a circular cross section of vessel is assumed which cannot be guaranteed. A small sample gate was used and sampling occurred from the centre of the vessel. This would increase the calculated volume flow. However we were interested in looking at relative changes over time in our normal range study. While the volume of blood flowing through the brachial artery may not be absolute, the trend did show a statistically significant increase in volume blood flow throughout pregnancy.

In conclusion, this technique was well tolerated by all the participants and is a safe, non-invasive test for the pregnant woman and her fetus. This large longitudinal study on a healthy pregnant population will provide a foundation for future studies assessing FMD in pre-eclampsia and gestational hypertension in pregnancy. This study has demonstrated that FMD in normal human pregnancy does not differ to the non-pregnant state. In pregnancy there is no change in FMD up to 32 weeks gestation then it decreases significantly at 36+weeks.

Chapter 3

The relationship between cigarette smoking, endothelial function and intrauterine growth restriction in human pregnancy.

3.1 Introduction.

Nearly a fifth of Australian women continue to smoke in pregnancy (686) despite the known dose dependent increased risk of vascular complications in otherwise low risk women (608, 632). Smoking in pregnancy results in complications at all stages. In early pregnancy, smoking amplifies the incidence of miscarriage (621, 624). These early miscarriages may occur because of apoptosis and decreased invasion of the cytotrophoblast with a resulting increase in placental dysfunction (620). Once a pregnancy is ongoing, smoking increases the risk of preterm birth (608), placental abruption (601) and placenta praevia (602). Women who smoke in pregnancy are also at increased risk of delivering babies with IUGR (603, 627) and increased perinatal morbidity and mortality (601, 603). All these complications are thought to result from a reduction in blood flow and decreased perfusion to the placenta with resultant placental injury. Indeed, epidemiological research suggests that smoking in pregnancy induces vascular dysfunction in the uterus (620).

Even though smoking has numerous deleterious effects on pregnancy it has been shown to have a protective effect for pre-eclampsia (602) with a 32% dose dependent reduction in risk (519). This is despite the fact that pregnant smokers who develop pre-eclampsia have a poorer outcome with increased rates of perinatal mortality, SGA and placental abruption (601). Smoking in pregnancy is also known to induce endothelial cell injury (651) and results in reduced NO levels (687). This presents an interesting conundrum on the effects of smoking on the vascular endothelium in pregnancy, whether it affects endothelial function and whether endothelial function would differ after chronic or acute cigarette smoke exposure.

In non-pregnant people smoking results in reduced dilatation of the vascular endothelium, that is endothelial dysfunction (177). Other studies have demonstrated no difference in endothelial function between chronic smokers and non-smokers, but after acute cigarette or nicotine product use reduced FMD occurred (183, 184). As pre-eclampsia is hypothesised to result in endothelial dysfunction (2) and smoking decreases the risk of pre-eclampsia it would seem reasonable to suggest that chronic or acute smoking in pregnancy may improve endothelial function.

We therefore hypothesised that smoking in pregnancy may provide a protective effect on the vascular endothelium by demonstrating no difference in vasodilatory endothelial function when compared to non-smoking pregnant women. The aim of this work was to compare the chronic and acute effects of cigarette smoking on endothelial function in pregnant women with non-smoking pregnant women.

3.2 Methodology.

This study recruited pregnant women who stated they smoked 10 or more cigarettes a day and compared them to pregnant women who did not smoke. The women were studied between 28-32 weeks gestation. All women were asked to have a low fat breakfast and refrain from caffeine drinks. Pregnant smokers were asked to abstain from cigarettes from midnight. Women with any existing medical conditions were not recruited.

Endothelial function was assessed by the ultrasound technique of flow-mediated dilatation (FMD) as described in chapter 2. The women had a third trimester fetal well-being ultrasound immediately before the study and their FMD test commenced at 9am. At the completion of the first test the women had a 10-15 minute break during which time the

smokers could choose to have none, one or two cigarettes. All the participants could have a non-caffeine drink. The FMD test was then repeated in the same manner on all volunteers after 10 minutes of rest to allow for acclimatisation. The break and the repeat test for the non-smoking women were to measure test-retest reliability. Height, weight and blood pressure measurements were taken prior to testing with blood pressure re-measured after the break. All studies and measurements were done by the author (AEQ). Approval for this project was given by both the Area Health Service and University Ethics Committees and written informed consent was obtained for enrolment.

3.3 Statistical analysis.

Statistics were performed using the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL). For normally distributed data, independent t-tests were used with data reported as mean \pm standard deviation ($m \pm SD$). A Mann-Whitney U analysis was used for continuous, non-normal variables and results reported as median and interquartile range ($m [IQR]$). For analysis of the test-retest in the two groups, a mixed between-within subjects analysis of variance was performed. Categorical data were analysed using Chi-square or a Fishers Exact Test if cell size was <5 . A significance value of $P \leq 0.05$ was used.

3.4 Results.

Twenty-two women who smoked in pregnancy and 20 non-smoking pregnant women were enrolled. One woman who smoked was excluded from analysis as she chose not to have a cigarette in the break. All participants stated no alcohol intake during pregnancy and only one woman (a smoker) used marijuana occasionally. Women confirmed their consumption of a caffeine free low fat breakfast. The smoking group also confirmed their smoking abstinence, with several women commenting they had remained awake to have a cigarette

and coffee at midnight. There was no financial or other incentive for the women to participate in this study.

There were no significant differences in participant characteristics, including physiological parameters, at enrolment between the women who smoked (n=21) and the women who did not smoke (n=20) except for parity (Table 3.1). There were less nulliparous women in the smoking group. In all, six women who smoked had complications in a previous pregnancy (Table 3.1). There were no significant differences in the fetal parameters of head circumference, abdominal circumference and umbilical artery Doppler waveforms at the time of study (Table 3.1). The self-reported mean number of cigarettes smoked per day was 15.7 ± 4.9 (range 10-30), with 19 of the women choosing to smoke one cigarette in the break, and two choosing to smoke two cigarettes.

Table 3.1: Basal participant characteristics in smokers and non-smokers. Values are reported as $m \pm SD$ or median [IQR].

	<i>Smoker (n=21)</i>	<i>Non-smoker (n=20)</i>	<i>P-value</i>
Maternal Age	26.6 \pm 5.8	27.5 \pm 5.1	ns
Nulliparous	7/21 (33%)	15/20 (75%)	0.007
Past obstetric history:			
Pre-eclampsia (PE)	3/14 (21%)	0	ns
IUGR#	4/14 (29%)	0	ns
Fetal death in-utero (FDIU)†	2/14 (14%)	0	ns
Body Mass Index	28.9 \pm 5.9	29.8 \pm 5.6	ns
Basal brachial artery diameter (mm)	3.5 \pm 0.4	3.4 \pm 0.4	ns
Maternal heart rate (bpm)	83 \pm 9.2	79 \pm 9.3	ns
Systolic BP (mmHg)	113 \pm 11.0	111 \pm 9.9	ns
Diastolic BP (mmHg)	70 \pm 9.5	71 \pm 8.3	ns
Gestational Age (weeks)	29.3 \pm 1.3	29.1 \pm 1.0	ns
Basal volume flow (mL/min)	346 [171-512]	252 [152-385]	ns
Umbilical artery Doppler Pulsatility Index	1.1 \pm 0.2	1.1 \pm 0.1	ns
Fetal head circumference (mm)	276 \pm 26	273 \pm 11	ns
Fetal abdominal circumference (mm)	250 \pm 14	257 \pm 15	ns

two fetuses IUGR plus PE. †one fetus FDIU plus IUGR plus PE.

In the women who smoked the baseline study FMD was significantly lower compared with the women who did not smoke ($P < 0.001$) (Table 3.2). At the retest, after the women in the smoking group had a cigarette/s, this significant difference in FMD persisted ($P < 0.001$) (Table 3.2). The initial FMD calculated when the women abstained from cigarettes for nine hours displayed no significant difference compared with the FMD after smoking 1-2 cigarettes (Table 3.2). There was also no significant difference in FMD in the non-smokers between the baseline study and after a 15 minute break (Table 3.2). Smoking a cigarette significantly increased the maternal physiological variables of heart rate and systolic blood pressure (Table 3.3). There was no significant difference in heart rate or blood pressure after the break in the non-smoking group (Table 3.4).

Table 3.2: FMD ($m \pm SD$) in pregnant women who smoke versus pregnant women who are non-smokers.

FMD %	Smokers (n=21)	Non-smokers (n=20)	P value
Test	4.0 \pm 2.3	9.7 \pm 4.0	<0.001*
Retest	4.4 \pm 2.5	9.6 \pm 3.8	<0.001*
P-value	ns#	ns#	

* Denotes between groups main effect. # denotes within group interaction effect.

Table 3.3: Maternal physiological variables before and after cigarette smoking ($m \pm SD$).

Maternal characteristics	Baseline (test)	Post cigarette (retest)	P-value
Heart rate (bpm)	83 \pm 9.2	94 \pm 10.5	<0.001
Systolic BP (mmHg)	113 \pm 11.2	117 \pm 11.9	0.009
Diastolic BP (mmHg)	70 \pm 9.5	73 \pm 11.6	ns

Table 3.4: Maternal physiological variables in non-smokers before and after break ($m \pm SD$).

Maternal characteristics	Baseline (test)	Post break (retest)	P-value
Heart rate (bpm)	79 \pm 9.3	78 \pm 10.5	ns
Systolic BP (mmHg)	111 \pm 9.9	111 \pm 10.0	ns
Diastolic BP (mmHg)	71 \pm 8.3	70 \pm 8.0	ns

At delivery there was no significant difference in gestational age between the two groups (smokers: 39.2 ± 1.6 versus non-smokers: 39.9 ± 1.2 weeks). However, birth weight was significantly decreased in the smoking group (3090 ± 596 g) compared to the non-smoking group (3501 ± 396 g) ($P=0.014$). Growth restriction was evident in the babies of smokers with 38% (8/21) less than the 10th percentile ($P=0.003$; Fishers Exact) and 24% (5/21) less than the 5th percentile ($P=0.048$; Fishers Exact). Of particular interest, babies whose birth weight was less than the 10th percentile were born to mothers with a significantly lower FMD of 4.7 ± 2.2 compared to those whose birth weight was greater than the 10th percentile (maternal FMD 7.3 ± 4.6 ($P=0.03$)). There were no small for gestational age babies in the non-smoking group. Only one woman (a smoker) developed gestational hypertension at term.

3.5 Discussion.

This study clearly demonstrates that smoking in pregnancy results in significantly reduced FMD indicating endothelial dysfunction. We believe this is the first study to use the technique of FMD to report the effects of smoking on endothelial function in pregnant women. Flow-mediated dilatation in the non-smoking women was the same as reported in the normal range study (Chapter 2).

The endothelium has many functions including control of vascular homeostasis by modulating vascular tone, vessel size and the regulation of blood flow. The endothelium also inhibits inflammation, counteracts the activation of platelets and inhibits proliferation of the vascular smooth muscle (26). The work in our study has concentrated on endothelial function in relation to regulation of vascular tone. Endothelial dysfunction occurs when there is a loss of the functional integrity of the endothelium with a reduction in NO

production. When this occurs, there is reduced vasodilatation, an increase in the vascular wall inflammatory response and an increase in thrombotic events (26).

Endothelial dysfunction can result from reduced availability of NO because of altered eNOS expression, or accelerated consumption of NO by ROS or RNOS (688). Reduced NOS (647) and eNOS levels (648) have been found in the placentas and umbilical veins taken from pregnant smokers respectively. The concentration of nitrite and nitrate, measured as an indicator of NO production was also found to be reduced in pregnant smokers compared with pregnant non-smokers (687).

A small number of studies have assessed the effects of smoking in pregnancy on the endothelium. Decreased endothelial dependent dilatation occurred in the aorta of rat pups after they were exposed to cigarette smoke in utero (652). Smoking was found to be harmful to the vascular endothelium in a pathological study of human umbilical arteries (651) and caused degeneration of villous capillary endothelial cells in human placentas (655). All these studies and our own demonstrate that smoking in pregnancy has a deleterious effect on the vascular endothelium.

Studies on non-pregnant individuals have demonstrated differing results. One study assessed the effect of smoking on endothelial function and demonstrated significantly reduced FMD, indicating endothelial dysfunction with similar FMD results to our work ($FMD = 4.0 \pm 3.9$) (177). Two studies have assessed the chronic and acute effect of cigarette smoking by using the test-retest method on non-pregnant participants. The first study demonstrated no difference in FMD between smokers and non-smokers (both genders) at baseline after an eight hour break from smoking. A reduced FMD was

recorded after both smokers and non-smokers smoked a cigarette. This study used a mixed study group and had small numbers (n=27) which were divided into four groups for analysis (183). The second study (184) also had small numbers to assess the effects of smoking a cigarette or using nicotine nasal spray on healthy smoking men (n=8) and women (n=8). This study demonstrated no effect from the chronic use of cigarettes on baseline FMD, with the FMD from smokers almost twice that of other studies (FMD = 10.2 ± 4.4 (nasal spray group) and 9.4 ± 3.8 (cigarette group)). A significant reduction in FMD after both treatments was reported. In this study, the BP cuff was placed on the upper arm (184) and this has been shown to reflect ischaemia (130), not solely NO mediated endothelial function.

Since the results of our study were reported other work has been published which is in agreement. A recent experiment (689) assessed vascular function in pregnant mice exposed to cigarette smoke and compared them to smoke exposed virgin mice. Both pregnant and virgin mice not exposed to cigarette smoke were controls. The endothelial dependent vasodilator metacholine was used to assess endothelial function in uterine, mesenteric and renal arteries in the pregnant and virgin mice. Fetal weight was significantly reduced in the mice exposed to cigarette smoke. Endothelial dependent vasodilatation was significantly reduced in the uterine and mesenteric but not renal arteries of pregnant mice. This work demonstrates endothelial dysfunction in pregnant mice exposed to smoke which varies according to the vessel studied.

Other recent work (673, 690) determined the effect of smoking on endothelial function using FMD in pregnant and non-pregnant women. The study group was comprised of 33 pregnant smokers and 47 pregnant non-smokers, 19 non-pregnant smokers and 34 non-

pregnant non-smokers. The brachial artery was scanned after lower arm cuff occlusion for five minutes at 200mmHg. Brachial artery was measured at rest, 30, 60, 90 and 120 seconds post cuff release and FMD calculated as the percentage increase in diameter from the basal and maximum artery diameter. The pregnant women were scanned between 24-28 weeks gestation and pregnant and non-pregnant women were of comparable age (between 20-30 years old) and size with none in the obese range (BMI between 24 and 25 for all groups). Smoking women were asked to not eat and refrain from smoking from 22:00 the night before. The time of day when they were assessed was not stated. There was no significant difference in diastolic BP and basal artery diameters between the four groups. Heart rate was significantly increased in both pregnant groups compared with non-pregnant groups. Systolic BP was significantly increased in the pregnant non-smokers compared with non-pregnant non-smokers and in non-pregnant smokers compared with non-pregnant non-smokers. A significant reduction in FMD was recorded in pregnancy in smokers compared with the non-smokers (8.74 ± 4.83 versus 11.5 ± 5.77 ; $P=0.03$). In the non-pregnant groups FMD was also significantly decreased in the smokers compared with the non-smokers (7.21 ± 5.57 versus 10.522 ± 4.76 ; $P=0.03$). There was no significant difference in FMD between pregnant and non-pregnant non-smokers ($P=0.42$) and pregnant and non-pregnant smokers ($P=0.30$). Maximum post occlusion arterial diameter was reached at 60 seconds in all groups (673, 690). This work agrees with the finding from our study demonstrating endothelial dysfunction in the form of reduced vasodilatation in pregnant women who smoke.

Cigarette smoke contains many compounds that damage the endothelium (177, 659, 691). Of these, nicotine the addictive component of cigarettes has been well studied (660, 661). Acute nicotine infusion has been shown to cause vasoconstriction and endothelial

dysfunction in hamster cheek arterioles with decreased dilatation noted at low nicotine levels and vasoconstriction at high levels (668). Nicotine exposure also results in uterine artery contractions in pregnant ewes (692). This vasoconstrictor effect is via inhibition of the NO pathway (671, 674, 692). As FMD is a NO mediated technique (17), the above studies suggest that the decreased dilatation seen in our and the other studies may be via the effect of nicotine on the NO pathway.

Nicotine also increases heart rate and BP in humans, with heart rate increasing maximally after the first cigarette of the day. Heart rate then plateaus as tolerance develops (663, 664). A study during pregnancy demonstrated nicotine but not carbon monoxide increased heart rate and BP after 12 hours of abstinence from smoking (665). Our study demonstrated no difference in basal BP or heart rate between smokers and non-smokers after smokers' abstinence from cigarettes. Consistent with the literature an increase in maternal heart rate and diastolic BP occurred after the women smoked their first cigarette of the day.

Nearly 40% of the babies of women who smoked in this study were growth restricted. Whilst this number may seem excessive, we did limit the study group to heavy smokers which could increase the incidence of IUGR. Of particular importance is our finding of a significant relationship between endothelial dysfunction and IUGR in the women who smoked. This supports the hypothesis that IUGR is related to endothelial dysfunction (355). Women who deliver an IUGR baby also have an increased risk for coronary artery disease in later life further substantiating the link with endothelial dysfunction (355, 693). Pregnant smokers who delivered a SGA baby have a dose dependent decrease in eNOS levels in human umbilical vein endothelial cells (648, 649). A quarter of the reduction in

weight in babies was attributed to the decrease in eNOS (649). These studies suggest reduced eNOS levels in fetal vessels from maternal smoking may result in vasoconstriction and partly explain the reduced fetal size.

To obtain ethical approval for this work, limitations were placed on the study design. The women could not be recruited until 28 weeks gestation because it was considered necessary to allow them time to quit or reduce their smoking. Recruitment was also limited to heavy smokers, defined as 10 or more cigarettes a day to ensure we were not asking the women to smoke more than they normally would. Women were also given the choice about whether to smoke or not at the break. Smoking status was known at the time the study was performed and could only have been blinded if someone else recruited the women. After the break, cigarette smoke was always evident on those who smoked. Bias regarding smoking status was reduced by measuring the brachial artery offline without smoking status being known. There was a significant difference in the number of nulliparous women in each group. In total, four women had a previous adverse pregnancy outcome (Table 3.1). Persistent endothelial dysfunction cannot be excluded from these pregnancies. Further studies should compare nulliparous smokers with nulliparous non-smokers.

Pregnant women who smoke have decreased nitrite and nitrate levels (687), decreased eNOS levels (692), and as NO is involved in the vasodilatation that occurs in normal pregnancy (7), a decrease in NO in pregnant smokers would most likely result in decreased vasodilatation and endothelial dysfunction (543, 612). This was demonstrated in our study as well as animal (668, 689) and other human studies (673, 690). With endothelial dysfunction in pregnant smokers, an increased risk of pre-eclampsia would be expected

(612). Endothelial dysfunction from cigarette smoking may be why pregnant smokers get worse disease when they do develop pre-eclampsia (601, 603). Women with chronic hypertension who smoke also have an increased risk of pre-eclampsia (superimposed) (694) and this may be because of pre-existing, underlying endothelial dysfunction. Our study, however does not answer the decreased risk of pre-eclampsia from smoking enigma. Recent work has suggested that carbon monoxide and angiogenic factors may play a role in this (612, 615, 683).

In conclusion this work has three significant findings. First, it demonstrates pregnant women who smoke have endothelial dysfunction. Second, using this test-retest method, this endothelial dysfunction was shown to be persistent. Specifically, FMD in the women who smoked was the same after a nine hour abstention from smoking as it was after smoking one or two cigarettes. Third, this work provides evidence of the relationship between endothelial dysfunction and IUGR. Finally, this study does not support the concept of smoking in pregnancy providing a protective effect on the vascular endothelium.

Chapter 4

Flow-mediated dilatation assessment in women with pre-eclampsia compared to women with gestational hypertension.

4.1 Introduction.

Pre-eclampsia is a serious disorder of pregnancy with an incidence of between 5-11% (594, 595). Even though pre-eclampsia results in increased morbidity and mortality to the mother and baby (272) the only treatment remains delivery (596). In Australia, the current consensus regarding hypertensive disorders of pregnancy is pre-eclampsia and gestational hypertension are different diseases (249, 251). The alternative hypothesis is that pre-eclampsia and gestational hypertension are similar diseases sharing a joint pathophysiology with pre-eclampsia being the more serious progression of the hypertensive disorder (236). Whilst the diagnosis of pre-eclampsia can be made on the basis of maternal hypertension and significant proteinuria or hypertension with certain other multi-system disorders (249) the diagnosis of gestational hypertension is often not made until after delivery when pre-eclampsia has been excluded. A recent systematic review demonstrated both pre-eclampsia and gestational hypertension result in long term sequelae in later life in the form of an increased risk of CVD and hypertension (262).

Pre-eclampsia is thought to be a two stage disorder which originates with the normal pregnancy specific inflammatory response. This then interacts with an abnormal maternal constitution where there are risk factors for pre-eclampsia. These risk factors result in or cause abnormal placentation and placental ischaemia (286). Alternatively normal placentation may occur and the interaction with maternal risk factors results in altered placental vascular remodelling (429). Factors are then released from the hypoxic placenta into the maternal circulation. These proposed factors which target the endothelium resulting in endothelial dysfunction and/or activation include cytokines (431, 432), STBM

(407), lipoproteins (433) or the anti-angiogenic factors sFlt-1 and sEng (444). Changes in the coagulation cascade and/or alterations in the amount of vasoregulators such as NO then occur resulting in the clinical picture of pre-eclampsia (444). The sub-classification of pre-eclampsia into early/late disease (265) or placental/maternal disease (415) to define severity of the syndrome or to explain its heterogeneity has been proposed.

A previous study reported endothelial dysfunction in pre-eclampsia when flow induced shear stress failed to produce vasodilatation (263). Others have shown increased vascular activity in women with pre-eclampsia (556). The inconclusive nature of these results could stem from the small numbers that have been recruited, the methodology used or the heterogeneous nature of pre-eclampsia. Interestingly, non-pregnant hypertensive adults have been shown to have endothelial dysfunction in the form of reduced vasodilatation (10).

Flow-mediated dilatation is a non-invasive method for assessing stimulated NO mediated endothelial function. Reduced FMD represents decreased vasodilatation and is considered an indicator of endothelial dysfunction (16). The basal release of NO is involved in the regulation of blood pressure and SVR. Endothelial dysfunction can occur when there is decreased availability of NO. Blood pressure has been shown to increase when NO synthesis is inhibited (44).

The technique of FMD has evolved since its inception by Celermajer and colleagues in 1992 when it was recommended that “peak” arterial dilatation be measured 45-60 seconds after cuff release (16). Although more recent work has established the validity of the basics of Celermajer’s work (such as scanning the brachial or radial artery, lower arm BP

cuff placement and five minutes occlusion time) in reflecting a mechanism that is primarily nitric oxide mediated (17), new parameters have been incorporated into the technique. These include measuring time to maximum dilatation for 90 seconds and normalising FMD to the shear stimulus response using shear rate (17) and haematocrit (105). Nevertheless, as with all new techniques, knowledge has evolved regarding the methodology with complete agreement still to be reached on what is considered best practice.

A study was designed to assess if women with pre-eclampsia have endothelial dysfunction in the form of reduced vasodilatation. We hypothesised that if women with gestational hypertension were also found to have endothelial dysfunction, similar in fact to the women with pre-eclampsia, then it would add further evidence to the theory that pre-eclampsia and gestational hypertension are related disorders. However, if endothelial function between the two groups proved to be different, a test that differentiates between pre-eclampsia and gestational hypertension could prove clinically useful. Therefore the aims of this work were, one to establish if women with pre-eclampsia have endothelial dysfunction presenting as vasoconstriction and if women with gestational hypertension have the same or different endothelial function as women with pre-eclampsia. A secondary aim was to determine if the FMD results would vary with the addition of the revised techniques. These revised techniques include measuring time to maximum dilatation for up to 90 seconds and normalising FMD using shear rate and haematocrit.

4.2 Methodology.

This was a prospective cross-sectional study of pregnant women who were recruited on admission to hospital with a hypertensive disorder of pregnancy. The women were classified as either having pre-eclampsia or gestational hypertension by the research criteria published in the ASSHP consensus statement. Hypertension was defined as blood

pressure $\geq 140/90$ mmHg. Women with the additional finding of proteinuria ≥ 300 mg/24 hours were defined as pre-eclampsia and the non-proteinuric group (proteinuria < 300 mg/24 hours) as gestational hypertension (249). Self-reported smoking status was recorded. Treatment with, and type of hypertensive medication at the time of study was documented. Exclusion criteria were pre-existing medical conditions and multiple pregnancy. All women were asked to refrain from caffeine and high fat foods and as they were all hospital inpatients, exercise was kept to a minimum.

The FMD technique that reflects mainly NO dependent endothelial dilatation was used (16, 17) as described in chapter 2. A pressure of 200 mmHg was used for BP cuff inflation unless BP was > 180 mmHg when the cuff would be inflated to 20 mmHg above systolic pressure. Flow-mediated dilatation which is the percentage difference in basal and PO brachial artery diameter was calculated. Post occlusion diameter was measured at 45-60 seconds post cuff release as originally described (16) and FMD-original (FMD_o) calculated. No normalisation of results was done for FMD_o in line with previous work (16, 534, 573-576). According to the most recent guidelines, PO brachial diameter was measured up to 90 seconds (17) with additional diameters averaged from four measurements at 60-75 seconds and 75-90 seconds post cuff release respectively. The FMD-maximum (FMD_{max}) was calculated using the maximum diameter obtained from between 45-90 seconds.

Pulsed Doppler was used to obtain a VTI at rest and post cuff release. Basal volume flow, PO volume flow and reactive hyperaemia were calculated as described in chapter 2. Current literature dictates normalising FMD to the shear stimulus. This is done using variations of Poiseuille's formula to calculate shear rate or shear stress. Shear rate (an

estimate of the shear stress placed on the endothelial cells from blood flow) was calculated by the formula: shear rate = velocity/diameter (17). Basal artery diameter was used to calculate basal and peak shear rates (215) as the artery does not begin to dilate during the shear stimulus period until approximately 45 seconds post cuff release. By this time the shear stimulus is decaying (223). Basal VTI and peak VTI were used to calculate basal and peak shear rates respectively. Shear stress was not calculated as it requires measurement of viscosity, however haematocrit which is the most significant “determinant of whole blood shear-dependent viscosity” (219) was obtained from a whole blood screen taken either on the day of FMD assessment or the preceding day and used to normalise FMD_{max} . Ethics approval was obtained from the local Area Health Ethics Committee and each woman provided written informed consent for enrolment. Recruitment of volunteers, all studies and measurements were performed by the author (AEQ).

Prior to the FMD test, uterine artery Doppler study was performed. This involves using a 3.5MHz curve linear transducer and the Philips Medical System HDI 5000 ultrasound machine (Philips Ultrasound, Bothell, WA, USA). With the woman lying in a semi-supine position, the ultrasound transducer was placed in the right iliac fossa at approximately 45 degrees. Colour Doppler imaging was activated. The maternal iliac artery and vein was visualised where the uterine artery appears to cross over the iliac vessels (Figure 4.1). The uterine artery was sampled at this point with pulsed Doppler ultrasound. Pulsed Doppler velocity was set at 60-80cm/s, the range gate at 5mm and a corrected Doppler angle between 0-60 degrees was used. Pulsatility index was measured and the absence (Figure 4.2) or presence (Figure 4.3) of diastolic notching was recorded. The process was repeated in the diametrically opposed left iliac fossa to assess the left uterine artery (695).

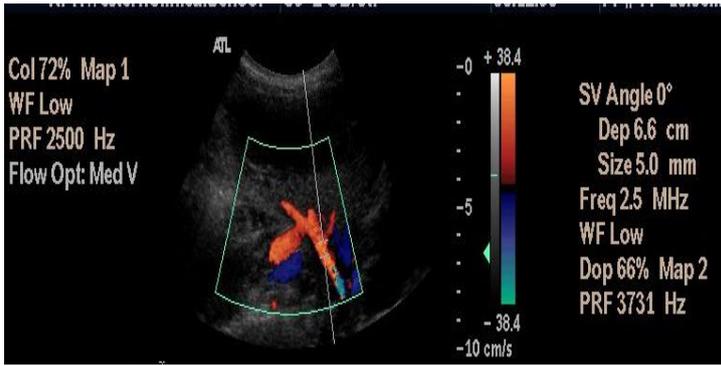


Figure 4.1: Colour Doppler image illustrating the uterine artery where it appears to cross the iliac vessels.

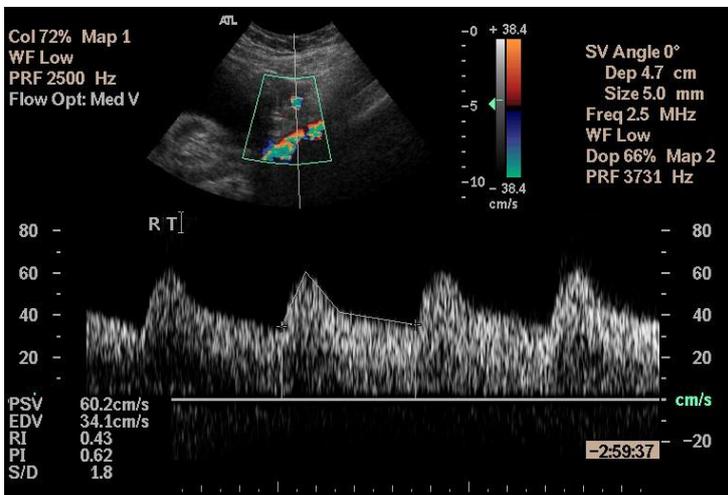


Figure 4.2: Uterine artery Doppler waveform demonstrating low resistance flow and no diastolic notch.

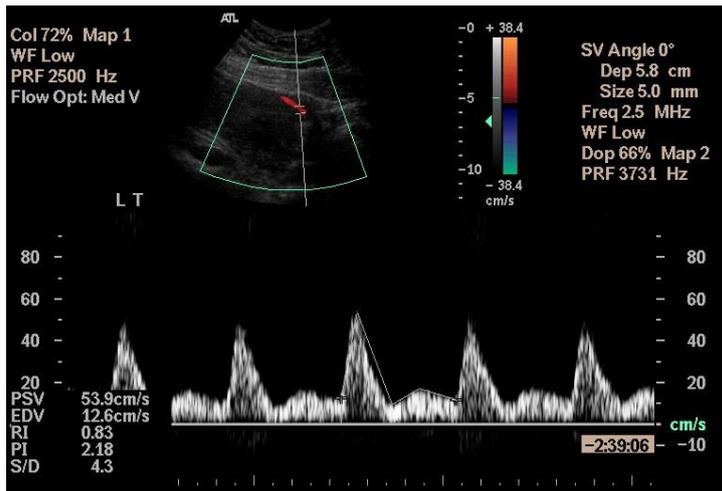


Figure 4.3: Uterine artery waveform demonstrating high resistance flow with increased PI and diastolic notch.

4.3 Statistical analysis.

Statistical analysis was performed using the Statistical Packages for Social Sciences SPSS 17.0 (SPSS Inc., Chicago, IL). Normally distributed data were analysed using independent sample t-tests or ANOVA with data reported as mean \pm standard deviation ($m \pm SD$). A factorial analysis of variance was performed for both FMD_o and FMD_{max} . Covariates added to the FMD_o and FMD_{max} models and removed in a stepwise fashion because of non-significance were systolic BP, diastolic BP, maternal age, BMI and gestational age at time of study. The FMD_{max} was analysed with the significant covariates of peak shear rate and haematocrit. A post hoc comparison was performed as appropriate. A chi-square test was used to analyse categorical data. A Mann-Whitney analysis or Kruskal-Wallis test was used for continuous, non-normal variables and results reported as median and interquartile range (m [IQR]). Correlation analysis was performed using Pearson Correlation test. The calculated P-values were 2-tailed and significance was assumed when $P \leq 0.05$. A Bonferroni correction was made when analysis was done across four groups with $P \leq 0.0125$ considered significant.

4.4 Results.

Ninety-two women were enrolled. We excluded 16 women, four for existing maternal disease (type 1 diabetes (n=2), antiphospholipid syndrome (n=1) and pre-existing renal disease (n=1)) and a further two, who, after review of their records did not meet the criteria for hypertension. Ten women who smoked were excluded because of small numbers and because we have shown pregnant women who smoke have endothelial dysfunction (Chapter 3). Of the 76 remaining women, 38 met the criteria for pre-eclampsia and 38 for gestational hypertension.

There were similar numbers of women with pre-eclampsia (25/38 (65.8%)) and gestational hypertension (23/38 (60.5%)) treated with hypertensive therapy at the time of study (P=0.41). Eighty-five percent (41/48) were solely prescribed methyldopa (a centrally acting anti-adrenergic); the remainder were treated with methyldopa combined with labetalol (an alpha-blocker and nonselective beta-blocker), oxprenolol (a beta-blocker) or hydralazine (a smooth muscle peripheral vasodilator). There was no difference in the number of nulliparous women in the pre-eclampsia (17/38 (44.7%)) and gestational hypertension (19/38 (50%)) groups (P=0.41). The participants in the two groups were evenly matched in baseline characteristics (Table 4.1). In particular there was no significant difference between the women with pre-eclampsia and gestational hypertension in BMI and gestational age at time of study. Proteinuria was significantly increased in the pre-eclampsia group as expected (Table 4.1).

Table 4.1: Participant characteristics ($m \pm SD$) or median [IQR] in pre-eclampsia and gestational hypertension women.

Characteristics	Pre-eclampsia (n=38)	Gestational Hypertension (n=38)	P-value
Gestational age at study	33.9 \pm 3.2	35.0 \pm 3.0	0.13
Maternal age	28.8 \pm 7.2	29.0 \pm 4.4	0.86
BMI	32.4 \pm 6.6	34.3 \pm 6.7	0.22
Proteinuria	850 [435-1170]	155 [0-223]	<0.0001
Highest recorded systolic BP (mmHg)	158 \pm 15.9	155 \pm 11.8	0.36
Highest recorded diastolic BP (mmHg)	102 \pm 10.0	100 \pm 8.4	0.46
Basal heart rate (bpm)	81.6 \pm 16.7	83.8 \pm 14.4	0.53
Basal artery diameter (mm)	3.7 \pm 0.4	3.9 \pm 0.5	0.11
Basal volume flow (mL/min)	395 [284-517]	472 [275-730]	0.11
Post occlusion volume flow (mL/min)	1840 [1533-2116]	2008 [1440-2480]	0.36
Reactive hyperaemia (%)	371 [220-501]	288 [205-440]	0.21
Basal shear rate (s⁻¹)	63.0 \pm 25.0	65.2 \pm 30.4	0.73
Peak shear rate (s⁻¹)	249 \pm 67	250 \pm 79	0.95
Haematocrit (%)	34.9 \pm 2.8	35.6 \pm 2.2	0.25

For FMDo, there was a significant interaction between pre-eclampsia and gestational hypertension and medication ($P < 0.0001$), so the diseases were compared separately in the medication and no medication groups. There was a significant difference at the $P < 0.0001$ level for the four groups. Post hoc comparisons using Tukey HSD test indicated FMDo was similar in the gestational hypertension-no medication and pre-eclampsia-no medication ($P = 0.83$) groups (Table 4.2). FMDo was significantly reduced in the gestational hypertension-medication versus the pre-eclampsia-medication ($P < 0.0001$)

groups (Table 4.2). All participant characteristics (except proteinuria) were similar when analysed across four groups (Table 4.3).

For FMD_{max} the interaction was not significant (both $P=0.08$) in either unadjusted analysis or analysis adjusted for the covariates haematocrit ($P=0.023$) and shear rate ($P=0.007$).

Therefore means averaged over medication are presented. The FMD_{max} was significantly reduced in the gestational hypertension group compared to the pre-eclampsia group ($P<0.0001$) (Table 4.2). Seventy nine percent (30/38) of the pre-eclamptic women reached maximum dilatation by 90 seconds compared to 63% (24/38) of gestational hypertension women (Chi-square, $P=0.16$). Dilatation was considered to have peaked at the measurement before the artery diameter began decreasing. This was seen in all but 8/38 (21%) of pre-eclampsia women and 14/38 (37%) of gestational hypertension women (Figure 4.4). A significant negative correlation between FMD_{max} and basal artery diameter in the pre-eclampsia ($r = -0.5$; $P=0.001$) and the gestational hypertension women ($r = -0.368$; $P=0.023$) was demonstrated.

Uterine artery Doppler studies were performed on 27 women with pre-eclampsia and 24 with gestational hypertension. Twenty-two percent (6/27) of pre-eclamptic women and 21% (5/24) of gestational hypertension women had bilateral uterine artery notching (Chi-square, $P=0.9$). There was no significant difference in uterine artery PI in the pre-eclampsia (1.1 ± 0.5) versus gestational hypertension (1.1 ± 0.4) groups ($P=0.69$). There was no correlation between uterine artery PI and FMD_{max} in the pre-eclamptic ($r = 0.023$; $P=0.915$) or gestational hypertension women ($r = 0.2$; $P=0.333$).

Birth outcomes were available for 34 women with pre-eclampsia and 35 women with gestational hypertension. The remaining women were lost to follow-up. Babies of mothers with pre-eclampsia were born significantly earlier than babies of mothers with gestational hypertension (36.3 [33-38] versus 37.9 [37-39] weeks) ($P < 0.0001$) and were significantly smaller (2428 [1702-2880] versus 3305 [2690-3545] grams) ($P = 0.001$). However when individual birth weight percentiles, corrected for sex and gestational age at delivery were calculated, there was no significant difference (33.2 [13-57] versus 61.4 [20-78]) ($P = 0.12$). There were 12 babies (six in each group) with birth weights $< 10^{\text{th}}$ percentile. Birth weight percentile and uterine artery Doppler PI correlated significantly and negatively in the gestational hypertension group ($r = -0.553$; $P = 0.014$) but not in the pre-eclamptic women ($r = -0.232$; $P = 0.286$). Birth weight percentile correlated significantly with maternal BMI in the pre-eclampsia women ($r = 0.339$; $P = 0.05$) but not in the gestational hypertension women ($r = 0.308$; $P = 0.072$).

Table 4.2: Comparison of FMD₀ and FMD_{max} in pre-eclampsia versus gestational hypertension (m ± SD).

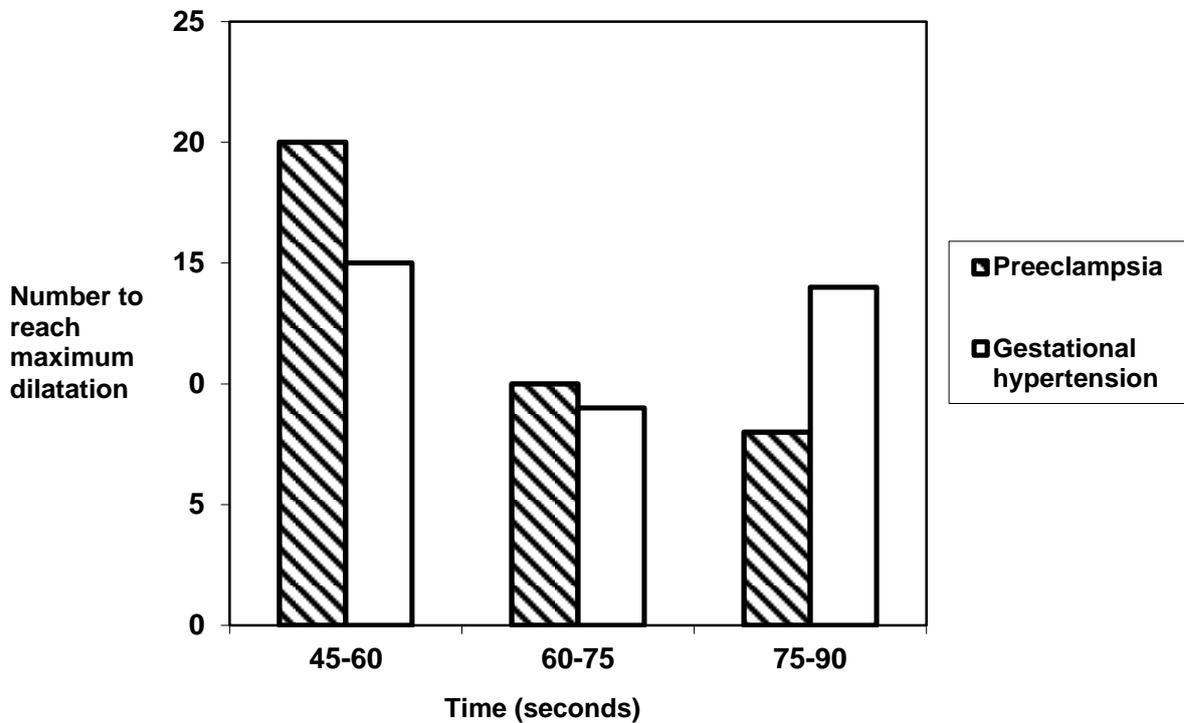
	<i>Pre-eclampsia</i>	<i>Gestational Hypertension</i>	<i>P value</i>
FMD₀, No medication (n)	6.5 ± 4.1 (13)	5.3 ± 3.2 (15)	0.83 [†]
FMD₀, Medication (n)	8.8 ± 4.3 (25)	3.7 ± 2.8 (23)	<0.0001 [†]
FMD_{max}, crude means (n)	9.2 ± 4.0 (38)	5.7 ± 4.0 (38)	<0.0001#
FMD_{max}, adjusted means (n)	9.3 ± 4.1 (36)	5.7 ± 3.4 (37)	<0.0001# [†]

FMD₀, peak dilatation measured at 45-60 seconds. FMD_{max}, peak dilatation measured up to 90 seconds. [†]Tukey HSD post hoc analysis. #ANOVA. [†]adjusted for haematocrit and shear rate.

Table 4.3. Participant characteristics across the four groups, (m ± SD) or median [IQR]).

Characteristics	Pre-eclampsia & medication (n=25)	Gestational Hypertension & medication (n=23)	Pre-eclampsia & no medication (n=13)	Gestational Hypertension & no medication (n=15)	P-value
Gestational age at study	33.8 ± 2.9	33.8 ± 3.6	34.7 ± 3.2	36.4 ± 1.9	0.044
Maternal age	26.8 ± 7.5	29.0 ± 4.1	32.2 ± 5.5	29.1 ± 4.8	0.061
BMI	31.1 ± 5.9	35.2 ± 7.6	34.6 ± 7.7	33.0 ± 4.8	0.165
Proteinuria	900 [420-1260]	145 [0-220]	810 [420-1025]	100 [0-230]	< 0.0001
Highest systolic BP (mmHg)	159 ± 15.5	159 ± 14.7	152 ± 11.5	152 ± 11.2	0.260
Highest diastolic BP (mmHg)	103 ± 9.3	102 ± 9.5	99 ± 10.7	97 ± 5.3	0.273
Basal heart rate (bpm)	77 ± 11.6	79 ± 13.7	90 ± 22.1	91 ± 12	0.008
Basal artery diameter (mm)	3.7 ± 0.4	3.8 ± 0.6	3.7 ± 0.5	4.0 ± 0.4	0.284
Basal volume flow (mL/min)	445 [289-525]	421 [256-625]	368 [262-502]	529 [406-783]	0.188
Post occlusion volume flow (mL/min)	1895 [1563-2111]	1772 [1261-2452]	1641 [1343-2116]	2054 [1698-2481]	0.520
Reactive hyperaemia (%)	382 [254-482]	358 [218-512]	338 [211-522]	256 [195-391]	0.474
Basal shear rate (s⁻¹)	63.9 ± 26.7	66.2 ± 28.1	59.7 ± 22.6	64.8 ± 34.0	0.929
Peak shear rate (s⁻¹)	250 ± 60	272 ± 85	237 ± 73	223 ± 65	0.203
Haematocrit (%)	35.0 ± 0.02	36.2 ± 0.02	34.2 ± 0.03	34.9 ± 0.02	0.117

Figure 4.4: Number of pre-eclampsia and gestational hypertension women to reach maximum dilatation between 45-90 seconds.



4.5 Discussion.

This study demonstrates how variations in technique can produce different results. FMD was similar in the no medication pre-eclampsia and gestational hypertension groups (Table 4.2). Reduced FMD in this pre-eclampsia group is similar to others who have reported low FMD as reflecting reduced NO bioavailability and endothelial dysfunction (53, 532, 534, 573-576, 578, 579). The methodology used in these studies varies considerably. Many used upper arm cuff placement (53, 534, 573, 575, 576, 579) which reflects an ischaemic effect and is not wholly NO mediated (130). Three studies used an occlusion time of less than five minutes (574-576) which is insufficient to produce significant dilatation (129). This demonstrates how the use of a technique that is still evolving can result in differing interpretations of data.

We found FMD₀ was significantly reduced in the medication gestational hypertension group compared with the medication pre-eclampsia group (Table 4.2). It is difficult to know if this result could be attributed to the effects of vasoactive medication. Best practice would involve studying the women before treatment was commenced although this was not always possible. To my knowledge there are no reported studies examining the effect of vasoactive medication on FMD in pregnancy. Some work has been done using different measures of endothelial function. No effect from medication was reported on the stable NO metabolites nitrate or nitrite in pre-eclamptic women (186). Methyldopa did decrease sFlt-1 and sEng levels in pre-eclamptic but not gestational hypertensive women. An effect of medication on the endothelium could not be excluded in this study (187). The FMD guidelines (96) state that vasoactive medication “be withheld for at least four half-lives, if possible”. Observational studies should collect data on the type of medication taken. Medication has been shown to have variable effects (none, increased or decreased FMD) on the endothelium in non-pregnant people (146, 188-191). The addition of medication into the FMD picture appears to add another layer of confounding information especially as it did not have a significant interaction in the FMD_{max} analysis. This would need to be addressed in any future studies undertaken.

The FMD_{max} demonstrated no interaction from the medication between pre-eclampsia and gestational hypertension women and altered the data interpretation. A significant reduction in FMD_{max} in the gestational hypertension group compared to the pre-eclampsia group (Table 4.2) was found. Both groups of women in our study were obese with the majority representing late onset disease (Table 4.1). Pre-eclampsia is a disease of contradictions and theories with endothelial dysfunction being the end point of a two stage hypothesis (2). Even though one of the functions of the endothelium is the control of vascular reactivity by

NO production (16), it is unclear whether NO deficiency actually occurs in pre-eclampsia with several studies demonstrating increased levels of nitrate and nitrite (67, 68, 186, 520, 524, 548) and others demonstrating decreased levels (449, 521, 549, 550) or no difference (433, 522, 523, 525, 551). The majority of studies using the FMD technique have reported reduced FMD before the clinical picture of pre-eclampsia becomes evident (Table 1.3) (353, 588-590, 592). All but one of these studies (589) recruited women who smoked and two recruited women with risk factors for pre-eclampsia and/or pre-existing medical conditions (589, 592). These factors may influence these results.

Reduced FMD was also observed in all the studies comparing pre-eclamptic women with normotensive pregnancy (Table 1.2) (53, 532, 534, 573-576, 578, 579). Direct comparison of these studies is difficult due to the variations in FMD methodology used and because the differences in pre-eclampsia definitions may result in women without pre-eclampsia being recruited. These studies also represent a mixed population sample with early (<34 weeks), late (≥ 34 weeks) (265) and mixed gestational age women recruited. Other studies using different techniques have had similar results to ours, with women having an increased micro-vasodilatory response before pre-eclampsia develops (557) and when the disease is present (556). The women recruited by Davis et al. were larger women (BMI 30 ± 2.0) and late pre-eclamptics (gestational age at delivery 35.6 ± 1.0) (556) and very similar in demographics to our group. The increased vasodilatation (FMD_{max}) in our pre-eclamptic women, similar to the normal range women (Chapter 2) may represent a compensatory vascular response of the endothelium as a rescue mechanism. Alternatively, the heterogeneity of the aetiology and multiple risk factors for pre-eclampsia may contribute to these differing results (3).

Most work has compared pre-eclampsia with normal pregnancy with very few studies assessing other hypertensive disorders of pregnancy (gestational hypertension, superimposed pre-eclampsia, chronic hypertension) using the FMD technique. The evaluation of FMD in chronic hypertension in pregnancy found reduced endothelial function, although to a less extent compared with pre-eclampsia (Table 1.2) (573). Another study could not differentiate between pre-eclampsia and superimposed pre-eclampsia probably due to small numbers as the pre-eclampsia FMD was more than double the superimposed pre-eclampsia FMD. Of interest, the women in this study were obese late pre-eclamptics (Table 1.2) (577) similar to our study.

The only other study that compared FMD in pre-eclampsia and gestational hypertension (prior to clinical disease) reported opposite findings to our study, that is increased FMD in gestational hypertension and reduced FMD in pre-eclampsia. None of these women were obese (Table 1.3) (592) and may therefore represent a different group of pre-eclamptic women to our study. Normal pregnancy results in increased cardiac output and decreased SVR (484). The endothelial dysfunction of pre-eclampsia is characterised by vasospasm, microthrombi and vascular permeability (542) which should result in decreased cardiac output and increased SVR as demonstrated in the study by Vasapollo et al. (497). The work by Easterling et al. (491) and Bosio and colleagues (496) demonstrated increased cardiac output and decreased SVR in pre-eclampsia. Closer inspection reveals the women in these studies were heavier late pre-eclamptics. The differences in these studies was explained when it was discovered that in early pre-eclampsia a low cardiac output high SVR state exists with the opposite occurring in late pre-eclampsia (499).

In our study the majority of pre-eclamptic women were obese late pre-eclamptics, most had normal uterine artery Doppler studies with no correlation between FMD and uterine artery Doppler PI. Persistent uterine artery notching at 23-24 weeks gestation has been associated with pre-eclampsia and growth restriction (695, 696) and is associated with an increased number of ischaemic lesions in the placenta which may represent a placental ischaemic form of pre-eclampsia (576). We suggest our study represents a different group of pre-eclamptics to the previously reported decreased FMD studies (Tables 1.2 and 1.3), similar in fact to the pre-eclamptic women in the studies by Easterling et al. (491), Bosio et al. (496), Davis et al. (556) and Filho et al. (577) and the pre-eclamptic women without uterine artery notching in Brodzki and colleagues study (576) (Table 1.2). The risk of pre-eclampsia increases linearly with increasing BMI (360) and metabolic syndrome is also a risk factor for pre-eclampsia (371). Our study adds further evidence to the hypothesis that different forms of pre-eclampsia exist. These results also suggest that late onset, obesity related pre-eclampsia may not be exclusively an endothelial disease resulting in reduced vasodilatation.

Reduced FMD_{max} in the gestational hypertension women suggests possible underlying, early stage or pre-existing hypertension and endothelial dysfunction. This may explain their increased risk of later developing CVD and hypertension (262). Our study suggests that endothelial dysfunction in the form of reduced FMD is not essential for the development of pre-eclampsia.

The difference between FMD_{0} and FMD_{max} analysis is due to the pre-eclampsia and gestational hypertension women taking longer than 60 seconds to reach maximum dilatation. The FMD technique is still evolving. The latest literature recommends

continuous recording of post occlusion arterial diameter for 180 seconds (100, 134). A number of pre-eclampsia studies measured PO arterial diameter for greater than 60 seconds with two reporting maximum mean dilatation at 60 seconds (534, 576) and others only that maximum dilatation was measured (573-575). Evaluating TTP dilatation as an adjunct measure to FMD is also suggested, not only because the endothelium has been shown to have two separate dilatory periods in normal healthy people (224) with two distinct mechanisms (endothelial and smooth muscle cell response) (224, 228), but also because TTP dilatation responds differently in pathological conditions with both early and late responders demonstrating endothelial dysfunction. The late responders may have later acting vasodilators other than NO acting as a “compensatory mechanism” when NO availability is reduced (230).

The FMD_{max} results may not be definitive as maximum dilatation was not reached in all women in either group although this was not significantly different. With longer monitoring, arterial diameter may continue to rise in the gestational hypertension group negating the significant difference in FMD_{max} between the pre-eclampsia and gestational hypertension women. As fewer women had reached maximum dilatation in the gestational hypertension group by 90 seconds (Figure 4.4), this could result in a longer TTP dilatation in this group indicating that pre-eclampsia and gestational hypertension are different disorders. Alternatively, if the significant difference remains, this would also indicate a different response to NO mediated dilatation in the two groups. We therefore hypothesise that pre-eclampsia may be a different disorder to gestational hypertension.

Although there is no consensus on normalising shear stimulus to FMD (222), in line with current literature we also “normalised” our data for the shear stimulus response in the

FMD_{max} analysis, that is we adjusted the FMD values to a common value of shear stress and haematocrit. This did not change the results (Table 4.2). Early work reported volume flow as an indicator of endothelial shear stimulus although this gives no indication of frictional force. However, if volume flow and artery diameter are similar (Table 4.1), then shear rate will be similar (17) as demonstrated by our results.

Limitations of this study are that PO artery diameter was not measured for three minutes. It is now recommended that continuous simultaneous Doppler velocity and artery diameter monitoring is done PO so that peak diameter and shear rate AUC can be measured for FMD normalisation (17). Even so the latest FMD technique consensus statement could not endorse this as the definitive method (100). Normalisation of FMD by this technique requires dedicated computer software (135) which our laboratory does not have. Studies are also needed to assess FMD longitudinally, before pre-eclampsia is evident and when the clinical diagnosis is made to ascertain when and if FMD changes. Future studies need to ensure pre-eclamptic women are divided into early and late groups and maternal demographics such as BMI are reported.

In conclusion this study demonstrates reduced FMD in gestational hypertension women compared to pre-eclamptic women. Strengths of this study are we excluded women with medical conditions and smokers, used strict criteria for the diagnosis of pre-eclampsia and used a NO mediated technique. We hypothesise that pre-eclampsia and gestational hypertension are different disorders based on the significant difference in FMD_{max}. We have assessed variations in the FMD technique in hypertensive pregnant women. Monitoring PO arterial diameter for more than 60 seconds has resulted in a difference in FMD₀ and FMD_{max}. Previous work assessing FMD in pre-eclamptic women may not be

definitive due to recent changes in an evolving technique. This study reinforces the multifactorial nature of pre-eclampsia adding further evidence to the hypothesis that pre-eclampsia due to maternal metabolic syndrome/obesity is different to the early/placental disorder.

Chapter 5

5.0 Conclusion.

5.1 Introduction.

The endothelium is a complex organ that is influenced and responds to differing factors in a variety of ways. The role of the endothelium in the physiology of pregnancy is now better understood. Nevertheless the relationship between endothelial function and pregnancy and hypertensive disorders of pregnancy continues to be a complicated one. A simple answer to the complex problem of the pathophysiology of pre-eclampsia is unlikely to be found in the near future.

When this work was commenced the aetiology of pre-eclampsia was unknown and this remains the status quo although enormous advances have been achieved in the last few years. Promising advances include the recognition of possible different types of pre-eclampsia. These different types are the differentiation of pre-eclampsia into early (<34 weeks) and late (≥ 34 weeks) syndromes. Other types are placental pre-eclampsia and maternal pre-eclampsia with the recognition of the involvement from underlying risk factors. These risk factors include the effect of obesity and cigarette smoking on the endothelium. This has enabled a better understanding of the often conflicting results reported in the pre-eclampsia literature. Scant literature exists on gestational hypertension. This may be because earlier literature tended to combine all hypertensive disorders of pregnancy into a single group called PIH (697). The recognition that gestational hypertension is not necessarily a benign disease and also results in long term morbidity may increase the number of studies performed.

5.2 Summary of this work.

5.2.1 Normal range study.

The first study developed normal range parameters of endothelial function as pregnancy progressed. Flow-mediated dilatation as a marker of stimulated endothelial function, changes in basal vessel diameter, brachial artery volume flow and reactive hyperaemia were measured in 47 healthy pregnant women and 18 non-pregnant healthy women. Endothelial function demonstrated no change from 11-32 weeks gestation. A significant decrease occurred at 36+ weeks gestation. There was no significant difference between pregnancy and postpartum values and non-pregnant values.

5.2.2 The effect of cigarette smoking in pregnancy on endothelial function.

The acute and chronic effects of tobacco cigarette smoking on endothelial function were assessed in 21 pregnant women who smoked and compared to 20 non-smoking pregnant women using a test-retest design. This study demonstrated that both chronic and acute smoking resulted in endothelial dysfunction compared with the non-smoking controls. The women who smoked in pregnancy and had growth restricted babies had decreased FMD compared with women who delivered babies weighing >10th percentile.

5.2.3 The assessment of endothelial function in women with pre-eclampsia and gestational hypertension.

This study on the hypertensive disorders of pre-eclampsia and gestational hypertension examined 38 women in each group. Women with gestational hypertension had decreased FMD which is considered indicative of endothelial dysfunction. The women with pre-eclampsia had FMD similar to the women in our normal range group. Both groups of

pregnant hypertensive women were in the obese range, the majority had normal uterine artery Doppler studies and were late gestational age pre-eclampsics.

5.3 Discussion.

The work in this thesis has concentrated on assessing endothelial function in pregnancy as measured by the ultrasound technique of FMD. The endothelium exerts control over vascular homeostasis by the production of numerous vasoconstrictors, vasodilators and by the various haemodynamic forces that occur. The normal range study demonstrated that whilst basal brachial artery vessel size increased progressively throughout pregnancy, endothelial function remained constant until the late third trimester when FMD decreased. The decrease in FMD in late pregnancy may be consistent with the decrease in eNOS and cGMP that occurs in the third trimester of pregnancy. The failure of FMD to increase throughout pregnancy may also be due to the relationship between vessel size and the shear stress placed upon the endothelium. The degree of FMD that occurs is inversely correlated with vessel size. Therefore the increase in basal artery diameter with increasing gestation would result in decreased shear stress and less dilatation of the artery.

Both chronic and acute smoking in pregnancy was shown to have a deleterious effect on the vascular endothelium in normal pregnancy in the form of reduced dilatation. Pregnant women with the lowest FMD also had smaller babies. The original hypothesis was that smoking may provide a protective effect on the vascular endothelium. This has been disproved. Although smoking reduces the risk of pre-eclampsia the results of this study demonstrate tobacco cigarettes do not protect the vascular endothelium. This further reinforces the message that smoking is harmful to both mother and baby and should be discouraged. None of the pregnant women in this study who smoked developed pre-

eclampsia. This suggests large vessel endothelial dysfunction in the form of reduced vasodilatation is not an essential precursor to pre-eclampsia.

The final study has demonstrated that women with gestational hypertension have endothelial dysfunction and this may explain the increased risk of future CVD in these women. These results suggest these women may be presenting with underlying, early stage or pre-existing hypertension. My study could not differentiate between obese women with a late form of pre-eclampsia and normal pregnancy. A careful review of the literature found similarities between the women in my study and in studies with previously unexplained findings such as decreased SVR and increased cardiac output or increased microvasodilatory response in pre-eclampsia. My study and others have demonstrated that late onset obesity related pre-eclampsia does not result in reduced FMD. The important effect of maternal characteristics such as obesity and metabolic syndrome and the differences made by gestational age are becoming apparent. The multifactorial nature of pre-eclampsia may contribute to these results. It has been suggested that the FMD test may be a useful predictive test for pre-eclampsia. My work would suggest this would not be the case in obese late onset pre-eclampsia.

5.4 Future directions.

Flow-mediated dilatation is an evolving technique with complete agreement on methodology still to be reached. Future studies should concentrate on using a NO dependent technique (monitoring of the brachial or radial artery with five minute lower arm cuff occlusion). The reporting of shear stress to ensure any differences found are not due to differences in the shear stimulus should occur. Monitoring of post occlusion dilatation also needs to be done for at least 180 seconds to ensure that maximum dilatation

is reached. Adherence to these principles would remove extraneous variables that may confound the results.

Further studies to determine if endothelial dysfunction persists in pregnant women who have ceased smoking prior to pregnancy or who have reduced their level of smoking to less than five cigarettes a day during pregnancy would be informative. A positive effect on the vascular endothelium in women who reduce or cease smoking would provide positive reinforcement to the public health message about the benefits of ceasing smoking.

Nicotine patches are used in pregnancy (698). A study assessing whether nicotine patches affect endothelial function in pregnancy may provide more information regarding the effect of nicotine on endothelial function in pregnancy.

It has become evident that many factors play a part in the hypertensive disorders of pregnancy. The hypertensive population in the Greater West area of Sydney appear to be distinctive due to their size. This could provide opportunities to study in more detail the effects of obesity and metabolic syndrome on the hypertensive disorders of pregnancy.

The comparison of obese women with normal weight women in both normal and hypertensive pregnancy would be educational. It would also be interesting to know what is occurring to their biochemical profiles (adiponectin, other adipocytokines and angiogenic factors) and correlate this with FMD. As pre-eclampsia is also thought to be a placental/early gestational age disorder or a late gestational age/maternal disorder, the effect on fetal growth, fetal Dopplers and maternal uterine arteries and the correlation with placental histology and FMD would be of interest.

In conclusion, more research is required regarding endothelial dependent vasodilatation in pregnancy. The work in this thesis supports the concept of pre-eclampsia being a multifactorial disease. This work also demonstrated that women with gestational hypertension may present because of underlying endothelial dysfunction. Although smoking provides a protective effect for the development of pre-eclampsia, it does not protect the endothelium from the deleterious effects of cigarette smoking.

References.

1. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*. 1998;91(10):3527-61.
2. Roberts JM. Endothelial Dysfunction in Preeclampsia. *Semin Reprod Endocrinol*. 1998;16(1):5-15.
3. Lopez-Jaramillo P, Arenas WD, Garcia RG, Rincon MY, Lopez M. The role of the L-arginine-nitric oxide pathway in preeclampsia. *Ther Adv Cardiovasc Dis*. 2008;2(4):261-75.
4. Pohl U, Busse R, Kuon E, Bassenge E. Pulsatile perfusion stimulates the release of endothelial autacoids. *J Appl Cardiol*. 1986;1(3):215-35.
5. Guyton AC, Hall JE. *Textbook of Medical Physiology*. 10th ed. Philadelphia: W.B. Saunders; 2000.
6. Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. *Am J Physiol*. 1997;272(2 Pt 2):R441-63.
7. Williams DJ, Vallance PJ, Neild GH, Spencer JA, Imms FJ. Nitric oxide-mediated vasodilation in human pregnancy. *Am J Physiol*. 1997;272(2 Pt 2):H748-52.
8. Carbillon L, Uzan M, Uzan S. Pregnancy, vascular tone, and maternal hemodynamics: a crucial adaptation. *Obstet Gynecol Surv*. 2000;55(9):574-81.
9. Sibai BM. Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol*. 2003;102(1):181-92.
10. Panza JA, Quyyumi AA, Brush JE, Jr., Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med*. 1990;323(1):22-7.

11. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol.* 1989;161(5):1200-4.
12. Roberts JM, Redman CWG. Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet.* 1993;341(8858):1447-51.
13. Lindheimer MD, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy.* 3rd ed. Boston: Elsevier Academic Press; 2009.
14. Sheppard SJ, Khalil RA. Risk factors and mediators of the vascular dysfunction associated with hypertension in pregnancy. *Cardiovasc Hematol Disord Drug Targets.* 2010;10(1):33-52.
15. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation: a diagnostic instrument, or an experimental tool? *Chest.* 2005;127(6):2254-63.
16. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet.* 1992;340(8828):1111-5.
17. Pyke KE, Tschakovsky ME. The relationship between shear stress and flow-mediated dilatation: implications for the assessment of endothelial function. *J Physiol.* 2005;568(Pt 2):357-69.
18. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* 1980;288(5789):373-6.
19. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* 1987;327(6122):524-6.
20. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A.* 1987;84(24):9265-9.

21. Vallance P, Patton S, Bhagat K, Macallister R, Radomski MW, Moncada S, et al. Direct measurement of nitric oxide in human beings. *Lancet*. 1995;345(8968):153-54.
22. Menshikova EB, Zenkov NK, Reutov VP. Nitric oxide and NO-synthases in mammals in different functional states. *Biochemistry (Moscow)*. 2000;65(4):409-26.
23. Harald HHW, Schmidt HH, Nau H, Wittfoht W, Gerlach J, Prescher KE, et al. Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol*. 1988;154(2):213-6.
24. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43(2):109-42.
25. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochemical Journal*. 1994;298(Pt 2):249-58.
26. Walford G, Loscalzo J. Nitric oxide in vascular biology. *J Thromb Haemost*. 2003;1(10):2112-8.
27. Martin E, Davis K, Bian K, Lee YC, Murad F. Cellular Signaling With Nitric Oxide and Cyclic Guanosine Monophosphate. *Semin Perinatol*. 2000;24(1):2-6.
28. Searle NR, Sahab P. Endothelial vasomotor regulation in health and disease. *Can J Anaesth*. 1992;39(8):838-57.
29. Vallance P. Nitric oxide in the human cardiovascular system--SKB lecture 1997. *Br J Clin Pharmacol*. 1998;45(5):433-9.
30. Vallance P, Collier J, Moncada S. Nitric oxide synthesised from L-arginine mediates endothelium dependent dilatation in human veins in vivo. *Cardiovasc Res*. 1989;23(12):1053-7.
31. Calver A, Collier J, Vallance P. Nitric oxide and cardiovascular control. *Exp Physiol*. 1993;78(3):303-26.

32. Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension*. 1986;8(1):37-44.
33. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol*. 1986;250(6 Pt 2):H1145-9.
34. Pyke KE, Dwyer EM, Tschakovsky ME. Impact of controlling shear rate on flow-mediated dilation responses in the brachial artery of humans. *J Appl Physiol*. 2004;97(2):499-508.
35. Chien S, Li S, Shyy YJ. Effects of mechanical forces on signal transduction and gene expression in endothelial cells. *Hypertension*. 1998;31(1 Pt 2):162-9.
36. Wink DA, Miranda KM, Espey MG. Effects of Oxidative and Nitrosative Stress in Cytotoxicity. *Semin Perinatol*. 2000;24(1):20-3.
37. Reutov VP. Nitric Oxide Cycle in Mammals and the Cyclicity Principle. *Biochemistry (Moscow)*. 2002;67(3):293-311.
38. Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol*. 2001;1(8):1397-406.
39. Durner J, Gow AJ, Stamler JS, Glazebrook J. Ancient Origins of Nitric Oxide Signaling in Biological Systems. *Proc Natl Acad Sci U S A*. 1999;96(25):14206-7.
40. Gross SS, Lane P. Physiological Reactions of Nitric Oxide and Hemoglobin: A Radical Rethink. *Proc Natl Acad Sci U S A*. 1999;96(18):9967-9.
41. Stamler JS, Jaraki O, Osbourne J, Simon DI, Keaney J, Vita J, et al. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci U S A*. 1992;89(16):7674-7.
42. Armstrong R. The physiological role and pharmacological potential of nitric oxide in neutrophil activation. *Int Immunopharmacol*. 2001;1(8):1501-12.

43. Kubes P, Suzuki M, Granger DN. Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A*. 1991;88(11):4651-5.
44. Stamler JS, Loh E, Roddy M-A, Currie KE, Creager MA. Nitric Oxide and Endothelin Effects: Nitric Oxide Regulates Basal Systemic and Pulmonary Vascular Resistance in Healthy Humans. *Circulation*. 1994;89(5):2035-40.
45. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377(6546):239-42.
46. Radomski MW, Palmer RMJ, Moncada S. Endogenous Nitric Oxide Inhibits Human Platelet Adhesion to Vascular Endothelium. *Lancet*. 1987;330(8567):1057-8.
47. Ignarro LJ. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res*. 1989;65(1):1-21.
48. Freedman JE, Sauter R, Battinelli EM, Ault K, Knowles C, Huang PL, et al. Deficient Platelet-Derived Nitric Oxide and Enhanced Hemostasis in Mice Lacking the NOSIII Gene. *Circ Res*. 1999;84(12):1416-21.
49. Tortora GJ, Grabowski SR. *Principles of Anatomy and Physiology*. 9th ed. New York: John Wiley & Sons, Inc.; 2000.
50. Galley HF, Webster NR. Physiology of the endothelium. *Br J Anaesth*. 2004;93(1):105-13.
51. Vane JR, Anggard EE, Botting RM. Regulatory functions of the vascular endothelium. *N Engl J Med*. 1990;323(1):27-36.
52. Zammit VC, Whitworth JA, Brown MA. Preeclampsia: the effects of serum on endothelial cell prostacyclin, endothelin, and cell membrane integrity. *Am J Obstet Gynecol*. 1996;174(2):737-43.

53. Yamamoto T, Suzuki Y, Kojima K, Suzumori K, Suzuki T. The biological investigation of prostacyclin in preeclamptic women seen reduced endothelial function. *Hypertens Pregnancy*. 2010;29:484-91.
54. Baker PN, Davidge ST, Barankiewicz J, Roberts JM. Plasma of preeclamptic women stimulates and then inhibits endothelial prostacyclin. *Hypertension*. 1996;27(1):56-61.
55. Bryan RM, Jr., You J, Golding EM, Marrelli SP. Endothelium-derived hyperpolarizing factor: a cousin to nitric oxide and prostacyclin. *Anesthesiology*. 2005;102(6):1261-77.
56. Luksha L, Nisell H, Kublickiene K. The mechanism of EDHF-mediated responses in subcutaneous small arteries from healthy pregnant women. *Am J Physiol Regul Integr Comp Physiol*. 2004;286(6):R1102-9.
57. Luksha L, Nisell H, Luksha N, Kublickas M, Hultenby K, Kublickiene K. Endothelium-derived hyperpolarizing factor in preeclampsia: heterogeneous contribution, mechanisms, and morphological prerequisites. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(2):R510.
58. Lorant DE, Zimmerman GA, McIntyre TM, Prescott SM. Platelet-activating factor mediates procoagulant activity on the surface of endothelial cells by promoting leukocyte adhesion. *Semin Cell Biol*. 1995;6(5):295-303.
59. Kim YD, Danchak RM, Heim KF, Lees DE, Myers AK. Constriction of canine coronary arteries by platelet activating factor after brief ischemia. *Prostaglandins*. 1993;46(3):269-76.
60. Yasuhara H, Hobson RW, 2nd, Duran WN. Platelet-activating factor causes venular constriction in the microcirculation. *Microvasc Res*. 1994;47(2):279-84.

61. Armstead WM, Pourcyrous M, Mirro R, Leffler CW, Busija DW. Platelet activating factor: a potent constrictor of cerebral arterioles in newborn pigs. *Circ Res.* 1988;62(1):1-7.
62. Juncos LA, Ren YL, Arima S, Ito S. Vasodilator and constrictor actions of platelet-activating factor in the isolated microperfused afferent arteriole of the rabbit kidney. Role of endothelium-derived relaxing factor/nitric oxide and cyclooxygenase products. *J Clin Invest.* 1993;91(4):1374-9.
63. Rowland BL, Vermillion ST, Roudebush WE. Elevated circulating concentrations of platelet activating factor in preeclampsia. *Am J Obstet Gynecol.* 2000;183(4):930-2.
64. Gu Y, Burlison SA, Wang Y. PAF levels and PAF-AH activities in placentas from normal and preeclamptic pregnancies. *Placenta.* 2006;27(6-7):744-9.
65. Taddei S, Viridis A, Ghiadoni L, Sudano I, Magagna A, Salvetti A. Role of endothelin in the control of peripheral vascular tone in human hypertension. *Heart Fail Rev.* 2001;6(4):277-85.
66. Lavallee M, Takamura M, Parent R, Thorin E. Crosstalk between endothelin and nitric oxide in the control of vascular tone. *Heart Fail Rev.* 2001;6(4):265-76.
67. Vural P. Nitric oxide/endothelin-1 in preeclampsia. *Clin Chim Acta.* 2002;317(1-2):65-70.
68. Nishikawa S, Miyamoto A, Yamamoto H, Ohshika H, Kudo R. The relationship between serum nitrate and endothelin-1 concentrations in preeclampsia. *Life Sci.* 2000;67(12):1447-54.
69. Ajne G, Ahlborg G, Wolff K, Nisell H. Contribution of endogenous endothelin-1 to basal vascular tone during normal pregnancy and preeclampsia. *Am J Obstet Gynecol.* 2005;193(1):234-40.

70. Goeschen K, Henkel E, Behrens O. Plasma prostacyclin and thromboxane concentrations in 160 normotensive, hypotensive, and preeclamptic patients during pregnancy, delivery, and the post partum period. *J Perinat Med.* 1993;21(6):481-9.
71. Mills JL, DerSimonian R, Raymond E, Morrow JD, Roberts LJ, 2nd, Clemens JD, et al. Prostacyclin and thromboxane changes predating clinical onset of preeclampsia: a multicenter prospective study. *JAMA.* 1999;282(4):356-62.
72. Malatyalioglu E, Adam B, Yanik FF, Kokcu A, Alvur M. Levels of stable metabolites of prostacyclin and thromboxane A2 and their ratio in normotensive and preeclamptic pregnant women during the antepartum and postpartum periods. *J Matern Fetal Med.* 2000;9(3):173-7.
73. Barac A, Campia U, Panza JA. Methods for evaluating endothelial function in humans. *Hypertension.* 2007;49(4):748.
74. Deanfield J, Donald A, Ferri C, Giannattasio C, Halcox J, Halligan S, et al. Endothelial function and dysfunction. Part I: Methodological issues for assessment in the different vascular beds: a statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension. *J Hypertens.* 2005;23(1):7.
75. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends Pharmacol Sci.* 2006;27(9):503-8.
76. Dupuis J. Noninvasive evaluation of endothelial vascular reactivity: should the quest continue? *Can J Cardiol.* 2005;21(12):1047-51.
77. Lockhart CJ, McVeigh GE, Cohn JN. Measuring endothelial function. *Curr Diab Rep.* 2006;6(4):267-73.

78. Donald AE, Charakida M, Cole TJ, Friberg P, Chowienczyk PJ, Millasseau SC, et al. Non-invasive assessment of endothelial function: which technique? *J Am Coll Cardiol*. 2006;48(9):1846-50.
79. Hansell J, Henareh L, Agewall S, Norman M. Non-invasive assessment of endothelial function - relation between vasodilatory responses in skin microcirculation and brachial artery. *Clin Physiol Funct Imaging*. 2004;24(6):317-22.
80. Kremkau FW. *Diagnostic Ultrasound: Principles and Instruments*. St Louis, Missouri: Saunders Elsevier; 2006.
81. Martins WP, Natri CO, Ferriani RA, Filho FM. Brachial artery pulsatility index change 1 minute after 5-minute forearm compression: comparison with flow-mediated dilatation. *J Ultrasound Med*. 2008;27(5):693-9.
82. Lima JC, Martins WP, Natri CO, Nicolau LGC, Filho FM. Pulsatility index change of brachial artery shows better reproducibility than flow-mediated vasodilation. *Ultrasound Med Biol*. 2010;36(12):2036-41.
83. Weissgerber TL, Davies GAL, Tschakovsky ME. Low flow-mediated constriction occurs in the radial but not the brachial artery in healthy pregnant and nonpregnant women. *J Appl Physiol*. 2010;108(5):1097-105.
84. Brunner H, Cockcroft JR, Deanfield J, Donald A, Ferrannini E, Halcox J, et al. Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension. *J Hypertens*. 2005;23(2):233-46.
85. http://www.ornl.gov/sci/techresources/Human_Genome/faq/snps.shtml. 2007 [cited 2008 19 March].
86. <http://www.superarray.com/>. 2008 [cited 2008 19 March].

87. Savvidou M, Vallance P, Nicolaides K, Hingorani A. Endothelial Nitric Oxide Synthase Gene Polymorphism and Maternal Vascular Adaptation to Pregnancy. *Hypertension*. 2001;38(6):1289-93.
88. Tsikas D. Review: Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. 2005;39(8):797 - 815.
89. Sugawara J, Mitsui-Saito M, Hoshiai T, Hayashi C, Kimura Y, Okamura K. Circulating endothelial progenitor cells during human pregnancy. *J Clin Endocrinol Metab*. 2005;90(3):1845-8.
90. Matsubara K, Abe E, Matsubara Y, Kameda K, Ito M. Circulating endothelial progenitor cells during normal pregnancy and pre-eclampsia. *Am J Reprod Immunol*. 2006;56(2):79-85.
91. Anderson EA, Mark AL. Flow-mediated and reflex changes in large peripheral artery tone in humans. *Circulation*. 1989;79(1):93-100.
92. Sinoway LI, Hendrickson C, Davidson WR, Jr., Prophet S, Zelis R. Characteristics of flow-mediated brachial artery vasodilation in human subjects. *Circ Res*. 1989;64(1):32-42.
93. Smiesko V, Kozik J, Dolezel S. Role of endothelium in the control of arterial diameter by blood flow. *Blood Vessels*. 1985;22(5):247-51.
94. Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*. 1989;334(8670):997-1000.
95. Davies PF, Tripathi SC. Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ Res*. 1993;72(2):239-45.
96. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-

mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol.* 2002;39(2):257-65.

97. Green D. Point: Flow-mediated dilation does reflect nitric oxide-mediated endothelial function. *J Appl Physiol.* 2005;99(3):1233-4.

98. Tschakovsky ME, Pyke KE. Counterpoint: Flow-mediated dilation does not reflect nitric oxide-mediated endothelial function. *J Appl Physiol.* 2005;99(3):1235-8.

99. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. *Hypertension.* 2010;55(5):1075-85.

100. Thijssen DHJ, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol.* 2011;300(1):H2-H12.

101. Herrington DM, Fan L, Drum M, Riley WA, Pusser BE, Crouse JR, et al. Brachial flow-mediated vasodilator responses in population-based research: methods, reproducibility and effects of age, gender and baseline diameter. *J Cardiovasc Risk.* 2001;8(5):319-28.

102. Walter JP. Physics of high-resolution ultrasound--practical aspects. *Radiol Clin North Am.* 1985;23(1):3-11.

103. Wendelhag I, Gustavsson T, Suurkula M, Berglund G, Wikstrand J. Ultrasound measurement of wall thickness in the carotid artery: fundamental principles and description of a computerized analysing system. *Clin Physiol.* 1991;11(6):565-77.

104. Potter K, Reed CJ, Green DJ, Hankey GJ, Arnolda LF. Ultrasound settings significantly alter arterial lumen and wall thickness measurements. *Cardiovascular Ultrasound.* 2008;6:6.

105. Parker BA, Ridout SJ, Proctor DN. Age and flow-mediated dilation: a comparison of dilatory responsiveness in the brachial and popliteal arteries. *Am J Physiol Heart Circ Physiol.* 2006;291(6):H3043-9.
106. Thijssen DH, van Bommel MM, Bullens LM, Dawson EA, Hopkins ND, Tinken TM, et al. The impact of baseline diameter on flow-mediated dilation differs in young and older humans. *Am J Physiol Heart Circ Physiol.* 2008;295(4):H1594-8.
107. Thijssen DH, Dawson EA, Black MA, Hopman MT, Cable NT, Green DJ. Heterogeneity in conduit artery function in humans: impact of arterial size. *Am J Physiol Heart Circ Physiol.* 2008;295(5):H1927-34.
108. Silber HA, Ouyang P, Bluemke DA, Gupta SN, Foo TK, Lima JA. Why is flow-mediated dilation dependent on arterial size? Assessment of the shear stimulus using phase-contrast magnetic resonance imaging. *Am J Physiol Heart Circ Physiol.* 2005;288(2):H822-8.
109. Stadler RW, Karl WC, Lees RS. New methods for arterial diameter measurement from B-mode images. *Ultrasound Med Biol.* 1996;22(1):25-34.
110. Wikstrand J, Wendelhag I. Methodological considerations of ultrasound investigation of intima-media thickness and lumen diameter. *J Intern Med.* 1994;236(5):555-9.
111. Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, et al. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. *Br Heart J.* 1995;74(3):247-53.
112. Kobayashi H, Yoshida A, Kobayashi M, Nakao S. Non-invasive detection of endothelial dysfunction with 30MHz transducer. *Lancet.* 1996;347(9011):1336-7.

113. Padilla J, Johnson BD, Newcomer SC, Wilhite DP, Mickleborough TD, Fly AD, et al. Normalization of flow-mediated dilation to shear stress area under the curve eliminates the impact of variable hyperemic stimulus. *Cardiovascular Ultrasound*. 2008;6:44.
114. Bots ML, Westerink J, Rabelink TJ, de Koning EJ. Assessment of flow-mediated vasodilatation (FMD) of the brachial artery: effects of technical aspects of the FMD measurement on the FMD response. *Eur Heart J*. 2005;26(4):363-8.
115. Eik-Nes SH, Marsal K, Kristoffersen K. Methodology and basic problems related to blood flow studies in the human fetus. *Ultrasound Med Biol*. 1984;10(3):329-37.
116. Kizhakekuttu TJ, Gutterman DD, Phillips SA, Jurva JW, Arthur EIL, Das E, et al. Measuring FMD in the brachial artery: how important is QRS gating? *J Appl Physiol*. 2010;109(4):959-65.
117. Association of Operating Room Nurses. Recommended practices for use of the pneumatic tourniquet. *AORN Journal*. 2002;75(2):379-82, 84-6.
118. Pedowitz RA, Gershuni DH, Botte MJ, Kuiper S, Rydevik BL, Hargens AR. The use of lower tourniquet inflation pressures in extremity surgery facilitated by curved and wide tourniquets and an integrated cuff inflation system. *Clin Orthop*. 1993(287):237-44.
119. Newman RJ, Muirhead A. A safe and effective low pressure tourniquet. A prospective evaluation. *J Bone Joint Surg Br*. 1986;68(4):625-8.
120. Reid HS, Camp RA, Jacob WH. Tourniquet hemostasis. A clinical study. *Clin Orthop*. 1983(177):230-4.
121. Levy O, David Y, Heim M, Eldar I, Chetrit A, Engel J. Minimal tourniquet pressure to maintain arterial closure in upper limb surgery. *J Hand Surg [Br]*. 1993;18(2):204-6.

122. Tuncali B, Karci A, Tuncali BE, Mavioglu O, Ozkan M, Bacakoglu AK, et al. A new method for estimating arterial occlusion pressure in optimizing pneumatic tourniquet inflation pressure. *Anesth Analg*. 2006;102(6):1752-7.
123. Peretz A, Leotta DF, Sullivan JH, Trenga CA, Sands FN, Aulet MR, et al. Flow mediated dilation of the brachial artery: an investigation of methods requiring further standardization. *BMC Cardiovasc Disord*. 2007;7:11.
124. Pyke K, Green DJ, Weisbrod C, Best M, Dembo L, O'Driscoll G, et al. Nitric oxide is not obligatory for radial artery flow-mediated dilation following release of 5 or 10 min distal occlusion. *Am J Physiol Heart Circ Physiol*. 2010;298(1):H119-26.
125. Mannion TC, Vita JA, Keaney JF, Jr., Benjamin EJ, Hunter L, Polak JF. Non-invasive assessment of brachial artery endothelial vasomotor function: the effect of cuff position on level of discomfort and vasomotor responses. *Vasc Med*. 1998;3(4):263-7.
126. Agewall S, Doughty RN, Bagg W, Whalley GA, Braatvedt G, Sharpe N. Comparison of ultrasound assessment of flow-mediated dilatation in the radial and brachial artery with upper and forearm cuff positions. *Clin Physiol*. 2001;21(1):9-14.
127. Berry KL, Skyrme-Jones RA, Meredith IT. Occlusion cuff position is an important determinant of the time course and magnitude of human brachial artery flow-mediated dilation. *Clin Sci*. 2000;99(4):261-7.
128. Betik AC, Luckham VB, Hughson RL. Flow-mediated dilation in human brachial artery after different circulatory occlusion conditions. *Am J Physiol Heart Circ Physiol*. 2004;286(1):H442-8.
129. Corretti MC, Plotnick GD, Vogel RA. Technical aspects of evaluating brachial artery vasodilatation using high-frequency ultrasound. *Am J Physiol*. 1995;268(4 Pt 2):H1397-404.

130. Doshi SN, Naka KK, Payne N, Jones CJ, Ashton M, Lewis MJ, et al. Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. *Clin Sci*. 2001;101(6):629-35.
131. Leeson P, Thorne S, Donald A, Mullen M, Clarkson P, Deanfield J. Non-invasive measurement of endothelial function: effect on brachial artery dilatation of graded endothelial dependent and independent stimuli. *Heart*. 1997;78(1):22-7.
132. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, et al. Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circ Res*. 2001;88(2):145-51.
133. Joannides R, Bakkali el H, Richard V, Benoist A, Moore N, Thuillez C. Evaluation of the determinants of flow-mediated radial artery vasodilatation in humans. *Clin Exp Hypertens*. 1997;19(5-6):813-26.
134. Black MA, Cable NT, Thijssen DH, Green DJ. Importance of measuring the time course of flow-mediated dilatation in humans. *Hypertension*. 2008;51(2):203-10.
135. Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, et al. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *J Appl Physiol*. 2001;91(2):929-37.
136. Craiem D, Chironi G, Simon A, Levenson J. New assessment of endothelium-dependent flow-mediated vasodilation to characterize endothelium dysfunction. *Am J Ther*. 2008;15(4):340-4.
137. Faita F, Masi S, Loukogeorgakis S, Gemignani V, Okorie M, Bianchini E, et al. Comparison of two automatic methods for the assessment of brachial artery flow-mediated dilation. *J Hypertens*. 2011;29(1):85-90.

138. Clarkson P, Celermajer DS, Donald AE, Sampson M, Sorensen KE, Adams M, et al. Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. *J Am Coll Cardiol.* 1996;28(3):573-9.
139. Leeson CP, Whincup PH, Cook DG, Donald AE, Papacosta O, Lucas A, et al. Flow-mediated dilation in 9- to 11-year-old children: the influence of intrauterine and childhood factors. *Circulation.* 1997;96(7):2233-8.
140. Benjamin EJ, Larson MG, Keyes MJ, Mitchell GF, Vasani RS, Keaney JF, Jr., et al. Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. *Circulation.* 2004;109(5):613-9.
141. Savvidou MD, Geerts L, Nicolaides KH, Savvidou MD, Geerts L, Nicolaides KH. Impaired vascular reactivity in pregnant women with insulin-dependent diabetes mellitus. *Am J Obstet Gynecol.* 2002;186(1):84-8.
142. Paradisi G, Biaggi A, Ferrazzani S. Abnormal Carbohydrate Metabolism During Pregnancy. Association with endothelial dysfunction. *Diabetes Care.* 2002;25(3):560-4.
143. Anastasiou E, Lekakis JP, Alevizaki M, Papamichael CM, Megas J, Souvatzoglou A, et al. Impaired endothelium-dependent vasodilatation in women with previous gestational diabetes. *Diabetes Care.* 1998;21(12):2111-5.
144. Iiyama K, Nagano M, Yo Y, Nagano N, Kamide K, Higaki J, et al. Impaired endothelial function with essential hypertension assessed by ultrasonography. *Am Heart J.* 1996;132(4):779-82.
145. Ghiadoni L, Huang Y, Magagna A, Buralli S, Taddei S, Salvetti A. Effect of acute blood pressure reduction on endothelial function in the brachial artery of patients with essential hypertension. *J Hypertens.* 2001;19(3 Pt 2):547-51.

146. Ghiadoni L, Magagna A, Versari D, Kardasz I, Huang Y, Taddei S, et al. Different effect of antihypertensive drugs on conduit artery endothelial function. *Hypertension*. 2003;41(6):1281-6.
147. Paradisi G, Biaggi A, Savone R, Ianniello F, Tomei C, Caforio L, et al. Cardiovascular risk factors in healthy women with previous gestational hypertension. *J Clin Endocrinol Metab*. 2006;91(4):1233-8.
148. Dimsdale JE. Psychological stress and cardiovascular disease. *J Am Coll Cardiol*. 2008;51(13):1237-46.
149. Hadi HAR, Carr CS, Al Suwaidi J. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc Health Risk Manage*. 2005;1(3):183-98.
150. Harris CW, Edwards JL, Baruch A, Riley WA, Pusser BE, Rejeski WJ, et al. Effects of mental stress on brachial artery flow-mediated vasodilation in healthy normal individuals. *Am Heart J*. 2000;139(3):405-11.
151. Ghiadoni L, Donald AE, Cropley M, Mullen MJ, Oakley G, Taylor M, et al. Mental stress induces transient endothelial dysfunction in humans. *Circulation*. 2000;102(20):2473-8.
152. Spieker LE, Hurlimann D, Ruschitzka F, Corti R, Enseleit F, Shaw S, et al. Mental stress induces prolonged endothelial dysfunction via endothelin-A receptors. *Circulation*. 2002;105(24):2817-20.
153. Mangos GJ, Walker BR, Kelly JJ, Lawson JA, Webb DJ, Whitworth JA. Cortisol inhibits cholinergic vasodilation in the human forearm. *Am J Hypertens*. 2000;13(11):1155-60.
154. Broadley AJ, Korszun A, Abdelaal E, Moskvina V, Jones CJ, Nash GB, et al. Inhibition of cortisol production with metyrapone prevents mental stress-induced endothelial dysfunction and baroreflex impairment. *J Am Coll Cardiol*. 2005;46(2):344-50.

155. Dyson KS, Shoemaker JK, Hughson RL. Effect of acute sympathetic nervous system activation on flow-mediated dilation of brachial artery. *Am J Physiol Heart Circ Physiol*. 2006;290(4):H1446-53.
156. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol*. 1994;24(2):471-6.
157. Black MA, Cable NT, Thijssen DH, Green DJ. Impact of age, sex, and exercise on brachial artery flow-mediated dilatation. *Am J Physiol Heart Circ Physiol*. 2009;297(3):H1109-16.
158. Jensen-Urstad K, Johansson J. Gender difference in age-related changes in vascular function. *J Intern Med*. 2001;250(1):29-36.
159. Lambert J, Stehouwer CD. Modulation of endothelium-dependent, flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation*. 1996;94(9):2319-20.
160. McCrohon JA, Adams MR, McCredie RJ, Robinson J, Pike A, Abbey M, et al. Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. *Clin Endocrinol (Oxf)*. 1996;45(4):435-41.
161. Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation*. 1995;92(12):3431-5.
162. Williams MR, Westerman RA, Kingwell BA, Paige J, Blombery PA, Sudhir K, et al. Variations in endothelial function and arterial compliance during the menstrual cycle. *J Clin Endocrinol Metab*. 2001 Nov;86(11):5389-95.

163. Kawano H, Motoyama T, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, et al. Menstrual cyclic variation of endothelium-dependent vasodilation of the brachial artery: possible role of estrogen and nitric oxide. *Proc Assoc Am Physicians*. 1996;108(6):473-80.
164. Giannattasio C, Failla M, Grappiolo A, Stella ML, Del Bo A, Colombo M, et al. Fluctuations of radial artery distensibility throughout the menstrual cycle. *Arterioscler Thromb Vasc Biol*. 1999;19(8):1925-9.
165. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. *Am J Cardiol*. 1997;79(3):350-4.
166. Raitakari OT, Lai N, Griffiths K, McCredie R, Sullivan D, Celermajer DS. Enhanced peripheral vasodilation in humans after a fatty meal. *J Am Coll Cardiol*. 2000;36(2):417-22.
167. Keogh JB, Grieger JA, Noakes M, Clifton PM. Flow-mediated dilatation is impaired by a high-saturated fat diet but not by a high-carbohydrate diet. *Arterioscler Thromb Vasc Biol*. 2005;25(6):1274-9.
168. Hall WL, Hall WL. Dietary saturated and unsaturated fats as determinants of blood pressure and vascular function. *Nutr Res Rev*. 2009;22(1):18-38.
169. Gosmanov AR, Smiley DD, Robalino G, Siquiera J, Khan B, Le N-A, et al. Effects of oral and intravenous fat load on blood pressure, endothelial function, sympathetic activity, and oxidative stress in obese healthy subjects. *Am J Physiol Endocrinol Metab*. 2010;299(6):E953-8.
170. Duffy SJ, Keaney JF, Jr., Holbrook M, Gokce N, Swerdloff PL, Frei B, et al. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation*. 2001;104(2):151-6.

171. Papamichael CM, Aznaouridis KA, Karatzis EN, Karatzi KN, Stamatelopoulos KS, Vamvakou G, et al. Effect of coffee on endothelial function in healthy subjects: the role of caffeine. *Clin Sci*. 2005;109(1):55-60.
172. Hodgson JM, Puddey IB, Burke V, Watts GF, Beilin LJ. Regular ingestion of black tea improves brachial artery vasodilator function. *Clin Sci*. 2002;102(2):195-201.
173. Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, et al. Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *J Am Coll Nutr*. 2004;23(3):197-204.
174. Vlachopoulos C, Aznaouridis K, Alexopoulos N, Economou E, Andreadou I, Stefanadis C. Effect of dark chocolate on arterial function in healthy individuals. *Am J Hypertens*. 2005;18(6):785-91.
175. Agewall S, Wright S, Doughty RN, Whalley GA, Duxbury M, Sharpe N. Does a glass of red wine improve endothelial function? *Eur Heart J*. 2000;21(1):74-8.
176. Hodgson JM. Effects of tea and tea flavonoids on endothelial function and blood pressure: a brief review. *Clin Exp Pharmacol Physiol*. 2006;33(9):838-41.
177. Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation*. 1993;88(5 Pt 1):2149-55.
178. Celermajer DS, Adams MR, Clarkson P, Robinson J, McCredie R, Donald A, et al. Passive smoking and impaired endothelium-dependent arterial dilatation in healthy young adults. *N Engl J Med*. 1996;334(3):150-4.
179. Raitakari OT, Adams MR, McCredie RJ, Griffiths KA, Celermajer DS. Arterial endothelial dysfunction related to passive smoking is potentially reversible in healthy young adults. *Ann Intern Med*. 1999;130(7):578-81.

180. Granberry MC, Smith ES, 3rd, Troillett RD, Eidt JF. Forearm endothelial response in smokeless tobacco users compared with cigarette smokers and nonusers of tobacco. *Pharmacotherapy*. 2003;23(8):974-8.
181. Papamichael CM, Aznaouridis KA, Stamatelopoulos KS, Karatzis EN, Protopgerou AD, Papaioannou TG, et al. Endothelial dysfunction and type of cigarette smoked: the impact of 'light' versus regular cigarette smoking. *Vasc Med*. 2004;9(2):103-5.
182. Karatzi K, Papamichael C, Karatzis E, Papaioannou TG, Stamatelopoulos K, Zakopoulos NA, et al. Acute smoke-induced endothelial dysfunction is more prolonged in smokers than in non-smokers. *Int J Cardiol*. 2007;120(3):404-6.
183. Lekakis J, Papamichael C, Vemmos C, Nanas J, Kontoyannis D, Stamatelopoulos S, et al. Effect of acute cigarette smoking on endothelium-dependent brachial artery dilatation in healthy individuals. *Am J Cardiol*. 1997;79(4):529-31.
184. Neunteufl T, Heher S, Kostner K, Mitulovic G, Lehr S, Khoschsorur G, et al. Contribution of nicotine to acute endothelial dysfunction in long-term smokers. *J Am Coll Cardiol*. 2002;39(2):251-6.
185. Jochmann N, Muller S, Kuhn C, Gericke C, Baumann G, Stangl K, et al. Chronic smoking prevents amelioration of endothelial function in the course of the menstrual cycle. *Circ J*. 2009;73(3):568-72.
186. Ranta V, Viinikka L, Halmesmaki E, Ylikorkala O. Nitric oxide production with preeclampsia. *Obstet Gynecol*. 1999;93(3):442-5.
187. Khalil A, Muttukrishna S, Harrington K, Jauniaux E. Effect of antihypertensive therapy with alpha methyl dopa on levels of angiogenic factors in pregnancies with hypertensive disorders. *PLoS ONE*. 2008;3(7):e2766.

188. Muiesan ML, Salvetti M, Monteduro C, Rizzoni D, Zulli R, Corbellini C, et al. Effect of treatment on flow-dependent vasodilation of the brachial artery in essential hypertension. *Hypertension*. 1999;33(1 Pt 2):575-80.
189. Gokce N, Holbrook M, Hunter LM, Palmisano J, Vigalok E, Keaney JF, Jr., et al. Acute effects of vasoactive drug treatment on brachial artery reactivity. *J Am Coll Cardiol*. 2002;40(4):761-5.
190. Lekakis JP, Protogerou A, Papamichael C, Vamvakou G, Iconomidis I, Fici F, et al. Effect of nebivolol and atenolol on brachial artery flow-mediated vasodilation in patients with coronary artery disease. *Cardiovasc Drugs Ther*. 2005;19(4):277-81.
191. Thuillez C, Richard V. Targeting endothelial dysfunction in hypertensive subjects. *J Hum Hypertens*. 2005;19(1):S21-5.
192. Arkin JM, Alsdorf R, Bigornia S, Palmisano J, Beal R, Istfan N, et al. Relation of cumulative weight burden to vascular endothelial dysfunction in obesity. *Am J Cardiol*. 2008;101(1):98-101.
193. Olson TP, Schmitz KH, Leon AS, Dengel DR. Vascular structure and function in women: relationship with body mass index. *Am J Prev Med*. 2006;30(6):487-92.
194. Etsuda H, Takase B, Uehata A, Kusano H, Hamabe A, Kuhara R, et al. Morning attenuation of endothelium-dependent, flow-mediated dilation in healthy young men: possible connection to morning peak of cardiac events? *Clin Cardiol*. 1999;22(6):417-21.
195. Gaenger H, Sturm W, Kirchmair R, Neumayr G, Ritsch A, Patsch J. Circadian variation of endothelium-dependent vasodilatation of the brachial artery as a confounding factor in the evaluation of endothelial function. *Atherosclerosis*. 2000;149(1):227-8.
196. Otto ME, Svatikova A, Barretto RB, Santos S, Hoffmann M, Khandheria B, et al. Early morning attenuation of endothelial function in healthy humans. *Circulation*. 2004;109(21):2507-10.

197. Ringqvist A, Caidahl K, Petersson AS, Wennmalm A. Diurnal variation of flow-mediated vasodilation in healthy premenopausal women. *Am J Physiol Heart Circ Physiol.* 2000;279(6):H2720-5.
198. ter Avest E, Holewijn S, Stalenhoef AF, de Graaf J. Variation in non-invasive measurements of vascular function in healthy volunteers during daytime. *Clin Sci.* 2005;108(5):425-31.
199. Agewall S, Hulthe J, Fagerberg B, Gottfridsson B, Wikstrand J. Post-occlusion brachial artery vasodilatation after ischaemic handgrip exercise is nitric oxide mediated. *Clin Physiol Funct Imaging.* 2002;22(1):18-23.
200. Wendelhag I, Fagerberg B, Wikstrand J. Adding ischaemic hand exercise during occlusion of the brachial artery increases the flow-mediated vasodilation in ultrasound studies of endothelial function. *Clin Physiol.* 1999;19(4):279-83.
201. Padilla J, Harris RA, Fly AD, Rink LD, Wallace JP. A comparison between active- and reactive-hyperaemia-induced brachial artery vasodilation. *Clin Sci.* 2006;110(3):387-92.
202. Padilla J, Harris RA, Wallace JP. Can the measurement of brachial artery flow-mediated dilation be applied to the acute exercise model? *Cardiovasc Ultrasound.* 2007;5:45.
203. Harvey PJ, Morris BL, Kubo T, Picton PE, Su WS, Notarius CF, et al. Hemodynamic after-effects of acute dynamic exercise in sedentary normotensive postmenopausal women. *J Hypertens.* 2005;23(2):285-92.
204. Gokce N, Holbrook M, Duffy SJ, Demissie S, Cupples LA, Biegelsen E, et al. Effects of race and hypertension on flow-mediated and nitroglycerin-mediated dilation of the brachial artery. *Hypertension.* 2001;38(6):1349-54.

205. Marchesi S, Lupattelli G, Sensini A, Lombardini R, Brozzetti M, Roscini AR, et al. Racial difference in endothelial function: role of the infective burden. *Atherosclerosis*. 2007;191(1):227-34.
206. Loehr LR, Espeland MA, Sutton-Tyrrell K, Burke GL, Crouse JR, 3rd, Herrington DM. Racial differences in endothelial function in postmenopausal women. *Am Heart J*. 2004;148(4):606-11.
207. Dorup I, Skajaa K, Sorensen KE. Normal pregnancy is associated with enhanced endothelium-dependent flow-mediated vasodilation. *Am J Physiol*. 1999;276(3pt2):H821-5.
208. Savvidou MD, Kametas NA, Donald AE, Nicolaides KH. Non-invasive assessment of endothelial function in normal pregnancy. *Ultrasound Obstet Gynecol*. 2000;15(6):502-7.
209. HDI 5000 Ultrasound System Reference Manual. Bothell, USA: ATL Ultrasound, A Philips Medical Systems Company; 2000.
210. Gill RW. Measurement of blood flow by ultrasound: Accuracy and sources of error. *Ultrasound Med Biol*. 1985;11(4):625-41.
211. Levenson JA, Peronneau PA, Simon A, Safar ME. Pulsed Doppler: determination of diameter, blood flow velocity, and volumic flow of brachial artery in man. *Cardiovasc Res*. 1981;15(3):164-70.
212. Burns PN, Jaffe CC. Quantitative flow measurements with Doppler ultrasound: techniques, accuracy, and limitations. *Radiol Clin North Am*. 1985;23(4):641-57.
213. Melkumyants AM, Balashov SA, Khayutin VM. Endothelium dependent control of arterial diameter by blood viscosity. *Cardiovasc Res*. 1989;23(9):741-7.
214. Widlansky ME. Shear stress and flow-mediated dilation: all shear responses are not created equally. *Am J Physiol Heart Circ Physiol*. 2009;296(1):H31-2.

215. Mitchell GF, Parise H, Vita JA, Larson MG, Warner E, Keaney JF, Jr., et al. Local shear stress and brachial artery flow-mediated dilation: the Framingham Heart Study. *Hypertension*. 2004;44(2):134-9.
216. Wootton DM, Ku DN. Fluid mechanics of vascular systems, diseases, and thrombosis. *Annu Rev Biomed Eng*. 1999;1:299-329.
217. Rosencranz R, Bogen SA. Clinical laboratory measurement of serum, plasma, and blood viscosity. *Am J Clin Pathol*. 2006;125 (Suppl):S78-86.
218. Burch GE, DePasquale NP. Hematocrit, viscosity and coronary blood flow. *Dis Chest*. 1965;48(3):225-32.
219. Katritsis D, Kaiktsis L, Chaniotis A, Pantos J, Efstathopoulos EP, Marmarelis V. Wall shear stress: theoretical considerations and methods of measurement. *Prog Cardiovasc Dis*. 2007;49(5):307-29.
220. Lee MY, Wu CM, Yu KH, Chu CS, Lee KT, Sheu SH, et al. Association between wall shear stress and carotid atherosclerosis in patients with never treated essential hypertension. *Am J Hypertens*. 2009;22(7):705-10.
221. Reneman RS, Arts T, Hoeks AP. Wall shear stress-an important determinant of endothelial cell function and structure-in the arterial system in vivo. Discrepancies with theory. *J Vasc Res*. 2006;43(3):251-69.
222. Parker BA, Trehearn TL, Meendering JR. Pick your poiseuille: normalizing the shear stimulus in studies of flow-mediated dilation. *J Appl Physiol*. 2009;107(4):1357-9.
223. Pyke KE, Tschakovsky ME. Peak vs. total reactive hyperemia: which determines the magnitude of flow-mediated dilation? *J Appl Physiol*. 2007;102(4):1510-9.
224. Pyke KE, Hartnett JA, Tschakovsky ME. Are the dynamic response characteristics of brachial artery flow-mediated dilation sensitive to the magnitude of increase in shear stimulus? *J Appl Physiol*. 2008;105(1):282-92.

225. Pyke KE, Poitras V, Tschakovsky ME. Brachial artery flow-mediated dilation during handgrip exercise: evidence for endothelial transduction of the mean shear stimulus. *Am J Physiol Heart Circ Physiol*. 2008;294(6):H2669-79.
226. Thijssen DH, Bullens LM, van Bommel MM, Dawson EA, Hopkins N, Tinken TM, et al. Does arterial shear explain the magnitude of flow-mediated dilation?: a comparison between young and older humans. *Am J Physiol Heart Circ Physiol*. 2009;296(1):H57-64.
227. Atkinson G, Batterham AM, Green DJ, Thijssen DH. Commentaries on viewpoint: pick your poiseuille: normalizing the shear stimulus in studies of flow-mediated dilation. *J Appl Physiol*. 2009;107(4):1362.
228. Johnson BD, Padilla J, Wallace JP. Normalization Of Flow Mediated Dilation To Time-to-Peak Improves Measurement Sensitivity. *Med Sci Sports Exerc*. 2009;41(5):78
229. Watanabe K, Oba K, Suzuki T, Ouchi M, Suzuki K, Futami-Suda S, et al. The Importance of Assessment of Endothelial Function According to the Time Course of Flow-mediated Dilation of the Brachial Artery. *J Nippon Med Sch*. 2010;77(1):59-61.
230. Irace C, Tschakovsky ME, Carallo C, Cortese C, Gnasso A. Endothelial dysfunction or dysfunctions? Identification of three different FMD responses in males with type 2 diabetes. *Atherosclerosis*. 2008;200(2):439-45.
231. Liuni A, Luca MC, Lisi M, Dragoni S, di Stolfo G, Mariani JA, et al. Observations of time-based measures of flow-mediated dilation of forearm conduit arteries: implications for the accurate assessment of endothelial function. *Am J Physiol Heart Circ Physiol*. 2010;299(3):H939-45.
232. Chesley LC. History and epidemiology of preeclampsia-eclampsia. *Clin Obstet Gynecol*. 1984;27(4):801-20.
233. Chesley LC. Hypertensive Disorders in Pregnancy. New York: Appleton-Century-Crofts; 1978.

234. Roberts JM, Hubel CA. The two stage model of preeclampsia: variations on the theme. *Placenta*. 2009;30 Suppl A:S32-7.
235. Roberts JM, Gammill HS. Preeclampsia: recent insights. *Hypertension*. 2005;46(6):1243-9.
236. Maynard S, Epstein FH, Karumanchi SA. Preeclampsia and angiogenic imbalance. *Annu Rev Med*. 2008;59:61-78.
237. Kanasaki K, Kalluri R. The biology of preeclampsia. *Kidney Int*. 2009;76(8):831-7.
238. Higgins JR, de Swiet M. Blood-pressure measurement and classification in pregnancy. *Lancet*. 2001;357(9250):131-5.
239. Page EW. The relation between hydatid moles, relative ischemia of the gravid uterus, and the placental origins of eclampsia. *Am J Obstet Gynecol*. 1939;37:291-3.
240. MacGillivray I. Hypertension in pregnancy and its consequences. *J Obstet Gynaecol Br Commonw*. 1961;68:557-69.
241. Helewa ME, Burrows RF, Smith J, Williams K, Brain P, Rabkin SW. Report of the Canadian Hypertension Society Consensus Conference: 1. Definitions, evaluation and classification of hypertensive disorders in pregnancy. *Can Med Assoc J*. 1997;157(6):715-25.
242. Redman CW, Jefferies M. Revised definition of pre-eclampsia. *Lancet*. 1988;1(8589):809-12.
243. Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders of pregnancy. *Am J Obstet Gynecol*. 1988;158(4):892-8.
244. Levine RJ, Ewell MG, Hauth JC, Curet LB, Catalano PM, Morris CD, et al. Should the definition of preeclampsia include a rise in diastolic blood pressure of ≥ 15 mm Hg to a level < 90 mm Hg in association with proteinuria? *Am J Obstet Gynecol*. 2000;183(4):787-92.

245. North RA, Taylor RS, Schellenberg JC. Evaluation of a definition of pre-eclampsia. *Br J Obstet Gynaecol.* 1999;106(8):767-73.
246. Harlow FH, Brown MA. The diversity of diagnoses of preeclampsia. *Hypertens Pregnancy.* 2001;20(1):57-67.
247. Chappell L, Poulton L, Halligan A, Shennan AH. Lack of consistency in research papers over the definition of pre-eclampsia. *Br J Obstet Gynaecol.* 1999;106(9):983-5.
248. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP) *Hypertens Pregnancy.* 2001;20(1):IX-XIV.
249. Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J, et al. The detection, investigation and management of hypertension in pregnancy: full consensus statement. *Aust N Z J Obstet Gynaecol.* 2000;40(2):139-55.
250. Lowe SA, Brown MA, Dekker G, Gatt S, McLintock C, McMahon L, et al. SOMANZ Guidelines for the Management of Hypertensive Disorders of Pregnancy 2008. 2008; Available from: www.somanz.org/pdfs/somanz_guidelines_2008.pdf.
251. Lowe SA, Brown MA, Dekker GA, Gatt S, McLintock CK, McMahon LP, et al. Guidelines for the management of hypertensive disorders of pregnancy 2008. *Aust N Z J Obstet Gynaecol.* 2009;49(3):242-6.
252. Policy Directive Maternity- Management of Hypertensive Disorders of Pregnancy. North Sydney 2011; Available from: <http://www.health.nsw.gov.au/policies/>.
253. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol.* 2000;183(1):S1-S22.

254. Roberts JM, Pearson GD, Cutler JA, Lindheimer MD. Summary of the NHLBI Working Group on Research on Hypertension During Pregnancy. *Hypertens Pregnancy*. 2003;22(2):109-27.
255. Magee LA, Helewa ME, Moutquin JM, Von Dadelszen P. Diagnosis, Evaluation and Management of the Hypertensive Disorders of Pregnancy. *JOGC*. 2008;30(3 Supplement 1):S1-S38.
256. Lindheimer MD, Taler SJ, Cunningham FG. ASH Position Article Hypertension in Pregnancy. *J Am Soc Hypertens*. 2008;2(6):484-94.
257. Thornton CE, Makris A, Ogle RF, Tooher JM, Hennessy A. Role of proteinuria in defining pre-eclampsia: clinical outcomes for women and babies. *Clin Exp Pharmacol Physiol*. 2010;37(4):466-70.
258. Villar J, Carroli G, Wojdyla D, Abalos E, Giordano D, Ba'aqueel H, et al. Preeclampsia, gestational hypertension and intrauterine growth restriction, related or independent conditions? *Am J Obstet Gynecol*. 2006;194(4):921-31.
259. Hauth JC, Ewell MG, Levine RJ, Esterlitz JR, Sibai B, Curet LB, et al. Pregnancy outcomes in healthy nulliparas who developed hypertension. Calcium for Preeclampsia Prevention Study Group. *Obstet Gynecol*. 2000;95(1):24-8.
260. Buchbinder A, Sibai BM, Caritis S, Macpherson C, Hauth J, Lindheimer MD, et al. Adverse perinatal outcomes are significantly higher in severe gestational hypertension than in mild preeclampsia. *Am J Obstet Gynecol*. 2002;186(1):66-71.
261. Barton JR, O'Brien JM, Bergauer NK, Jacques DL, Sibai BM. Mild gestational hypertension remote from term: progression and outcome. *Am J Obstet Gynecol*. 2001;184(5):979-83.

262. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ*. 2007;335(7627):974-85.
263. Cockell AP, Poston L. Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. *Hypertension*. 1997;30(2 Pt 1):247-51.
264. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. *Lancet*. 2005;365(9461):785-99.
265. von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. *Hypertens Pregnancy*. 2003;22(2):143-8.
266. Kenneth L, Hall DR, Gebhardt S, Grove D. Late onset preeclampsia is not an innocuous condition. *Hypertens Pregnancy*. 2010;29(3):262-70.
267. Huppertz B. Placental origins of preeclampsia: challenging the current hypothesis. *Hypertension*. 2008;51(4):970-5.
268. Tuffnell DJ, Shennan AH, Waugh JJS, Walker JJ. The Management of Severe Pre-eclampsia/Eclampsia (Green-top 10A). London: Royal College of Obstetrics and Gynaecology; 2006 [cited 2009 15 June]; Available from: www.rcog.org.uk/womens-health/clinical-guidance/management-severe-pre-eclampsiaeclampsia-green-top-10a.
269. Phillips JK, Janowiak M, Badger GJ, Bernstein IM. Evidence for distinct preterm and term phenotypes of preeclampsia. *J Matern Fetal Neonatal Med*. 2010;23(7):622-6.
270. von Dadelszen P, Menzies JM, Payne B, Magee LA. Predicting adverse outcomes in women with severe pre-eclampsia. *Semin Perinatol*. 2009;33(3):152-7.
271. Laws P, Sullivan E. Australia's mothers and babies 2007 (Perinatal Statistics Series No. 23; Cat. No. PER 48). Sydney: Australian Institute of Health and Welfare, National Perinatal Statistics Unit. 2009.

272. World Health Organization Collaboration. World Health Report: Make Every Mother and Child Count. Geneva: Department of Reproductive Health and Research, WHO. 2005.
273. Sullivan E, Hall B, King J. Maternal deaths in Australia 2003–2005. (Maternal deaths series no. 3. Cat. no. PER 42). Sydney, Australia: AHW National Perinatal Statistics Unit. 2008.
274. Douglas KA, Redman CW. Eclampsia in the United Kingdom. *BMJ*. 1994;309(6966):1395-400.
275. MacKay AP, Berg CJ, Atrash HK. Pregnancy-related mortality from preeclampsia and eclampsia. *Obstet Gynecol*. 2001;97(4):533-8.
276. Kuklina EV, Ayala C, Callaghan WM. Hypertensive disorders and severe obstetric morbidity in the United States. *Obstet Gynecol*. 2009;113(6):1299-306.
277. Heard AR, Dekker GA, Chan A, Jacobs DJ, Vreeburg SA, Priest KR. Hypertension during pregnancy in South Australia, part 1: pregnancy outcomes. *Aust N Z J Obstet Gynaecol*. 2004;44(5):404-9.
278. Sibai BM. Preeclampsia as a cause of preterm and late preterm (near-term) births. *Semin Perinatol*. 2006;30(1):16-9.
279. Lain KY, Krohn MA, Roberts JM. Second pregnancy outcomes following preeclampsia in a first pregnancy. *Hypertens Pregnancy*. 2005;24(2):159-69.
280. Gaugler-Senden IPM, Berends AL, de Groot CJM, Steegers EAP. Severe, very early onset preeclampsia: subsequent pregnancies and future parental cardiovascular health. *Eur J Obstet Gynecol Reprod Biol*. 2008;140(2):171-7.
281. Nisell H, Lintu H, Lunell NO, Mollerstrom G, Pettersson E. Blood pressure and renal function seven years after pregnancy complicated by hypertension. *Br J Obstet Gynaecol*. 1995;102(11):876-81.

282. Haukkamaa L, Salminen M, Laivuori H, Leinonen H, Hiilesmaa V, Kaaja R. Risk for subsequent coronary artery disease after preeclampsia. *Am J Cardiol.* 2004;93(6):805-8.
283. Wilson BJ, Watson MS, Prescott GJ, Sunderland S, Campbell DM, Hannaford P, et al. Hypertensive diseases of pregnancy and risk of hypertension and stroke in later life: results from cohort study. *BMJ.* 2003;326(7394):845.
284. Ray JG, Vermeulen MJ, Schull MJ, Redelmeier DA. Cardiovascular health after maternal placental syndromes (CHAMPS): population-based retrospective cohort study. *Lancet.* 2005;366(9499):1797-803.
285. Berends AL, de Groot CJ, Sijbrands EJ, Sie MP, Benneheij SH, Pal R, et al. Shared constitutional risks for maternal vascular-related pregnancy complications and future cardiovascular disease. *Hypertension.* 2008;51(4):1034-41.
286. Redman CW, Sargent IL. Preeclampsia and the systemic inflammatory response. *Semin Nephrol.* 2004;24(6):565-70.
287. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol.* 1999;180(2 Pt 1):499-506.
288. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med.* 1999;340(6):448-54.
289. Redman CWG, Sargent IL. Placental stress and pre-eclampsia: a revised view. *Placenta.* 2009;30 Suppl A:S38-42.
290. Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol.* 1998;179(1):80-6.
291. Perry KG, Jr., Martin JN, Jr. Abnormal hemostasis and coagulopathy in preeclampsia and eclampsia. *Clin Obstet Gynecol.* 1992;35(2):338-50.

292. Haeger M, Unander M, Norder-Hansson B, Tylman M, Bengtsson A. Complement, neutrophil, and macrophage activation in women with severe preeclampsia and the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Obstet Gynecol.* 1992;79(1):19-26.
293. Lehmann K. *Eklampsien i Danmark i Aarene, 1918-1927*. Copenhagen, Denmark: Busck; 1933.
294. MacGillivray I. Some Observations on the Incidence of Pre-eclampsia. *J Obstet Gynaecol Br Emp.* 1958;65:536-9.
295. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ.* 2005;330(7491):565.
296. Wolf M, Shah A, Lam C, Martinez A, Smirnakis KV, Epstein FH, et al. Circulating levels of the antiangiogenic marker sFLT-1 are increased in first versus second pregnancies. *Am J Obstet Gynecol.* 2005;193(1):16-22.
297. Campbell DM, MacGillivray I, Carr-Hill R. Pre-eclampsia in second pregnancy. *Br J Obstet Gynaecol.* 1985;92(2):131-40.
298. Hnat MD, Sibai BM, Caritis S, Hauth J, Lindheimer MD, MacPherson C, et al. Perinatal outcome in women with recurrent preeclampsia compared with women who develop preeclampsia as nulliparas. *Am J Obstet Gynecol.* 2002;186(3):422-6.
299. Xiong X, Fraser WD, Demianczuk NN. History of abortion, preterm, term birth, and risk of preeclampsia: a population-based study. *Am J Obstet Gynecol.* 2002;187(4):1013-8.
300. Saftlas AF, Levine RJ, Klebanoff MA, Martz KL, Ewell MG, Morris CD, et al. Abortion, changed paternity, and risk of preeclampsia in nulliparous women. *Am J Epidemiol.* 2003;157(12):1108-14.

301. Trogstad L, Magnus P, Skjaerven R, Stoltenberg C. Previous abortions and risk of pre-eclampsia. *Int J Epidemiol.* 2008;37(6):1333-40.
302. Marti JJ, Herrmann U. Immunogestosis: a new etiologic concept of "essential" EPH gestosis, with special consideration of the primigravid patient; preliminary report of a clinical study. *Am J Obstet Gynecol.* 1977;128(5):489-93.
303. Tubbergen P, Lachmeijer AM, Althuisius SM, Vlak ME, van Geijn HP, Dekker GA. Change in paternity: a risk factor for preeclampsia in multiparous women? *J Reprod Immunol.* 1999;45(1):81-8.
304. Trupin LS, Simon LP, Eskenazi B. Change in paternity: a risk factor for preeclampsia in multiparas. *Epidemiology.* 1996;7(3):240-4.
305. Deen ME, Ruurda LG, Wang J, Dekker GA. Risk factors for preeclampsia in multiparous women: primipaternity versus the birth interval hypothesis. *J Matern Fetal Neonatal Med.* 2006;19(2):79-84.
306. Robillard PY, Hulsey TC, Perianin J, Janky E, Miri EH, Papiernik E. Association of pregnancy-induced hypertension with duration of sexual cohabitation before conception. *Lancet.* 1994;344(8928):973-5.
307. Robillard PY, Hulsey TC. Association of pregnancy-induced-hypertension, pre-eclampsia, and eclampsia with duration of sexual cohabitation before conception. *Lancet.* 1996;347(9001):619.
308. Peters B, Whittall T, Babaahmady K, Gray K, Vaughan R, Lehner T. Effect of heterosexual intercourse on mucosal alloimmunisation and resistance to HIV-1 infection. *Lancet.* 2004;363(9408):518-24.
309. Kho EM, McCowan LM, North RA, Roberts CT, Chan E, Black MA, et al. Duration of sexual relationship and its effect on preeclampsia and small for gestational age perinatal outcome. *J Reprod Immunol.* 2009;82(1):66-73.

310. Sibai BM. Diagnosis, prevention, and management of eclampsia. *Obstet Gynecol.* 2005;105(2):402-10.
311. Trogstad LI, Eskild A, Magnus P, Samuelsen SO, Nesheim BI. Changing paternity and time since last pregnancy; the impact on pre-eclampsia risk. A study of 547 238 women with and without previous pre-eclampsia. *Int J Epidemiol.* 2001;30(6):1317-22.
312. Skjaerven R, Wilcox AJ, Lie RT. The interval between pregnancies and the risk of preeclampsia. *N Engl J Med.* 2002;346(1):33-8.
313. Dekker G, Robillard P-Y. The birth interval hypothesis-does it really indicate the end of the primipaternity hypothesis. *J Reprod Immunol.* 2003;59(2):245-51.
314. Dekker G, Robillard P-Y. Pre-eclampsia: Is the immune maladaptation hypothesis still standing? An epidemiological update. *J Reprod Immunol.* 2007;76(1-2):8-16.
315. Long PA, Oats JN. Preeclampsia in twin pregnancy-severity and pathogenesis. *Aust N Z J Obstet Gynaecol.* 1987;27(1):1-5.
316. Sibai BM, Hauth J, Caritis S, Lindheimer MD, MacPherson C, Klebanoff M, et al. Hypertensive disorders in twin versus singleton gestations. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol.* 2000;182(4):938-42.
317. Wen SW, Demissie K, Yang Q, Walker MC. Maternal morbidity and obstetric complications in triplet pregnancies and quadruplet and higher-order multiple pregnancies. *Am J Obstet Gynecol.* 2004;191(1):254-8.
318. Lynch A, McDuffie R, Jr., Murphy J, Faber K, Orleans M. Preeclampsia in multiple gestation: the role of assisted reproductive technologies. *Obstet Gynecol.* 2002;99(3):445-51.

319. Bdolah Y, Lam C, Rajakumar A, Shivalingappa V, Mutter W, Sachs BP, et al. Twin pregnancy and the risk of preeclampsia: bigger placenta or relative ischemia? *Am J Obstet Gynecol.* 2008;198(4):428.e1-6.
320. Maynard SE, Moore Simas TA, Solitro MJ, Rajan A, Crawford S, Soderland P, et al. Circulating angiogenic factors in singleton vs multiple-gestation pregnancies. *Am J Obstet Gynecol.* 2008;198(2):200.e1-7.
321. Kohorn EI. Molar pregnancy: presentation and diagnosis. *Clin Obstet Gynecol.* 1984;27(1):181-91.
322. Soto-Wright V, Bernstein M, Goldstein DP, Berkowitz RS. The changing clinical presentation of complete molar pregnancy. *Obstet Gynecol.* 1995;86(5):775-9.
323. Koga K, Osuga Y, Tajima T, Hirota Y, Igarashi T, Fujii T, et al. Elevated serum soluble fms-like tyrosine kinase 1 (sFlt1) level in women with hydatidiform mole. *Fertil Steril.* 2009;94(1):305-8.
324. Boyd PA, Lindenbaum RH, Redman C. Pre-eclampsia and trisomy 13: a possible association. *Lancet.* 1987;2(8556):425-7.
325. Tuohy JF, James DK. Pre-eclampsia and trisomy 13. *Br J Obstet Gynaecol.* 1992;99(11):891-4.
326. Bdolah Y, Palomaki GE, Yaron Y, Bdolah-Abram T, Goldman M, Levine RJ, et al. Circulating angiogenic proteins in trisomy 13. *Am J Obstet Gynecol.* 2006;194(1):239-45.
327. Jacobs DJ, Vreeburg SA, Dekker GA, Heard AR, Priest KR, Chan A. Risk factors for hypertension during pregnancy in South Australia. *Aust N Z J Obstet Gynaecol.* 2003;43(6):421-8.
328. Catov JM, Ness RB, Kip KE, Olsen J. Risk of early or severe pre-eclampsia related to pre-existing conditions. *Int J Epidemiol.* 2007;36(2):412-9.

329. Caritis S, Sibai B, Hauth J, Lindheimer M, VanDorsten P, Klebanoff M, et al. Predictors of pre-eclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol.* 1998;179(4):946-51.
330. Ramin SM, Vadaeff AC, Yeomans ER, Gilstrap LC, 3rd. Chronic renal disease in pregnancy. *Obstet Gynecol.* 2006;108(6):1531-9.
331. Maynard SE, Thadhani R. Pregnancy and the kidney. *J Am Soc Nephrol.* 2009;20(1):14-22.
332. Wolfberg AJ, Lee-Parritz A, Peller AJ, Lieberman ES. Association of rheumatologic disease with preeclampsia. *Obstet Gynecol.* 2004;103(6):1190-3.
333. Morrison ER, Miedzybrodzka ZH, Campbell DM, Haites NE, Wilson BJ, Watson MS, et al. Prothrombotic genotypes are not associated with pre-eclampsia and gestational hypertension: results from a large population-based study and systematic review. *Thromb Haemost.* 2002;87(5):779-85.
334. Kupferminc MJ. Thrombophilia and preeclampsia: the evidence so far. *Clin Obstet Gynecol.* 2005;48(2):406-15.
335. Said JM, Higgins JR, Moses EK, Walker SP, Borg AJ, Monagle PT, et al. Inherited thrombophilia polymorphisms and pregnancy outcomes in nulliparous women. *Obstet Gynecol.* 2010;115(1):5-13.
336. Persson M, Norman M, Hanson U. Obstetric and perinatal outcomes in type 1 diabetic pregnancies: A large, population-based study. *Diabetes Care.* 2009;32(11):2005-9.
337. Scioscia M, Gumaa K, Rademacher TW. The link between insulin resistance and preeclampsia: new perspectives. *J Reprod Immunol.* 2009;82(2):100-5.

338. Mastrogiannis DS, Spiliopoulos M, Mulla W, Homko CJ. Insulin resistance: the possible link between gestational diabetes mellitus and hypertensive disorders of pregnancy. *Curr Diab Rep.* 2009;9(4):296-302.
339. Hopkinson ZE, Sattar N, Fleming R, Greer IA. Polycystic ovarian syndrome: the metabolic syndrome comes to gynaecology. *BMJ.* 1998;317(7154):329-32.
340. Ramsay JE, Greer I, Sattar N. ABC of obesity. *Obesity and reproduction. BMJ.* 2006;333(7579):1159-62.
341. Global database on body mass index. WHO BMI classification World Health Organization; 2006 [updated 21 January, 2011].
342. Gustat J, Elkasabany A, Srinivasan S, Berenson GS. Relation of Abdominal Height to Cardiovascular Risk Factors in Young Adults. *Am J Epidemiol.* 2000;151(9):885-91.
343. Callaway LK, O'Callaghan M, McIntyre HD. Obesity and the hypertensive disorders of pregnancy. *Hypertens Pregnancy.* 2009;28(4):473-93.
344. Roberts JM, Bodnar LM, Patrick TE, Powers RW. The role of obesity in preeclampsia. *Pregnancy Hypertens.* 2011;1(1):6-16.
345. Nutrition during pregnancy, part 1: Weight gain. Washington (DC): Institute of Medicine. National Academy Press; 1990. Available from: <http://www.nap.edu/catalog/1451.html>.
346. Cnossen JS, Leeflang MMG, De Haan EEM, Mol BWJ, Van Der Post JAM, Khan KS, et al. Systematic review: Accuracy of body mass index in predicting pre-eclampsia: bivariate meta-analysis. *BJOG.* 2007;114(12):1477-85.
347. Denison FC, Roberts KA, Barr SM, Norman JE. Obesity, pregnancy, inflammation, and vascular function. *Reproduction.* 2010;140(3):373-85.

348. Zebekakis PE, Nawrot T, Thijs L, Balkestein EJ, van der Heijden-Spek J, Van Bortel LM, et al. Obesity is associated with increased arterial stiffness from adolescence until old age. *J Hypertens*. 2005;23(10):1839-46.
349. Sturm W, Sandhofer A, Engl J, Laimer M, Molnar C, Kaser S, et al. Influence of visceral obesity and liver fat on vascular structure and function in obese subjects. *Obesity*. 2009;17(9):1783-8.
350. Ramsay JE, Ferrell WR, Crawford L, Wallace AM, Greer IA, Sattar N. Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. *J Clin Endocrinol Metab*. 2002;87(9):4231-7.
351. Myers J, Hall C, Wareing M, Gillham J, Baker P. The effect of maternal characteristics on endothelial-dependent relaxation of myometrial arteries. *Eur J Obstet Gynecol Reprod Biol*. 2006;124(2):158-63.
352. Stewart FM, Freeman DJ, Ramsay JE, Greer IA, Caslake M, Ferrell WR. Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers. *J Clin Endocrinol Metab*. 2007;92(3):969-75.
353. Savvidou MD, Hingorani AD, Tsikas D, Frolich JC, Vallance P, Nicolaides KH. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. *Lancet*. 2003;361(9368):1511-7.
354. Herrmann W, Isber S, Obeid R, Herrmann M, Jouma M. Concentrations of homocysteine, related metabolites and asymmetric dimethylarginine in preeclamptic women with poor nutritional status. *Clin Chem Lab Med*. 2005;43(10):1139-46.
355. Ness RB, Sibai BM. Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. *Am J Obstet Gynecol*. 2006;195(1):40-9.

356. Stone JL, Lockwood CJ, Berkowitz GS, Alvarez M, Lapinski R, Berkowitz RL. Risk factors for severe preeclampsia. *Obstet Gynecol.* 1994;83(3):357-61.
357. Cedergren MI. Maternal morbid obesity and the risk of adverse pregnancy outcome. *Obstet Gynecol.* 2004;103(2):219-24.
358. Castro LC, Avina RL. Maternal obesity and pregnancy outcomes. *Curr Opin Obstet Gynecol.* 2002;14(6):601-6.
359. Rosenberg TJ, Garbers S, Chavkin W, Chiasson MA. Prepregnancy weight and adverse perinatal outcomes in an ethnically diverse population. *Obstet Gynecol.* 2003;102(5 Pt 1):1022-7.
360. O'Brien TE, Ray JG, Chan WS. Maternal body mass index and the risk of preeclampsia: a systematic overview. *Epidemiology.* 2003;14(3):368-74.
361. Alanis MC, Goodnight WH, Hill EG, Robinson CJ, Villers MS, Johnson DD. Maternal super-obesity (body mass index \geq 50) and adverse pregnancy outcomes. *Acta Obstet Gynecol Scand.* 2010;89(7):924-30.
362. Australian Bureau of Statistics: Australian Social Trends [database on the Internet]. Australian Government. 2009 [cited 16 February 2011].
363. Hossain P, Kavar B, El Nahas M. Obesity and Diabetes in the Developing World - A Growing Challenge. *N Engl J Med.* 2007;356(3):213-5.
364. Doherty DA, Magann EF, Francis J, Morrison JC, Newnham JP. Pre-pregnancy body mass index and pregnancy outcomes. *Int J Gynaecol Obstet.* 2006;95(3):242-7.
365. Athukorala C, Rumbold A, Willson K, Crowther C. The risk of adverse pregnancy outcomes in women who are overweight or obese. *BMC Pregnancy and Childbirth.* 2010;10(1):56.
366. Hauger MS, Gibbons L, Vik T, Belizán JM. Prepregnancy weight status and the risk of adverse pregnancy outcome. *Acta Obstet Gynecol Scand.* 2008;87(9):953-9.

367. Mahomed K, Williams MA, Woelk GB, Jenkins-Woelk L, Mudzamiri S, Longstaff L, et al. Risk factors for pre-eclampsia among Zimbabwean women: maternal arm circumference and other anthropometric measures of obesity. *Paediatr Perinat Epidemiol.* 1998;12(3):253-62.
368. Fortner RT, Pekow P, Solomon CG, Markenson G, Chasan-Taber L. Prepregnancy body mass index, gestational weight gain, and risk of hypertensive pregnancy among Latina women. *Am J Obstet Gynecol.* 2009;200(2):167.e1-e7.
369. Chen Z, Du J, Shao L, Zheng L, Wu M, Ai M, et al. Prepregnancy body mass index, gestational weight gain, and pregnancy outcomes in China. *Int J Gynaecol Obstet.* 2010;109(1):41-4.
370. Bodnar LM, Catov JM, Klebanoff MA, Ness RB, Roberts JM. Prepregnancy body mass index and the occurrence of severe hypertensive disorders of pregnancy. *Epidemiology.* 2007;18(2):234-9.
371. Sattar N, Greer IA. Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? *BMJ.* 2002;325(7356):157-60.
372. Mazar RM, Srinivas SK, Sammel MD, Andrela CM, Elovitz MA. Metabolic score as a novel approach to assessing preeclampsia risk. *Am J Obstet Gynecol.* 2007;197(4):411.e1-5.
373. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA.* 2001;285(19):2486-97.
374. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation.* 2002;106(25):3143-421.

375. Dane B, Dane C, Kiray M, Koldas M, Cetin A. A new metabolic scoring system for analyzing the risk of hypertensive disorders of pregnancy. *Arch Gynecol Obstet*. 2009;280(6):921-4.
376. Bartha JL, Gonzalez-Bugatto F, Fernandez-Macias R, Gonzalez-Gonzalez NL, Comino-Delgado R, Hervias-Vivancos B. Metabolic syndrome in normal and complicated pregnancies. *Eur J Obstet Gynecol Reprod Biol*. 2008;137(2):178-84.
377. Ray JG, Diamond P, Singh G, Bell CM. Brief overview of maternal triglycerides as a risk factor for pre-eclampsia. *BJOG*. 2006;113(4):379-86.
378. Wiznitzer A, Mayer A, Novack V, Sheiner E, Gilutz H, Malhotra A, et al. Association of lipid levels during gestation with preeclampsia and gestational diabetes mellitus: a population-based study. *Am J Obstet Gynecol*. 2009;201(5):482.e1-8.
379. Baker AM, Klein RL, Moss KL, Haeri S, Boggess K. Maternal serum dyslipidemia occurs early in pregnancy in women with mild but not severe preeclampsia. *Am J Obstet Gynecol*. 2009;201(3):293.e1-4.
380. Lampinen KH, Ronnback M, Groop P-H, Kaaja RJ. A relationship between insulin sensitivity and vasodilation in women with a history of preeclamptic pregnancy. *Hypertension*. 2008;52(2):394-401.
381. Stekkinger E, Zandstra M, Peeters LLH, Spaanderman MEA. Early-onset preeclampsia and the prevalence of postpartum metabolic syndrome. *Obstet Gynecol*. 2009;114(5):1076-84.
382. von Dadelszen P, Magee LA. Could an infectious trigger explain the differential maternal response to the shared placental pathology of preeclampsia and normotensive intrauterine growth restriction? *Acta Obstet Gynecol Scand*. 2002;81(7):642-8.
383. Mittendorf R, Lain KY, Williams MA, Walker CK. Preeclampsia. A nested, case-control study of risk factors and their interactions. *J Reprod Med*. 1996;41(7):491-6.

384. Boggess KA, Lief S, Murtha AP, Moss K, Beck J, Offenbacher S. Maternal periodontal disease is associated with an increased risk for preeclampsia. *Obstet Gynecol.* 2003;101(2):227-31.
385. Chesley LC, Annitto JE, Cosgrove RA. The familial factor in toxemia of pregnancy. *Obstet Gynecol.* 1968;32(3):303-11.
386. Nilsson E, Salonen Ros H, Cnattingius S, Lichtenstein P. The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG.* 2004;111(3):200-6.
387. Esplin MS, Fausett MB, Fraser A, Kerber R, Mineau G, Carrillo J, et al. Paternal and maternal components of the predisposition to preeclampsia. *N Engl J Med.* 2001;344(12):867-72.
388. Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E, Irgens LM. Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ.* 1998;316(7141):1343-7.
389. Skjaerven R, Vatten LJ, Wilcox AJ, Ronning T, Irgens LM, Lie RT. Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort. *BMJ.* 2005;331(7521):877.
390. Carr DB, Epplen M, Johnson CO, Easterling TR, Critchlow CW. A sister's risk: family history as a predictor of preeclampsia. *Am J Obstet Gynecol.* 2005;193(3 Pt 2):965-72.
391. Genetics of Pre-eclampsia Consortium. Babies, pre-eclamptic mothers and grandparents: a three-generation phenotyping study. *J Hypertens.* 2007;25(4):849-54.
392. Berends AL, Steegers EA, Isaacs A, Aulchenko YS, Liu F, de Groot CJ, et al. Familial aggregation of preeclampsia and intrauterine growth restriction in a genetically isolated population in The Netherlands. *Eur J Hum Genet.* 2008;16(12):1437-42.

393. Sezik M, Ozkaya O, Sezik HT, Yapar EG, Kaya H. Does marriage between first cousins have any predictive value for maternal and perinatal outcomes in pre-eclampsia? *J Obstet Gynaecol Res.* 2006;32(5):475-81.
394. Badria LF, Amarin ZO, Badria LF. Does consanguinity affect the severity of pre-eclampsia? *Arch Gynecol Obstet.* 2003;268(2):117-20.
395. Laivuori H, Lahermo P, Ollikainen V, Widen E, Haiva-Mallinen L, Sundstrom H, et al. Susceptibility loci for preeclampsia on chromosomes 2p25 and 9p13 in Finnish families. *Am J Hum Genet.* 2003;72(1):168-77.
396. Oudejans CB, Mulders J, Lachmeijer AM, van Dijk M, Konst AA, Westerman BA, et al. The parent-of-origin effect of 10q22 in pre-eclamptic females coincides with two regions clustered for genes with down-regulated expression in androgenetic placentas. *Mol Hum Reprod.* 2004;10(8):589-98.
397. Mutze S, Rudnik-Schoneborn S, Zerres K, Rath W. Genes and the preeclampsia syndrome. *J Perinat Med.* 2008;36(1):38-58.
398. Williams TN, Mwangi TW, Wambua S, Alexander ND, Kortok M, Snow RW, et al. Sickle Cell Trait and the Risk of Plasmodium falciparum Malaria and Other Childhood Diseases. *J Infect Dis.* 2005;192(1):178-86.
399. Pijnenborg R, Vercruyse L, Hanssens M. Fetal-maternal conflict, trophoblast invasion, preeclampsia, and the red queen. *Hypertens Pregnancy.* 2008;27(2):183-96.
400. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science.* 2005;308(5728):1592-4.
401. Shembrey MA, Noble AD. An instructive case of abdominal pregnancy. *Aust N Z J Obstet Gynaecol.* 1995;35(2):220-1.

402. Matsuo K, Kooshesh S, Dinc M, Sun C-CJ, Kimura T, Baschat AA. Late postpartum eclampsia: report of two cases managed by uterine curettage and review of the literature. *Am J Perinatol.* 2007;24(4):257-66.
403. Pijnenborg R, Vercruyse L. Shifting concepts of the fetal-maternal interface: a historical perspective. *Placenta.* 2008;29 Suppl A:S20-5.
404. Pijnenborg R, Vercruyse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta.* 2006;27(9-10):939-58.
405. Brosens I, Robertson WB, Dixon HG. The physiological response of the vessels of the placental bed to normal pregnancy. *J Pathol Bacteriol.* 1967;93(2):569-79.
406. Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu.* 1972;1:177-91.
407. Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response-a review. *Placenta.* 2003;24(Suppl A):S21-7.
408. Brosens IA. Morphological changes in the utero-placental bed in pregnancy hypertension. *Clin Obstet Gynaecol.* 1977;4(3):573-93.
409. Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol.* 1994;101(8):669-74.
410. De Wolf F, Robertson WB, Brosens I. The ultrastructure of acute atherosclerosis in hypertensive pregnancy. *Am J Obstet Gynecol.* 1975;123(2):164-74.
411. Salafia CM, Pezzullo JC, Ghidini A, Lopez-Zeno JA, Whittington SS. Clinical correlations of patterns of placental pathology in preterm pre-eclampsia. *Placenta.* 1998;19(1):67-72.

412. Egbor M, Ansari T, Morris N, Green CJ, Sibbons PD. Morphometric placental villous and vascular abnormalities in early- and late-onset pre-eclampsia with and without fetal growth restriction. *BJOG*. 2006;113(5):580-9.
413. Moldenhauer JS, Stanek J, Warshak C, Khoury J, Sibai B. The frequency and severity of placental findings in women with preeclampsia are gestational age dependent. *Am J Obstet Gynecol*. 2003;189(4):1173-7.
414. van der Merwe JL, Hall DR, Wright C, Schubert P, Grove D. Are Early and Late Preeclampsia Distinct Subclasses of the Disease-What Does the Placenta Reveal? *Hypertens Pregnancy*. 2010;29(4):457-67.
415. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. *Am J Obstet Gynecol*. 1996;175(5):1365-70.
416. Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Preeclampsia and fetal growth. *Obstet Gynecol*. 2000;96(6):950-5.
417. Rasmussen S, Irgens LM. Fetal growth and body proportion in preeclampsia. *Obstet Gynecol*. 2003;101(3):575-83.
418. Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *Br J Obstet Gynaecol*. 1986;93(10):1049-59.
419. Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC. Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. *J Pathol*. 2006;208(4):535-42.
420. Arias F, Rodriguez L, Rayne SC, Kraus FT. Maternal placental vasculopathy and infection: two distinct subgroups among patients with preterm labor and preterm ruptured membranes. *Am J Obstet Gynecol*. 1993;168(2):585-91.

421. Hutchinson ES, Brownbill P, Jones NW, Abrahams VM, Baker PN, Sibley CP, et al. Utero-placental haemodynamics in the pathogenesis of pre-eclampsia. *Placenta*. 2009;30(7):634-41.
422. Brosens JJ, Pijnenborg R, Brosens IA. The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature. *Am J Obstet Gynecol*. 2002;187(5):1416-23.
423. Lyall F. Mechanisms regulating cytotrophoblast invasion in normal pregnancy and pre-eclampsia. *Aust N Z J Obstet Gynaecol*. 2006;46(4):266-73.
424. Hung T-H, Burton GJ. Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia. *Taiwanese J Obstet Gynecol*. 2006;45(3):189-200.
425. Borzychowski AM, Sargent IL, Redman CWG. Inflammation and pre-eclampsia. *Semin Fetal Neonatal Med*. 2006;11(5):309-16.
426. Myatt L, Webster RP. Vascular biology of preeclampsia. *J Thromb Haemost*. 2009;7(3):375-84.
427. Hung T-H, Skepper JN, Charnock-Jones DS, Burton GJ. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. *Circ Res*. 2002;90(12):1274-81.
428. Makris A, Thornton C, Thompson J, Thomson S, Martin R, Ogle R, et al. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. *Kidney Int*. 2007;71(10):977-84.
429. Ayuk PTY, Matijevic R. Placental ischaemia is a consequence rather than a cause of pre-eclampsia. *Med Hypotheses*. 2006;67(4):792-5.
430. Assali NS, Prystowsky H. Studies on autonomic blockade. I. Comparison between the effects of tetraethylammonium chloride (TEAC) and high selective spinal anesthesia on

blood pressure of normal and toxemic pregnancy. *Clinical Investigation*.

1950;29(10):1354-66.

431. Greer IA, Lyall F, Perera T, Boswell F, Macara LM. Increased concentrations of cytokines interleukin-6 and interleukin-1 receptor antagonist in plasma of women with preeclampsia: a mechanism for endothelial dysfunction? *Obstet Gynecol*. 1994;84(6):937-40.

432. Rinehart BK, Terrone DA, Lagoo-Deenadayalan S, Barber WH, Hale EA, Martin JN, Jr., et al. Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. *Am J Obstet Gynecol*. 1999;181(4):915-20.

433. Davidge ST, Signorella AP, Hubel CA, Lykins DL, Roberts JM. Distinct factors in plasma of preeclamptic women increase endothelial nitric oxide or prostacyclin. *Hypertension*. 1996;28(5):758-64.

434. Smarason AK, Sargent IL, Redman CW. Endothelial cell proliferation is suppressed by plasma but not serum from women with preeclampsia. *Am J Obstet Gynecol*. 1996;174(2):787-93.

435. Knight M, Redman CW, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *Br J Obstet Gynaecol*. 1998;105(6):632-40.

436. Karumanchi SA, Bdolah Y. Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. *Endocrinology*. 2004;145(11):4835-7.

437. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature*. 2000;407(6801):242-8.

438. Dunk C, Shams M, Nijjar S, Rhaman M, Qiu Y, Bussolati B, et al. Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development. *Am J Pathol.* 2000;156(6):2185-99.
439. Kayisli UA, Cayli S, Seval Y, Tertemiz F, Huppertz B, Demir R. Spatial and temporal distribution of Tie-1 and Tie-2 during very early development of the human placenta. *Placenta.* 2006;27(6-7):648-59.
440. Seval Y, Sati L, Celik-Ozenci C, Taskin O, Demir R. The distribution of angiopoietin-1, angiopoietin-2 and their receptors tie-1 and tie-2 in the very early human placenta. *Placenta.* 2008;29(9):809-15.
441. Goldman-Wohl D, Greenfield C, Haimov-Kochman R, Ariel I, Anteby EY, Hochner-Celnikier D, et al. Eph and ephrin expression in normal placental development and preeclampsia. *Placenta.* 2004;25(7):623-30.
442. Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol.* 2006;7(5):359-71.
443. Zachary I, Glikli G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res.* 2001;49(3):568-81.
444. Wang A, Rana S, Karumanchi SA. Preeclampsia: the role of angiogenic factors in its pathogenesis. *Physiology.* 2009;24:147-58.
445. Papapetropoulos A, García-Cardeña G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest.* 1997;100(12):3131-9.
446. Grummer MA, Sullivan JA, Magness RR, Bird IM. Vascular endothelial growth factor acts through novel, pregnancy-enhanced receptor signalling pathways to stimulate

- endothelial nitric oxide synthase activity in uterine artery endothelial cells. *Biochem J*. 2009;417(2):501-11.
447. Brockelsby JC, Anthony FW, Johnson IR, Baker PN. The effects of vascular endothelial growth factor on endothelial cells: a potential role in preeclampsia. *Am J Obstet Gynecol*. 2000;182(1 Pt 1):176-83.
448. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol*. 1998;274(3 Pt 2):H1054-8.
449. Sandrim VC, Palei ACT, Metzger IF, Gomes VA, Cavalli RC, Tanus-Santos JE. Nitric oxide formation is inversely related to serum levels of antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endogline in preeclampsia. *Hypertension*. 2008;52(2):402-7.
450. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. *Hum Reprod*. 1996;11(5):1090-8.
451. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, et al. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod*. 1998;59(6):1540-8.
452. Zhou Y, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T, et al. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol*. 2002;160(4):1405-23.
453. Maynard SE, Min J-Y, Merchan J, Lim K-H, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest*. 2003;111(5):649-58.

454. Venkatesha S, Toporsian M, Lam C, Hanai J-i, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med*. 2006;12(6):642-9.
455. Koga K, Osuga Y, Yoshino O, Hirota Y, Ruimeng X, Hirata T, et al. Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. *J Clin Endocrinol Metab*. 2003;88(5):2348-51.
456. Levine RJ, Maynard SE, Qian C, Lim K-H, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*. 2004;350(7):672-83.
457. Lyall F, Greer IA, Boswell F, Fleming R. Suppression of serum vascular endothelial growth factor immunoreactivity in normal pregnancy and in pre-eclampsia. *Br J Obstet Gynaecol*. 1997;104(2):223-8.
458. Livingston JC, Chin R, Haddad B, McKinney ET, Ahokas R, Sibai BM. Reductions of vascular endothelial growth factor and placental growth factor concentrations in severe preeclampsia. *Am J Obstet Gynecol*. 2000;183(6):1554-7.
459. Ogge G, Romero R, Kusanovic JP, Chaiworapongsa T, Dong Z, Mittal P, et al. Serum and plasma determination of angiogenic and anti-angiogenic factors yield different results: the need for standardization in clinical practice. *J Matern Fetal Neonatal Med*. 2010;23(8):820-7.
460. Taylor RN, Grimwood J, Taylor RS, McMaster MT, Fisher SJ, North RA. Longitudinal serum concentrations of placental growth factor: evidence for abnormal placental angiogenesis in pathologic pregnancies. *Am J Obstet Gynecol*. 2003;188(1):177-82.
461. Tidwell SC, Ho HN, Chiu WH, Torry RJ, Torry DS. Low maternal serum levels of placenta growth factor as an antecedent of clinical preeclampsia. *Am J Obstet Gynecol*. 2001;184(6):1267-72.

462. Levine RJ, Thadhani R, Qian C, Lam C, Lim K-H, Yu KF, et al. Urinary placental growth factor and risk of preeclampsia. *JAMA*. 2005;293(1):77-85.
463. ten Dijke P, Goumans M-J, Pardali E. Endoglin in angiogenesis and vascular diseases. *Angiogenesis*. 2008;11(1):79-89.
464. Karumanchi SA, Epstein FH. Placental ischemia and soluble fms-like tyrosine kinase 1: cause or consequence of preeclampsia? *Kidney Int*. 2007;71(10):959-61.
465. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med*. 2006;355(10):992-1005.
466. Romero R, Nien JK, Espinoza J, Todem D, Fu W, Chung H, et al. A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. *J Matern Fetal Neonatal Med*. 2008;21(1):9-23.
467. Espinoza J, Chaiworapongsa T, Romero R, Kim YM, Kim GJ, Nien JK, et al. Unexplained fetal death: another anti-angiogenic state. *J Matern Fetal Neonatal Med*. 2007;20(7):495-507.
468. Espinoza J, Romero R, Nien JK, Kusanovic JP, Richani K, Gomez R, et al. A role of the anti-angiogenic factor sVEGFR-1 in the 'mirror syndrome' (Ballantyne's syndrome). *J Matern Fetal Neonatal Med*. 2006;19(10):607-13.
469. Kusanovic JP, Romero R, Espinoza J, Nien JK, Kim CJ, Mittal P, et al. Twin-to-twin transfusion syndrome: an antiangiogenic state? *Am J Obstet Gynecol*. 2008;198(4):382.e1-8.
470. Roberts JM, Rajakumar A. Preeclampsia and soluble fms-like tyrosine kinase 1. *J Clin Endocrinol Metab*. 2009;94(7):2252-4.

471. Suwaki N, Masuyama H, Nakatsukasa H, Masumoto A, Sumida Y, Takamoto N, et al. Hypoadiponectinemia and circulating angiogenic factors in overweight patients complicated with pre-eclampsia. *Am J Obstet Gynecol.* 2006;195(6):1687-92.
472. Masuyama H, Segawa T, Sumida Y, Masumoto A, Inoue S, Akahori Y, et al. Different profiles of circulating angiogenic factors and adipocytokines between early- and late-onset pre-eclampsia. *BJOG.* 2010;117(3):314-20.
473. Iwashima Y, Katsuya T, Ishikawa K, Ouchi N, Ohishi M, Sugimoto K, et al. Hypoadiponectinemia is an independent risk factor for hypertension. *Hypertension.* 2004;43(6):1318-23.
474. Tan KCB, Xu A, Chow WS, Lam MCW, Ai VHG, Tam SCF, et al. Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. *J Clin Endocrinol Metab.* 2004;89(2):765-9.
475. Shimabukuro M, Higa N, Asahi T, Oshiro Y, Takasu N, Tagawa T, et al. Hypoadiponectinemia is closely linked to endothelial dysfunction in man. *J Clin Endocrinol Metab.* 2003;88(7):3236-40.
476. Hendler I, Blackwell SC, Mehta SH, Whitty JE, Russell E, Sorokin Y, et al. The levels of leptin, adiponectin, and resistin in normal weight, overweight, and obese pregnant women with and without preeclampsia. *Am J Obstet Gynecol.* 2005;193(3 Pt 2):979-83.
477. Clapp JF, Seaward BL, Sleamaker RH, Hiser J. Maternal physiologic adaptations to early human pregnancy. *Am J Obstet Gynecol.* 1988;159(6):1456-60.
478. Flanagan B, Muldowney F, Cannon P. The relationships of circulating red cell mass, basal oxygen consumption and lean body mass during normal human pregnancy. *Clin Sci.* 1966;30(3):439.
479. Lund CJ, Donovan JC. Blood volume during pregnancy. Significance of plasma and red cell volumes. *Am J Obstet Gynecol.* 1967;98(3):394-403.

480. Taylor D, Lind T. Red cell mass during and after normal pregnancy. *Br J Obstet Gynaecol.* 1979;86(5):364-70.
481. Whittaker PG, Macphail S, Lind T. Serial hematologic changes and pregnancy outcome. *Obstet Gynecol.* 1996;88(1):33-9.
482. O'Day MP. Cardio-respiratory physiological adaptation of pregnancy. *Semin Perinatol.* 1997;21(4):268-75.
483. Clapp JF. Maternal heart rate in pregnancy. *Am J Obstet Gynecol.* 1985 659-60;15(152):6 Pt 1.
484. Robson SC, Hunter S, Boys RJ, Dunlop W. Serial study of factors influencing changes in cardiac output during human pregnancy. *Am J Physiol.* 1989;256(4 Pt 2):H1060-5.
485. Walters WA, MacGregor WG, Hills M. Cardiac output at rest during pregnancy and the puerperium. *Clin Sci.* 1966;30(1):1-11.
486. MacGillivray I, Rose GA, Rowe B. Blood pressure survey in pregnancy. *Clin Sci.* 1969;32(2):394-407.
487. Gallery EDM, Hunyor SN, Ross M, Györy AZ. Predicting the development of pregnancy-associated hypertension. The place of standardised blood-pressure measurement. *Lancet.* 1977;309(8025):1273-5.
488. van Oppen AC, van der Tweel I, Alsbach GP, Heethaar RM, Bruinse HW. A longitudinal study of maternal hemodynamics during normal pregnancy. *Obstet Gynecol.* 1996;88(1):40-6.
489. Clark SL, Cotton DB, Lee W, Bishop C, Hill T, Southwick J, et al. Central hemodynamic assessment of normal term pregnancy. *Am J Obstet Gynecol.* 1989;161(6):1439-42.

490. Chapman AB, Abraham WT, Zamudio S, Coffin C, Merouani A, Young D, et al. Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. *Kidney Int.* 1998;54(6):2056-63.
491. Easterling TR, Benedetti TJ, Schmucker BC, Millard SP. Maternal Hemodynamics in Normal and Preeclamptic Pregnancies: A Longitudinal Study. *Obstet Gynecol.* 1990;76(6):1061-9.
492. Mesa A, Jessurun C, Hernandez A, Adam K, Brown D, Vaughn WK, et al. Left ventricular diastolic function in normal human pregnancy. *Circulation.* 1999;99(4):511-7.
493. Poppas A, Shroff S, Korcarz C, Hibbard JU, Berger DS, Lindheimer M, et al. Serial Assessment of the Cardiovascular System in Normal Pregnancy: Role of Arterial Compliance and Pulsatile Arterial Load. *Circulation.* 1997;95(10):2407-15.
494. Fairlie FM, Walker JJ. Does the brachial artery Doppler flow velocity waveform reflect changes in downstream impedance? *Am J Obstet Gynecol.* 1991;165(6 Pt 1):1741-4.
495. Schiessl B, Strasburger CJ, Spannagl M, Kainer F. Decreasing peripheral resistance during pregnancy monitored at the cubital artery. *Eur J Clin Invest.* 2003;33(4):346-51.
496. Bosio PM, McKenna PJ, Conroy R, O'Herlihy C. Maternal central hemodynamics in hypertensive disorders of pregnancy. *Obstet Gynecol.* 1999;94(6):978-84.
497. Vasapollo B, Novelli GP, Valensise H. Total vascular resistance and left ventricular morphology as screening tools for complications in pregnancy. *Hypertension.* 2008;51(4):1020-6.
498. Rang S, van Montfrans GA, Wolf H. Serial hemodynamic measurement in normal pregnancy, preeclampsia, and intrauterine growth restriction. *Am J Obstet Gynecol.* 2008;198(5):519.e1-9.

499. Valensise H, Vasapollo B, Gagliardi G, Novelli GP. Early and late preeclampsia: two different maternal hemodynamic states in the latent phase of the disease. *Hypertension*. 2008;52(5):873-80.
500. Bernstein IM, Ziegler W, Badger GJ. Plasma volume expansion in early pregnancy. *Obstet Gynecol*. 2001;97(5 Pt 1):669-72.
501. Pirani BB, Campbell DM, MacGillivray I. Plasma volume in normal first pregnancy. *J Obstet Gynaecol Br Commonw*. 1973;80(10):884-7.
502. Brown MA, Mitar DA, Whitworth JA. Measurement of plasma volume in pregnancy. *Clin Sci*. 1992;83(1):29-34.
503. Salas SP, Marshall G, Gutierrez BL, Rosso P. Time course of maternal plasma volume and hormonal changes in women with preeclampsia or fetal growth restriction. *Hypertension*. 2006;47(2):203.
504. Silver HM, Seebeck M, Carlson R. Comparison of total blood volume in normal, preeclamptic, and nonproteinuric gestational hypertensive pregnancy by simultaneous measurement of red blood cell and plasma volumes. *Am J Obstet Gynecol*. 1998;179(1):87-93.
505. Zeeman GG, Cunningham FG, Pritchard JA. The magnitude of hemoconcentration with eclampsia. *Hypertens Pregnancy*. 2009;28(2):127-37.
506. Lyall F, von Dadelszen P. A note on the publication of the magnitude of hemoconcentration with eclampsia. *Hypertens Pregnancy*. 2009;28(2):123.
507. Mahowald MB. Changing grounds for equipoise? *Hypertens Pregnancy*. 2009;28(2):124-6.
508. Ganzevoort W, Rep A, Bonsel GJ, de Vries JIP, Wolf H. Plasma volume and blood pressure regulation in hypertensive pregnancy. *J Hypertens*. 2004;22(7):1235-42.

509. Luft FC, Gallery EDM, Lindheimer MD. Normal and Abnormal Volume Homeostasis. In: Lindheimer MD, Roberts JM, Cunningham FG, editors. Chesley's Hypertensive Disorders in Pregnancy (Third Edition). San Diego: Academic Press; 2009. p. 269-85.
510. Schobel HP, Fischer T, Heuszer K, Geiger H, Schmieder RE. Preeclampsia - a state of sympathetic overactivity. *N Engl J Med.* 1996;335(20):1480-5.
511. Gallery EDM, Hunyor SN, Gyory AZ. Plasma volume contraction: a significant factor in both pregnancy-associated hypertension (pre-eclampsia) and chronic hypertension in pregnancy. *Q J Med.* 1979;48(192):593-602.
512. Lurie S, Mamet Y. Red blood cell survival and kinetics during pregnancy. *Eur J Obstet Gynecol Reprod Biol.* 2000;93(2):185-92.
513. Hobbs JB, Oats JN, Palmer AA, Long PA, Mitchell GM, Lou M, et al. Whole blood viscosity in preeclampsia. *Am J Obstet Gynecol.* 1982;142(3):288-92.
514. Thorburn J, Drummond MM, Whigham KA, Lowe GD, Forbes CD, Prentice CR, et al. Blood viscosity and haemostatic factors in late pregnancy, pre-eclampsia and fetal growth retardation. *Br J Obstet Gynaecol.* 1982;89(2):117-22.
515. Pepple DJ, Reid HL, Mullings AM. Is there hyperviscosity in pre-eclampsia? *West Indian Med J.* 2000;49(3):229-31.
516. Hershkovitz R, Ohel I, Sheizaf B, Nathan I, Erez O, Sheiner E, et al. Erythropoietin concentration among patients with and without preeclampsia. *Arch Gynecol Obstet.* 2005;273(3):140-3.
517. Magann EF, Martin JN, Jr. The laboratory evaluation of hypertensive gravidas. *Obstet Gynecol Surv.* 1995;50(2):138-45.
518. Heilmann L, Rath W, Pollow K. Hemorheological changes in women with severe preeclampsia. *Clin Hemorheol Microcirc.* 2004;31(1):49-58.

519. Conde-Agudelo A, Althabe F, Belizan JM, Kafury-Goeta AC. Cigarette smoking during pregnancy and risk of preeclampsia: a systematic review. *Am J Obstet Gynecol.* 1999;181(4):1026-35.
520. Shaamash AH, Elsnosy ED, Makhlouf AM, Zakhari MM, Ibrahim OA, EL-dien HM. Maternal and fetal serum nitric oxide (NO) concentrations in normal pregnancy, pre-eclampsia and eclampsia. *Int J Gynaecol Obstet.* 2000;68(3):207-14.
521. Choi JW, Im MW, Pai SH. Nitric oxide production increases during normal pregnancy and decreases in preeclampsia. *Ann Clin Lab Sci.* 2002;32(3):257-63.
522. Conrad KP, Kerchner LJ, Mosher MD. Plasma and 24-h NO(x) and cGMP during normal pregnancy and preeclampsia in women on a reduced NO(x) diet. *Am J Physiol.* 1999;277(1 Pt 2):F48-57.
523. Brown MA, Tibben E, Zammit VC, Cario GM, Carlton MA. Nitric oxide excretion in normal and hypertensive pregnancies. *Hypertens Pregnancy.* 1995;14(3):319-26.
524. Smarason AK, Allman KG, Young D, Redman CW. Elevated levels of serum nitrate, a stable end product of nitric oxide, in women with pre-eclampsia. *Br J Obstet Gynaecol.* 1997;104(5):538-43.
525. Teran E, Escudero C, Vivero S, Enriquez A, Calle A. Intraplatelet cyclic guanosine-3',5'-monophosphate levels during pregnancy and preeclampsia. *Hypertens Pregnancy.* 2004;23(3):303-8.
526. Buhimschi I, Yallampalli C, Dong YL, Garfield RE. Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. *Am J Obstet Gynecol.* 1995;172(5):1577-84.
527. Telfer JF, Itoh H, Thomson AJ, Norman JE, Nakao K, Campa JS, et al. Activity and expression of soluble and particulate guanylate cyclases in myometrium from

- nonpregnant and pregnant women: down-regulation of soluble guanylate cyclase at term. *J Clin Endocrinol Metab.* 2001;86(12):5934-43.
528. Norman JE, Thompson AJ, Telfer JF, Young A, Greer IA, Cameron IT. Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy. *Mol Hum Reprod.* 1999;5(2):175-81.
529. Learmont JG, Cockell AP, Knock GA, Poston L. Myogenic and flow-mediated responses in isolated mesenteric small arteries from pregnant and nonpregnant rats. *Am J Obstet Gynecol.* 1996;174(5):1631-6.
530. Hennessy A, Whitworth JA, Raymond CJ, Phippard AF, Thompson JF, Horvath JS. Haemodynamic actions of a nitric oxide (EDRF) synthesis inhibitor in conscious baboons (*Papio hamadryas*). *Clin Exp Pharmacol Physiol.* 1994;21(9):695-700.
531. Anumba DO, Robson SC, Boys RJ, Ford GA. Nitric oxide activity in the peripheral vasculature during normotensive and preeclamptic pregnancy. *Am J Physiol.* 1999;277(2 Pt 2):H848-54.
532. Yoshida A, Nakao S, Kobayashi H, Kobayashi M. Noninvasive Assessment of Flow-Mediated Vasodilation with 30-MHz Transducer in Pregnant Women. *Hypertension.* 1998;31(5):1200.
533. Faber-Swensson AP, O'Callaghan SP, Walters WA. Endothelial cell function enhancement in a late normal human pregnancy. *Aust N Z J Obstet Gynaecol.* 2004;44(6):525-9.
534. Yamamoto T, Suzuki Y, Kojima K, Suzumori K. Reduced flow-mediated vasodilation is not due to a decrease in production of nitric oxide in preeclampsia. *Am J Obstet Gynecol.* 2005;192(2):558-63.

535. Saarelainen H, Laitinen T, Raitakari OT, Juonala M, Heiskanen N, Lyyra-Laitinen T, et al. Pregnancy-related hyperlipidemia and endothelial function in healthy women. *Circ J*. 2006;70(6):768-72.
536. Kametas NA, Savvidou MD, Donald AE, McAuliffe F, Nicolaides KH. Flow-mediated dilatation of the brachial artery in pregnancy at high altitude. *BJOG*. 2002;109(8):930-7.
537. Saarelainen H, Valtonen P, Punnonen K, Laitinen T, Raitakari OT, Juonala M, et al. Subtle changes in ADMA and l-arginine concentrations in normal pregnancies are unlikely to account for pregnancy-related increased flow-mediated dilatation. *Clin Physiol Funct Imaging*. 2008;28(2):120-4.
538. Saarelainen H, Valtonen P, Punnonen K, Laitinen T, Raitakari OT, Juonala M, et al. Flow mediated vasodilation and circulating concentrations of high sensitive C-reactive protein, interleukin-6 and tumor necrosis factor-alpha in normal pregnancy-The Cardiovascular Risk in Young Finns Study. *Clin Physiol Funct Imaging*. 2009;29(5):347-52.
539. Valtonen P, Laitinen T, Lyyra-Laitinen T, Raitakari OT, Juonala M, Viikari JSA, et al. Serum L-homoarginine concentration is elevated during normal pregnancy and is related to flow-mediated vasodilatation. *Circ J*. 2008;72(11):1879-84.
540. Sierra-Laguado J, Garcia RG, Lopez-Jaramillo P. Flow-mediated dilatation of the brachial artery in pregnancy. *Int J Gynaecol Obstet*. 2006;93(1):60-1.
541. Accini JL, Sotomayor A, Trujillo F, Barrera JG, Bautista L, Lopez-Jaramillo P. Colombian study to assess the use of noninvasive determination of endothelium-mediated vasodilatation (CANDEV). Normal values and factors associated. *Endothelium: Journal of Endothelial Cell Research*. 2001;8(2):157-66.

542. Taylor RN, Davidge ST, Roberts JM. Endothelial Cell Dysfunction and Oxidative Stress. In: Lindheimer MD, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy (Third Edition)*. San Diego: Academic Press; 2009. p. 143-67.
543. Karumanchi SA, Levine RJ. How does smoking reduce the risk of preeclampsia? *Hypertension*. 2010;55(5):1100-1.
544. Campbell DM, Campbell AJ. Evans Blue disappearance rate in normal and pre-eclamptic pregnancy. *Hypertens Pregnancy*. 1983;2(1):163-9.
545. Roberts JM, Lain KY. Recent Insights into the pathogenesis of pre-eclampsia. *Placenta*. 2002;23(5):359-72.
546. Redman CW, Bonnar J, Beilin L. Early platelet consumption in pre-eclampsia. *Br Med J*. 1978;1(6111):467-9.
547. Hutt R, Ogunniyi SO, Sullivan MH, Elder MG. Increased platelet volume and aggregation precede the onset of preeclampsia. *Obstet Gynecol*. 1994;83(1):146-9.
548. Rowe J, Campbell S, Gallery ED. Nitric oxide production by decidual endothelial cells is not reduced in preeclampsia. *Hypertens Pregnancy*. 2003;22(1):63-75.
549. Schiessl B, Strasburger CJ, Bidlingmaier M, Spannagl M, Ugele B, Kainer F. Decreasing resistance in the maternal uterine and peripheral arterial system is apparently unrelated to plasma and urinary levels of nitrite/nitrate and cyclic-guanosinmonophosphate during the course of normal pregnancies. *J Perinat Med*. 2003;31(4):281-6.
550. Mao D, Che J, Li K, Han S, Yue Q, Zhu L, et al. Association of homocysteine, asymmetric dimethylarginine, and nitric oxide with preeclampsia. *Arch Gynecol Obstet*. 2010;282(4):371-5.
551. Lyall F, Young A, Greer IA. Nitric oxide concentrations are increased in the fetoplacental circulation in preeclampsia. *Am J Obstet Gynecol*. 1995;173(3 Pt 1):714-8.

552. McCarthy AL, Woolfson RG, Raju SK, Poston L. Abnormal endothelial cell function of resistance arteries from women with preeclampsia. *Am J Obstet Gynecol.* 1993;168(4):1323-30.
553. Kublickiene KR, Lindblom B, Kruger K, Nisell H. Preeclampsia: evidence for impaired shear stress-mediated nitric oxide release in uterine circulation. *Am J Obstet Gynecol.* 2000;183(1):160-6.
554. Kenny LC, Baker PN, Kendall DA, Randall MD, Dunn WR. Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. *Clin Sci.* 2002;103(1):67-73.
555. Myers J, Mires G, Macleod M, Baker P. In preeclampsia, the circulating factors capable of altering in vitro endothelial function precede clinical disease. *Hypertension.* 2005;45(2):258-63.
556. Davis KR, Ponnampalam J, Hayman R, Baker PN, Arulkumaran S, Donnelly R. Microvascular vasodilator response to acetylcholine is increased in women with pre-eclampsia. *BJOG.* 2001;108(6):610-4.
557. Khan F, Belch JJ, MacLeod M, Mires G. Changes in endothelial function precede the clinical disease in women in whom preeclampsia develops. *Hypertension.* 2005;46(5):1123-8.
558. Anim-Nyame N, Gamble J, Sooranna SR, Johnson MR, Sullivan MH, Steer PJ. Evidence of impaired microvascular function in pre-eclampsia: a non-invasive study. *Clin Sci.* 2003;104(4):405-12.
559. Anim-Nyame N, Sooranna SR, Johnson MR, Gamble J, Steer PJ. A longitudinal study of resting peripheral blood flow in normal pregnancy and pregnancies complicated by chronic hypertension and pre-eclampsia. *Cardiovasc Res.* 2001;50(3):603-9.

560. Bowyer L, Brown MA, Jones M. Forearm blood flow in pre-eclampsia. *BJOG*. 2003;110(4):383-91.
561. Veille J-C, Gorsuch L, Weeks W, Zaccaro D. Hyperemic Response of the Brachial Artery During the Second Half of Pregnancy. *J Soc Gynecol Investig*. 1998;5(38):38-43.
562. Williams K, Kocer C. Vascular reactivity in preeclampsia assessed noninvasively using maternal brachial artery hyperemic response. *Obstet Gynecol*. 2004;104(5 Pt 1):1025-9.
563. Chambers JC, Fusi L, Malik I, Haskard D, De Swiet M, Kooner J. Association of Maternal Endothelial Dysfunction With Preeclampsia. *JAMA*. 2001;285(12):1607-12.
564. Ramsay JE, Stewart F, Greer IA, Sattar N. Microvascular dysfunction: a link between pre-eclampsia and maternal coronary heart disease. *BJOG*. 2003;110(11):1029-31.
565. Agatista PK, Ness RB, Roberts JM, Costantino JP, Kuller LH, McLaughlin MK. Impairment of endothelial function in women with a history of preeclampsia: an indicator of cardiovascular risk. *Am J Physiol Heart Circ Physiol*. 2004;286(4):H1389-93.
566. Lampinen KH, Ronnback M, Kaaja RJ, Groop P-H. Impaired vascular dilatation in women with a history of pre-eclampsia. *J Hypertens*. 2006;24(4):751-6.
567. Blaauw J, Graaff R, van Pampus MG, van Doormaal JJ, Smit AJ, Rakhorst G, et al. Abnormal endothelium-dependent microvascular reactivity in recently preeclamptic women. *Obstet Gynecol*. 2005;105(3):626-32.
568. Lommerse T, Aardenburg R, Houben A, Peeters LL. Endothelium-dependent vasodilatation in formerly preeclamptic women correlates inversely with body mass index and varies independently of plasma volume. *Reprod Sci*. 2007;14(8):765-70.

569. Germain AM, Romanik MC, Guerra I, Solari S, Reyes MS, Johnson RJ, et al. Endothelial dysfunction: a link among preeclampsia, recurrent pregnancy loss, and future cardiovascular events? *Hypertension*. 2007;49(1):90-5.
570. Hamad RR, Eriksson MJ, Silveira A, Hamsten A, Bremme K. Decreased flow-mediated dilation is present 1 year after a pre-eclamptic pregnancy. *J Hypertens*. 2007;25(11):2301-7.
571. Paez O, Alfie J, Gorosito M, Puleio P, de Maria M, Prieto N, et al. Parallel decrease in arterial distensibility and in endothelium-dependent dilatation in young women with a history of pre-eclampsia. *Clin Exp Hypertens*. 2009;31(7):544-52.
572. Yinon Y, Kingdom JCP, Odutayo A, Moineddin R, Drewlo S, Lai V, et al. Vascular dysfunction in women with a history of preeclampsia and intrauterine growth restriction: insights into future vascular risk. *Circulation*. 2010;122(18):1846-53.
573. Yoshida A, Nakao S, Kobayashi M, Kobayashi H. Flow-mediated vasodilation and plasma fibronectin levels in preeclampsia. *Hypertension*. 2000;36(3):400-4.
574. Takata M, Nakatsuka M, Kudo T. Differential blood flow in uterine, ophthalmic, and brachial arteries of preeclamptic women. *Obstet Gynecol*. 2002;100(5 Pt 1):931-9.
575. Kuscuk NK, Kurhan Z, Yildirim Y, Tavli T, Koyuncu F. Detection of endothelial dysfunction in preeclamptic patients by using color Doppler sonography. *Arch Gynecol Obstet*. 2003;268(2):113-6.
576. Brodzki J, Lanne T, Laurini R, Strevens H, Wide-Svensson D, Marsal K. Vascular mechanical properties and endothelial function in pre-eclampsia with special reference to bilateral uterine artery notch. *Acta Obstet Gynecol Scand*. 2008;87(2):154-62.
577. Filho EV, Mohr C, Filho BJ, Gadonski G, Paula LG, Antonello IC, et al. Flow-mediated dilatation in the differential diagnosis of preeclampsia syndrome. *Arq Bras Cardiol*. 2010;94(2):182-6.

578. Mori T, Shinohara K, Wakatsuki A, Watanabe K, Fujimaki A. Adipocytokines and endothelial function in preeclamptic women. *Hypertens Res - Clin Exp.* 2010;33(3):250-4.
579. Adali E, Kurdoglu M, Adali F, Cim N, Yildizhan R, Kulusari A. The relationship between brachial artery flow-mediated dilatation, high sensitivity C-reactive protein, and uterine artery doppler velocimetry in women with pre-eclampsia. *J Clin Ultrasound.* 2011;39(4):191-7.
580. Matsubara K, Matsubara Y, Hyodo S, Katayama T, Ito M. Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. *J Obstet Gynaecol Res.* 2010;36(2):239-47.
581. ACOG technical bulletin. Hypertension in pregnancy. Number 219-January 1996 (replaces no. 91, February 1986). Committee on Technical Bulletins of the American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet.* 1996;53(2):175-83.
582. Lenfant C, W. GR, Zuspan FP. National high blood pressure education program working group report on high blood pressure in pregnancy. *Am J Obstet Gynecol.* 1990;163(5 Pt 1):1961-712.
583. Gilstrap LC, Ramin SM. ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet.* 2002;77(1):67-75.
584. Harper AC, Holman CDJ, Dawes VP. The health of populations. An introduction. 2nd ed. Melbourne: Churchill Livingstone; 1997.
585. Conde-Agudelo A, Romero R, Lindheimer MD. Tests to Predict Preeclampsia. In: Lindheimer MD, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy (Third Edition)*. San Diego: Academic Press; 2009. p. 189-211.
586. Meads CA, Cnossen JS, Meher S, Juarez-Garcia A, ter Riet G, Duley L, et al. Methods of prediction and prevention of pre-eclampsia: systematic reviews of accuracy

and effectiveness literature with economic modelling. *Health Technol Assess.*

2008;12(6):1-270.

587. Cnossen JS, Morris RK, ter Riet G, Mol BWJ, van der Post JAM, Coomarasamy A, et al. Use of uterine artery Doppler ultrasonography to predict pre-eclampsia and intrauterine growth restriction: a systematic review and bivariable meta-analysis. *CMAJ.* 2008;178(6):701-11.

588. Takase B, Goto T, Hamabe A, Uehata A, Kuroda K, Satomura K, et al. Flow-mediated dilation in brachial artery in the second half of pregnancy and prediction of pre-eclampsia. *J Hum Hypertens.* 2003;17(10):697-704.

589. Garcia RG, Celedon J, Sierra-Laguado J, Alarcon MA, Luengas C, Silva F, et al. Raised C-reactive protein and impaired flow-mediated vasodilation precede the development of preeclampsia. *Am J Hypertens.* 2007;20(1):98-103.

590. Savvidou MD, Noori M, Anderson JM, Hingorani AD, Nicolaides KH. Maternal endothelial function and serum concentrations of placental growth factor and soluble endoglin in women with abnormal placentation. *Ultrasound Obstet Gynecol.* 2008;32(7):871-6.

591. Kamat R, Jain V, Bahl A. Serial estimation of flow mediated dilatation in women at risk of hypertensive disorders of pregnancy. *Int J Cardiol.* 2011 (EPub date 2009);149(1):17-22.

592. Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ. Prospective study of placental angiogenic factors and maternal vascular function before and after preeclampsia and gestational hypertension. *Circulation.* 2010;122(5):478-87.

593. Donald AE, Halcox JP, Charakida M, Storry C, Wallace SML, Cole TJ, et al. Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. *J Am Coll Cardiol.* 2008;51(20):1959-64.

594. Villar J, Say L, Shennan A, Lindheimer M, Duley L, Conde-Agudelo A, et al. Methodological and technical issues related to the diagnosis, screening, prevention, and treatment of pre-eclampsia and eclampsia. *Int J Gynaecol Obstet.* 2004;85 Suppl 1:S28-41.
595. Duley L. The Global Impact of Pre-eclampsia and Eclampsia. *Semin Perinatol.* 2009;33(3):130-7.
596. Rath W, Fischer T. The diagnosis and treatment of hypertensive disorders of pregnancy: new findings for antenatal and inpatient care. *Deutsches Arzteblatt Int.* 2009;106(45):733-8.
597. Xu H, Perez-Cuevas R, Xiong X, Reyes H, Roy C, Julien P, et al. An international trial of antioxidants in the prevention of preeclampsia (INTAPP). *Am J Obstet Gynecol.* 2010;202(3):239.e1-.e10.
598. Sexton M, Hebel JR. A clinical trial of change in maternal smoking and its effect on birth weight. *JAMA.* 1984;251(7):911-5.
599. Cnattingius S. Smoking habits in early pregnancy. *Addict Behav.* 1989;14(4):453-7.
600. Haug K, Foss OP, Kvamme JM. Do pregnant women who report a reduction in cigarette consumption consume less tobacco? *Scand J Prim Health Care.* 1994;12(4):269-75.
601. Cnattingius S, Mills JL, Yuen J, Eriksson O, Salonen H. The paradoxical effect of smoking in preeclamptic pregnancies: smoking reduces the incidence but increases the rates of perinatal mortality, abruptio placentae, and intrauterine growth restriction. *Am J Obstet Gynecol.* 1997;177(1):156-61.
602. Underwood PB, Kesler KF, O'Lane JM, Callagan DA. Parental smoking empirically related to pregnancy outcome. *Obstet Gynecol.* 1967;29(1):1-8.

603. Duffus GM, MacGillivray I. The incidence of pre-eclamptic toxemia in smokers and non-smokers. *Lancet*. 1968;1(7550):994-5.
604. Zhang J, Klebanoff MA, Levine RJ, Puri M, Moyer P. The puzzling association between smoking and hypertension during pregnancy. *Am J Obstet Gynecol*. 1999;181(6):1407-13.
605. England LJ, Levine RJ, Qian C, Morris CD, Sibai BM, Catalano PM, et al. Smoking before pregnancy and risk of gestational hypertension and preeclampsia. *Am J Obstet Gynecol*. 2002;186(5):1035-40.
606. Lain KY, Powers RW, Krohn MA, Ness RB, Crombleholme WR, Roberts JM. Urinary cotinine concentration confirms the reduced risk of preeclampsia with tobacco exposure. *Am J Obstet Gynecol*. 1999;181(5 Pt 1):1192-6.
607. Janakiraman V, Gantz M, Maynard S, El-Mohandes A. Association of cotinine levels and preeclampsia among African-American women. *Nicotine Tob Res*. 2009;11(6):679-84.
608. Hammoud AO, Bujold E, Sorokin Y, Schild C, Krapp M, Baumann P. Smoking in pregnancy revisited: findings from a large population-based study. *Am J Obstet Gynecol*. 2005;192(6):1856-62.
609. Yang Q, Wen SW, Smith GN, Chen Y, Krewski D, Chen XK, et al. Maternal cigarette smoking and the risk of pregnancy-induced hypertension and eclampsia. *Int J Epidemiol*. 2006;35(2):288-93.
610. Pipkin FB. Genetics of Preeclampsia Consortium. Smoking in moderate/severe preeclampsia worsens pregnancy outcome, but smoking cessation limits the damage. *Hypertension*. 2008;51(4):1042-6.

611. Leeners B, Neumaier-Wagner P, Kuse S, Rath W. Smoking and the risk of developing hypertensive diseases in pregnancy: what is the effect on HELLP syndrome? *Acta Obstet Gynecol Scand.* 2006;85(10):1217-24.
612. England L, Zhang J. Smoking and risk of preeclampsia: a systematic review. *Front Biosci.* 2007;12:2471-83.
613. Miller EC, Cao H, Wen SW, Yang Q, Lafleche J, Walker M. The risk of adverse pregnancy outcomes is increased in preeclamptic women who smoke compared with nonpreeclamptic women who do not smoke. *Am J Obstet Gynecol.* 2010;203(4):334.e1-8.
614. Peltier MR, Ananth CV. Is the association of maternal smoking and pregnancy-induced hypertension dependent on fetal growth? *Am J Obstet Gynecol.* 2007;196(6):532.e1-6.
615. Wikstrom A-K, Stephansson O, Chattingius S. Tobacco use during pregnancy and preeclampsia risk: effects of cigarette smoking and snuff. *Hypertension.* 2010;55(5):1254-9.
616. Stone CD, Diallo O, Shyken J, Leet T. The combined effect of maternal smoking and obesity on the risk of preeclampsia. *J Perinat Med.* 2007;35(1):28-31.
617. Ness RB, Zhang J, Bass D, Klebanoff MA. Interactions between Smoking and Weight in Pregnancies Complicated by Preeclampsia and Small-for-Gestational-Age Birth. *Am J Epidemiol.* 2008;168(4):427-33.
618. Aagaard-Tillery KM, Porter TF, Lane RH, Varner MW, Lacoursiere DY. In utero tobacco exposure is associated with modified effects of maternal factors on fetal growth. *Am J Obstet Gynecol.* 2008;198(1):66.e1-6.
619. Shiverick KT, Salafia C. Cigarette smoking and pregnancy I: ovarian, uterine and placental effects. *Placenta.* 1999;20(4):265-72.

620. Salafia C, Shiverick K. Cigarette smoking and pregnancy II: vascular effects. *Placenta*. 1999;20(4):273-9.
621. Himmelberger DU, Brown BW, Jr., Cohen EN. Cigarette smoking during pregnancy and the occurrence of spontaneous abortion and congenital abnormality. *Am J Epidemiol*. 1978;108(6):470-9.
622. Windham GC, Swan SH, Fenster L. Parental cigarette smoking and the risk of spontaneous abortion. *Am J Epidemiol*. 1992;135(12):1394-403.
623. Windham GC, Von Behren J, Waller K, Fenster L. Exposure to environmental and mainstream tobacco smoke and risk of spontaneous abortion. *Am J Epidemiol*. 1999;149(3):243-7.
624. Kline J, Levin B, Kinney A, Stein Z, Susser M, Warburton D. Cigarette smoking and spontaneous abortion of known karyotype. Precise data but uncertain inferences. *Am J Epidemiol*. 1995;141(5):417-27.
625. Ventura SJ, Hamilton BE, Mathews TJ, Chandra A. Trends and variations in smoking during pregnancy and low birth weight: evidence from the birth certificate, 1990-2000. *Pediatrics*. 2003;111(5 Part 2):1176-80.
626. Bai J, Wong FW, Gyaneshwar R, Stewart HC. Profile of maternal smokers and their pregnancy outcomes in south western Sydney. *J Obstet Gynaecol Res*. 2000;26(2):127-32.
627. Olafsdottir AS, Skuladottir GV, Thorsdottir I, Hauksson A, Steingrimsdottir L. Combined effects of maternal smoking status and dietary intake related to weight gain and birth size parameters. *BJOG*. 2006;113(11):1296-302.
628. Villalbi JR, Salvador J, Cano-Serral G, Rodriguez-Sanz MC, Borrell C. Maternal smoking, social class and outcomes of pregnancy. *Paediatr Perinat Epidemiol*. 2007;21(5):441-7.

629. Jackson DJ, Batiste E, Rendall-Mkosi K. Effect of smoking and alcohol use during pregnancy on the occurrence of low birthweight in a farming region in South Africa. *Paediatr Perinat Epidemiol.* 2007;21(5):432-40.
630. McCowan LME, Roberts CT, Dekker GA, Taylor RS, Chan EHY, Kenny LC, et al. Risk factors for small-for-gestational-age infants by customised birthweight centiles: data from an international prospective cohort study. *BJOG.* 2010;117(13):1599-607.
631. Ananth CV, Smulian JC, Vintzileos AM. Incidence of placental abruption in relation to cigarette smoking and hypertensive disorders during pregnancy: a meta-analysis of observational studies. *Obstet Gynecol.* 1999;93(4):622-8.
632. Cnattingius S, Axelsson O, Eklund G, Lindmark G. Smoking, maternal age, and fetal growth. *Obstet Gynecol.* 1985;66(4):449-52.
633. Peacock JL, Bland JM, Anderson HR, Brooke OG. Cigarette smoking and birthweight: type of cigarette smoked and a possible threshold effect. *Int J Epidemiol.* 1991;20(2):405-12.
634. Li CQ, Windsor RA, Perkins L, Goldenberg RL, Lowe JB. The impact on infant birth weight and gestational age of cotinine-validated smoking reduction during pregnancy. *JAMA.* 1993;269(12):1519-24.
635. Vreeburg SA, Jacobs DJ, Dekker GA, Heard AR, Priest KR, Chan A. Hypertension during pregnancy in South Australia, part 2: risk factors for adverse maternal and/or perinatal outcome - results of multivariable analysis. *Aust N Z J Obstet Gynaecol.* 2004;44(5):410-8.
636. Pirani BB, MacGillivray I. Smoking during pregnancy. Its effect on maternal metabolism and fetoplacental function. *Obstet Gynecol.* 1978;52(3):257-63.

637. Burton GJ, Palmer ME, Dalton KJ. Morphometric differences between the placental vasculature of non-smokers, smokers and ex-smokers. *Br J Obstet Gynaecol.* 1989;96(8):907-15.
638. Rosso P, Donoso E, Braun S, Espinoza R, Fernandez C, Salas S. Maternal hemodynamic adjustments in idiopathic fetal growth retardation. *Gynecol Obstet Invest.* 1993;35(3):162-5.
639. Sontag LW, Wallace RF. The Effect of Cigaret Smoking During Pregnancy Upon the Fetal Heart Rate. *Am J Obstet Gynecol.* 1935;29:77-83.
640. Kelly J, Mathews KA, O'Connor M. Smoking in pregnancy: effects on mother and fetus. *Br J Obstet Gynaecol.* 1984;91(2):111-7.
641. Lehtovirta P, Forss M. The acute effect of smoking on intervillous blood flow of the placenta. *Br J Obstet Gynaecol.* 1978;85(10):729-31.
642. Lehtovirta P, Forss M. The acute effect of smoking on uteroplacental blood flow in normotensive and hypertensive pregnancy. *Int J Gynaecol Obstet.* 1980;18(3):208-11.
643. Newnham JP, Patterson L, James I, Reid SE. Effects of maternal cigarette smoking on ultrasonic measurements of fetal growth and on Doppler flow velocity waveforms. *Early Hum Dev.* 1990;24(1):23-36.
644. Morrow RJ, Ritchie JW, Bull SB. Maternal cigarette smoking: the effects on umbilical and uterine blood flow velocity. *Am J Obstet Gynecol.* 1988;159(5):1069-71.
645. Castro LCM, Allen RM, Ogunyemi DM, Roll KR, Platt LDM. Cigarette Smoking During Pregnancy: Acute Effects on Uterine Flow Velocity Waveforms. *Obstet Gynecol.* 1993;81(4):551-5.
646. Kho EM, North RA, Chan E, Stone PR, Dekker GA, McCowan LME. Changes in Doppler flow velocity waveforms and fetal size at 20 weeks gestation among cigarette smokers. *BJOG.* 2009;116(10):1300-6.

647. Sooranna SR, Morris NH, Steer PJ. Placental nitric oxide metabolism. *Reprod Fertil Dev.* 1995;7(6):1525-31.
648. Andersen MR, Walker LR, Stender S. Reduced endothelial nitric oxide synthase activity and concentration in fetal umbilical veins from maternal cigarette smokers. *Am J Obstet Gynecol.* 2004;191(1):346-51.
649. Andersen MR, Simonsen U, Uldbjerg N, Aalkjaer C, Stender S. Smoking cessation early in pregnancy and birth weight, length, head circumference, and endothelial nitric oxide synthase activity in umbilical and chorionic vessels: an observational study of healthy singleton pregnancies. *Circulation.* 2009;119(6):857-64.
650. Lain KY, Wilson JW, Crombleholme WR, Ness RB, Roberts JM. Smoking during pregnancy is associated with alterations in markers of endothelial function. *Am J Obstet Gynecol.* 2003;189(4):1196-201.
651. Asmussen I, Kjeldsen K. Intimal ultrastructure of human umbilical arteries. Observations on arteries from newborn children of smoking and nonsmoking mothers. *Circ Res.* 1975;36(5):579-89.
652. Hutchison SJ, Glantz SA, Zhu BQ, Sun YP, Chou TM, Chatterjee K, et al. In-utero and neonatal exposure to secondhand smoke causes vascular dysfunction in newborn rats. *J Am Coll Cardiol.* 1998;32(5):1463-7.
653. Kaminsky LM, Ananth CV, Prasad V, Nath C, Vintzileos AM. The influence of maternal cigarette smoking on placental pathology in pregnancies complicated by abruption. *Am J Obstet Gynecol.* 2007;197(3):275.e1-5.
654. Naeye RL. Effects of maternal cigarette smoking on the fetus and placenta. *Br J Obstet Gynaecol.* 1978;85(10):732-7.
655. van der Veen F, Fox H. The effects of cigarette smoking on the human placenta: a light and electron microscopic study. *Placenta.* 1982;3(3):243-56.

656. Clausen HV, Larsen LG, Jorgensen A, Bzorek M. The human placenta from heavy smokers: evaluation of vasoactive peptides by immunohistochemistry. *APMIS*. 2007;115(1):22-9.
657. Bush PG, Mayhew TM, Abramovich DR, Aggett PJ, Burke MD, Page KR. Maternal cigarette smoking and oxygen diffusion across the placenta. *Placenta*. 2000;21(8):824-33.
658. Vogt Isaksen C. Maternal smoking, intrauterine growth restriction, and placental apoptosis. *Pediatr Dev Pathol*. 2004;7(5):433-42.
659. Powell JT. Vascular damage from smoking: disease mechanisms at the arterial wall. *Vasc Med*. 1998;3(1):21-8.
660. Lambers DS, Clark KE. The maternal and fetal physiologic effects of nicotine. *Semin Perinatol*. 1996;20(2):115-26.
661. Leone A. Biochemical markers of cardiovascular damage from tobacco smoke. *Curr Pharm Des*. 2005;11(17):2199-208.
662. Dempsey D, Jacob P, Benowitz NL. Accelerated metabolism of nicotine and cotinine in pregnant smokers. *J Pharmacol Exp Ther*. 2002;301(2):594-8.
663. Benowitz NL. Drug therapy. Pharmacologic aspects of cigarette smoking and nicotine addiction. *N Engl J Med*. 1988;319(20):1318-30.
664. West RJ, Russell MA. Cardiovascular and subjective effects of smoking before and after 24 h of abstinence from cigarettes. *Psychopharmacology (Berl)*. 1987;92(1):118-21.
665. Lindblad A, Marsal K, Anersson KE. Effect of nicotine on human fetal blood flow. *Obstet Gynecol*. 1988;72(3):371.
666. Zimmerman M, McGeachie J. The effect of nicotine on aortic endothelial cell turnover. An autoradiographic study. *Atherosclerosis*. 1985;58(1-3):39-47.

667. Zimmerman M, McGeachie J. The effect of nicotine on aortic endothelium. A quantitative ultrastructural study. *Atherosclerosis*. 1987;63(1):33-41.
668. Mayhan WG, Patel KP. Effect of nicotine on endothelium-dependent arteriolar dilatation in vivo. *Am J Physiol*. 1997;272(5 Pt 2):H2337-42.
669. Mayhan WG, Sharpe GM. Chronic exposure to nicotine alters endothelium-dependent arteriolar dilatation: effect of superoxide dismutase. *J Appl Physiol*. 1999;86(4):1126-34.
670. Black CE, Huang N, Neligan PC, Levine RH, Lipa JE, Lintlop S, et al. Effect of nicotine on vasoconstrictor and vasodilator responses in human skin vasculature. *Am J Physiol Regul Integr Comp Physiol*. 2001;281(4):R1097-104.
671. Chalon S, Moreno H, Jr., Benowitz NL, Hoffman BB, Blaschke TF. Nicotine impairs endothelium-dependent dilatation in human veins in vivo. *Clin Pharmacol Ther*. 2000;67(4):391-7.
672. Cirillo P, De Rosa S, Pacileo M, Gargiulo A, Leonardi A, Angri V, et al. Nicotine induces tissue factor expression in cultured endothelial and smooth muscle cells. *J Thromb Haemost*. 2006;4(2):453-8.
673. Nicolau LGC, Martins WP, Gallarreta FMP, Lima JC, Mauad Filho F. Influence of pregnancy and smoking on brachial artery flow-mediated dilation values and time until maximum response. *Arch Gynecol Obstet*. 2010.
674. Tonnessen BH, Severson SR, Hurt RD, Miller VM. Modulation of nitric-oxide synthase by nicotine. *J Pharmacol Exp Ther*. 2000;295(2):601-6.
675. Zhang JY, Cao YX, Xu CB, Edvinsson L. Lipid-soluble smoke particles damage endothelial cells and reduce endothelium-dependent dilatation in rat and man. *BMC Cardiovasc Disord*. 2006;6(1):3.

676. Li Z, Barrios V, Buchholz JN, Glenn TC, Duckles SP. Chronic nicotine administration does not affect peripheral vascular reactivity in the rat. *J Pharmacol Exp Ther.* 1994;271(3):1135-42.
677. Krishna K. Tobacco chewing in pregnancy. *Br J Obstet Gynaecol.* 1978;85(10):726-8.
678. Zdravkovic T, Genbacev O, McMaster MT, Fisher SJ. The adverse effects of maternal smoking on the human placenta: a review. *Placenta.* 2005;26 Suppl A:S81-6.
679. Genbacev O, Bass KE, Joslin RJ, Fisher SJ. Maternal smoking inhibits early human cytotrophoblast differentiation. *Reprod Toxicol.* 1995;9(3):245-55.
680. Genbacev O, McMaster MT, Zdravkovic T, Fisher SJ. Disruption of oxygen-regulated responses underlies pathological changes in the placentas of women who smoke or who are passively exposed to smoke during pregnancy. *Reprod Toxicol.* 2003;17(5):509-18.
681. Mimura K, Tomimatsu T, Sharentuya N, Tskitishvili E, Kinugasa-Taniguchi Y, Kanagawa T, et al. Nicotine restores endothelial dysfunction caused by excess sFlt1 and sEng in an in vitro model of preeclamptic vascular endothelium: a possible therapeutic role of nicotinic acetylcholine receptor (nAChR) agonists for preeclampsia. *Am J Obstet Gynecol.* 2010;202(5):464.e1-6.
682. Cooke JP, Bitterman H. Nicotine and angiogenesis: a new paradigm for tobacco-related diseases. *Ann Med.* 2004;36(1):33-40.
683. Mehendale R, Hibbard J, Fazleabas A, Leach R. Placental angiogenesis markers sFlt-1 and PlGF: response to cigarette smoke. *Am J Obstet Gynecol.* 2007;197(4):363.e1-5.
684. Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev.* 2005;57(4):585.

685. Bainbridge SA, Sidle EH, Smith GN. Direct placental effects of cigarette smoke protect women from pre-eclampsia: the specific roles of carbon monoxide and antioxidant systems in the placenta. *Med Hypotheses*. 2005;64(1):17-27.
686. Laws PJ, Grayson N, Sullivan EA. Smoking and pregnancy. National Perinatal Statistics Unit, Australian Institute of Health and Welfare. University of New South Wales AIHW Cat. No. PER 33. National Perinatal Statistics Unit; 2006.
687. Ozerol E, Ozerol I, Gökdeniz R, Temel I, Akyol O. Effect of smoking on serum concentrations of total homocysteine, folate, vitamin B12, and nitric oxide in pregnancy: a preliminary study. *Fetal Diagn Ther*. 2004;19(2):145.
688. Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol*. 2004;15(8):1983-92.
689. Gandley RE, Jeyabalan A, Desai K, McGonigal S, Rohland J, DeLoia JA. Cigarette exposure induces changes in maternal vascular function in a pregnant mouse model. *Am J Physiol Regul Integr Comp Physiol*. 2010;298(5):R1249-56.
690. Nicolau LGC, Martins WP, Ferreira AC, Gallarreta FMP, Lima JC, Barra DA, et al. Maximum dilation of the brachial artery in smoking and nonsmoking pregnant and non-pregnant women. *Radiologia Brasileira*. 2010;43(2):85-9.
691. Costa F, Soares R. Nicotine: a pro-angiogenic factor. *Life Sci*. 2009;84(23-24):785-90.
692. Xiao DL, Huang X, Yang S, Zhang L. Direct effects of nicotine on contractility of the uterine artery in pregnancy. *J Pharmacol Exp Ther*. 2007;322(1):180.
693. Smith GD, Harding S, Rosato M. Relation between infants' birth weight and mothers' mortality: prospective observational study. *BMJ*. 2000;320(7238):839-40.

694. Chappell LC, Enye S, Seed P, Briley AL, Poston L, Shennan AH. Adverse perinatal outcomes and risk factors for preeclampsia in women with chronic hypertension: a prospective study. *Hypertension*. 2008;51(4):1002-9.
695. Harrington K, Cooper D, Lees C, Hecher K, Campbell S. Doppler ultrasound of the uterine arteries: the importance of bilateral notching in the prediction of pre-eclampsia, placental abruption or delivery of a small-for-gestational-age baby. *Ultrasound Obstet Gynecol*. 1996;7(3):182-8.
696. Albaiges G, Missfelder-Lobos H, Lees C, Parra M, Nicolaides KH. One-stage screening for pregnancy complications by color Doppler assessment of the uterine arteries at 23 weeks' gestation. *Obstet Gynecol*. 2000;96(4):559-64.
697. Lindheimer MD, Roberts JM, Cunningham FG, Chesley LC. Introduction, History, Controversies and Definitions. In: Lindheimer MD, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy (Third edition)*. San Diego: Academic Press; 2009. p. 1-23.
698. Ogburn Jr PL, Hurt RD, Croghan IT, Schroeder DR, Ramin KD, Offord KP, et al. Nicotine patch use in pregnant smokers: nicotine and cotinine levels and fetal effects. *Am J Obstet Gynecol*. 1999;181(3):736-43.

Appendix.

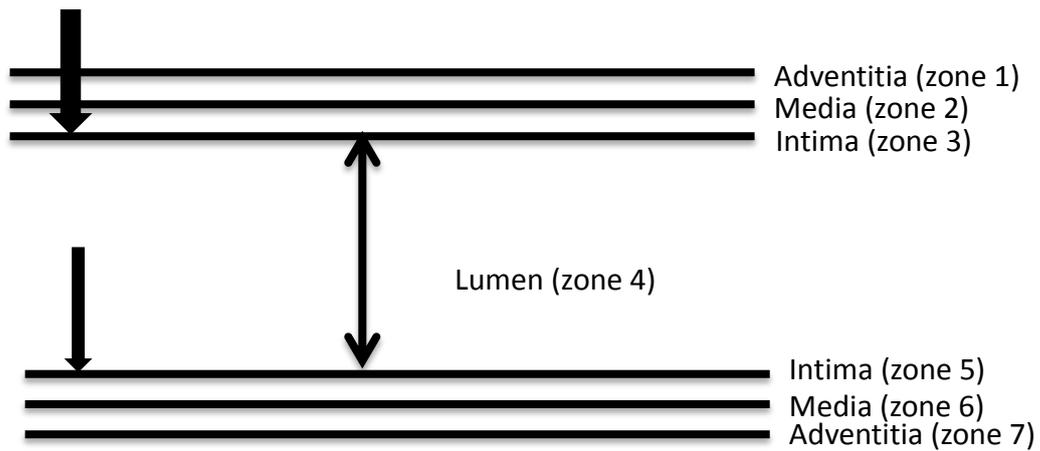


Figure: Schematic diagram of an artery demonstrating the layers of the vessel wall and lumen as seen by ultrasound. To measure the lumen diameter of a vessel (double head arrow), it is necessary to measure from the leading edge of echo zone 3 (thick arrow) to the leading edge of echo zone 5 (thin arrow), that is from the media–intima interface to the intima–media complex.