AN INVESTIGATION OF THE MATERNAL VASCULAR RESPONSE TO ISCHAEMIC PRE-CONDITIONING IN PRE-ECLAMPSIA

Thesis presented for the degree of Doctor of Philosophy
at the Institute for Women's Health,
University College London

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Signed Declaration

I, Tamara Kubba confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgements

Firstly, I would like to thank my principal supervisor Professor David Williams for the opportunity to undertake and complete this PhD. I have worked as part of the Maternal Medicine Research Group for a decade and I am enormously grateful for David's guidance and support. He has helped me develop both as an individual and as a clinical academic. I am also very grateful to my subsidiary supervisor Professor Sean Davidson who has steered me through my time at The Hatter Cardiovascular Institute and has helped keep my PhD focussed. I am extremely thankful for mentoring both David and Sean have given me and I could not have completed this PhD without their encouragement and supervision.

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Abstract

Pre-eclampsia is a syndrome of pregnancy, classically defined by the gestational-onset of hypertension and proteinuria. Pre-eclampsia complicates 4% of first-time pregnancies and contributes to maternal and neonatal morbidity and mortality globally. Women with pre-eclampsia have reduced utero-placental blood flow, compromising placental and fetal growth. This triggers maternal endothelial dysfunction and variable organ dysfunction.

Outside pregnancy, repeated episodes of transient organ ischaemia, (ischaemic pre-conditioning: IPC) has improved endothelial function and blood flow to remote organs. IPC has not previously been tested in pregnancy.

The main hypothesis is that local IPC improves endothelial function in women with established pre-eclampsia, as measured by brachial artery flow-mediated dilatation (FMD). To investigate how IPC may improve endothelial function, levels of vasoactive factors were assayed.

I first investigated healthy non-pregnant women of childbearing age (n=24) to determine the time-interval following IPC that gave the greatest improvement in FMD. Having identified a 24-hour study interval, I carried out FMD before and after IPC in healthy normotensive pregnant women (n=42), women at risk of pre-eclampsia (n=20), and women with established pre-eclampsia (n=10).

The results demonstrate that IPC significantly improves endothelial function in women with pre-eclampsia and at risk of pre-eclampsia (3.5% to 5.8% and 7.6% to 8.9% respectively, vs 10.3% to 10.5% in healthy women). IPC also shortened the time to reach peak FMD in healthy pregnant women.

As expected, women with pre-eclampsia had higher sFlt-1 and lower PlGF levels, and higher sFlt1:PlGF ratios. Following IPC, there were no changes to these levels, nor consistent changes to levels of SDF-1α, DPP-4 or VEGF.

In conclusion, my results suggest that local IPC improves endothelial function in women with pre-eclampsia and at risk of pre-eclampsia. The relative simplicity and cost-effectiveness of IPC, make it an attractive intervention worthy of further investigation as a prophylaxis or treatment of pre-eclampsia.

Impact Statement

Pre-eclampsia is a syndrome of pregnancy, classically defined by the gestational-onset of hypertension and proteinuria. Pre-eclampsia complicates 4% of first-time pregnancies and is a major contributor to maternal and neonatal morbidity and mortality globally. A simple, effective and cheap prophylaxis against pre-eclampsia would be invaluable to low-resource nations where maternal and perinatal morbidity is highest.

In this thesis I report the first investigation to test whether local ischaemic pre-conditioning (IPC) in women with pre-eclampsia can improve their endothelial function, as measured by brachial artery flow-mediated dilatation (FMD). I also measured changes in a panel of blood biomarkers. The participants included: healthy normotensive pregnant women (n=42), pregnant women at risk of pre-eclampsia (n=20) and women with established pre-eclampsia (n=10).

The most significant and important finding from my thesis was that IPC significantly improved endothelial function in pregnant women with pre-eclampsia and those at risk of pre-eclampsia.

My results support the need for further investigation of local IPC, remote IPC and repeated IPC in preventing the development of pre-eclampsia or ameliorating the severity of disease.

My study was not powered to investigate whether IPC improved blood pressure in women with pre-eclampsia or at risk of pre-eclampsia. If further research discovered that IPC was able to improve blood pressure in hypertensive pregnant women, then this would be of clinical significance. It would have the potential to change clinical practice and would impact the care pregnant women receive both nationally and internationally.

Despite 30 years of research the mechanism underlying IPC still remains unclear. Although some significant changes were detected in the levels of SDF-1α, DPP-4 and VEGF in different groups, the precise mechanism underlying IPC remains unresolved.

Some of these findings have been presented at local and international meetings, and I am preparing a paper for submission on these findings.

Ultimately if IPC could prevent pre-eclampsia, or safely prolong pregnancy in women with pre-term pre-eclampsia, it would not only improve pregnancy outcomes for these women, but may also reduce their life-long risk of future cardiovascular disease.

Presentations and Publications

UCL EGA Institute for Women's Health 15th Annual Meeting, London, 7th June 2019

"Ischaemic pre-conditioning: a potential non-invasive therapy to improve endothelial function in women at risk of pre-eclampsia and with established pre-eclampsia"

Kubba T, Davidson S, Williams D

Oral Presentation (overall winner of session prize awarded)

American Heart Association Scientific Sessions, Virtual Event, 13th-17th November 2020

"Ischaemic pre-conditioning: A non-invasive intervention that improves maternal endothelial function in women with preeclampsia and at risk of preeclampsia"

Kubba T, Davidson S, Williams D

Poster Presentation

Kubba T, Davidson S, Williams D

"Ischaemic pre-conditioning: A non-invasive intervention that improves maternal endothelial function in women with preeclampsia and at risk of preeclampsia".

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Abbreviations

ANOVA	Analysis of Variance
BMI	Body Mass Index
BP	Blood Pressure
COVID-19	Severe Acute Respiratory Syndrome Coronavirus 2
CRF	Clinical Research Facility
DPP-4	Dipeptidyl Peptidase-4
EDTA	Ethylenediaminetetraacetic Acid
EGA	Elizabeth Garrett Anderson
ELISA	Enzyme-Linked Immunosorbent Assay
EPCs	Endothelial Progenitor Cells
FMD	Flow-Mediated Dilatation
HIF	Hypoxia-Inducible Factor
HUVEC	Human Umbilical Vein Endothelial Cell
IL	Interleukin
IPC	Ischaemic Pre-Conditioning
ISSHP	International Society for the Study of Hypertension in Pregnancy
LVEF	Left Ventricular Ejection Fraction
MAP	Mean Arterial Pressure
MI	Myocardial Infarction
mRNA	Messenger Ribonucleic Acid
NIHR	National Institute for Health Research
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PCR	Protein Creatinine Ratio
PI	Pulsatility Index
PlGF	Placental Growth Factor
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SD	Standard Deviation
SDF-1α(1-67)	Stromal Derived Factor-1α(1-67)

SDF-1α	Stromal Derived Factor-1α
sFlt-1	Soluble FMS-like tyrosine kinase-1
TK	Tamara Kubba
UCL	University College London
UCLH	University College London Hospital
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Chapter 1 - Introduction

1.1 Pre-eclampsia

Pre-eclampsia is a multi-system syndrome of pregnancy, defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP) as the presence of de novo hypertension after 20 weeks gestation accompanied by proteinuria and/or evidence of maternal acute kidney injury, liver dysfunction, neurological features, haemolysis or thrombocytopenia, and/or fetal growth restriction (1).

Pre-eclampsia complicates 4% of first time pregnancies worldwide, and is a major contributor to maternal mortality globally (2-8). Pre-eclampsia is also a major cause of severe maternal morbidity, including: renal failure, cerebrovascular events, cardiac arrest, liver failure, coagulation disorders and pulmonary oedema (9, 10). Pre-eclampsia is associated with significant neonatal morbidity and mortality, due to the sequelae of iatrogenic pre-term delivery and fetal growth restriction (3, 6) and is responsible for one-sixth of all pre-term births (10, 11).

Women who have had pre-eclampsia have an increased risk of developing and dying from cardiovascular disease in later life (12-14). Women who have developed pre-eclampsia are more likely to develop hypertension (x4), cardiovascular and cerebrovascular disease (x2) in the future (15). The American Heart Association recommends that obstetric history is part of the evaluation of cardiovascular risk in women (16). Furthermore, growth restricted babies, born to women with pre-eclampsia, have themselves an increased likelihood of cardiovascular disease in adulthood (17). Therefore, preventing the onset of pre-eclampsia or ameliorating the severity of disease is beneficial for both women and their offspring in the short and long-term.

There is no cure for pre-eclampsia, and the only definitive ‘treatment’ is delivery of the placenta (5, 18). However, in the United Kingdom, the mortality

from pre-eclampsia has decreased in recent years. This has been partly due to daily low-dose aspirin, which is an effective prophylaxis against pre-term pre-eclampsia, for women who are at risk of developing pre-eclampsia (5, 6, 19-21).

Women at risk of pre-eclampsia can be identified from their medical and obstetric history, as well as their maternal characteristics (22). Pre-pregnancy risk factors for pre-eclampsia include nulliparity, pre-existing hypertension, having had pre-eclampsia in a previous pregnancy, obesity, renal disease, pre-existing diabetes and advanced maternal age (5, 23, 24). Some obstetric departments combine these risk factors with clinical measures, such as mean arterial pressure, uterine artery Doppler pulsatility index (PI) and serum placental growth factor (PlGF) in order to further identify women at risk of pre-eclampsia (22, 25).

A recent multicentre, stepped-wedge cluster randomised controlled trial provided evidence for the clinical adoption of PlGF-based testing to aid the exclusion/diagnosis of pre-eclampsia amongst women with suspected pre-eclampsia (26). Since the publication of this study, one-time PlGF testing between 20 and 34+6 weeks gestation has been used in clinical practice to support the diagnosis of pre-eclampsia and inform the level of risk of pre-term delivery or fetal or maternal complications within 14 days. It is worth noting that clinically PlGF - or in some units the ratio of sFlt-1:PlGF - is used to aid the diagnosis of pre-eclampsia, rather than the use of sFlt-1 alone.

1.2 Pathogenesis of pre-eclampsia

The pathogenesis of pre-eclampsia is complex and not fully understood (2, 6, 7, 9, 27). One author has termed pre-eclampsia a “disease of theories” as extensive research has not yet been able to fully explain its complex aetiology and pathophysiology (28).

Maternal cardiovascular adaptations

During a normotensive pregnancy, the maternal cardiovascular system adapts early on with peripheral vasodilation, which is mediated by endothelium-dependent vasodilatory factors (29-33). This then leads to a reduction in systemic vascular resistance (32). This reduction in systemic vascular resistance, alongside an increase in circulating volume and sinus tachycardia, leads to an increased maternal stroke volume and cardiac output (34-36).

The vascular endothelium produces prostacyclin and nitric oxide (NO), which are vasodilatory (31, 34), and contribute to the fall in systemic vascular resistance seen in normotensive pregnancies. Nitric oxide largely regulates the decrease in systemic vascular resistance seen in normotensive pregnancies (37). The vascular endothelium also produces the vasoconstrictors endothelin and thromboxane (31, 34, 38). In the pre-eclamptic state there is an increase in these vasoconstrictors, which contributes to the development of maternal hypertension (39-41). Women who have a pregnancy complicated by pre-eclampsia have a diminished fall in systemic vascular resistance, and early-onset pre-eclampsia (<34 weeks of gestation) is associated with low cardiac output and high vascular resistance (42), which is thought to be related to vasoconstriction at the level of the resistance arteries and arterioles (43).

In a normotensive pregnancy, another adaptation that occurs is that all components of the renin-angiotensin-aldosterone system are upregulated, but resistance to the pressor effects of angiotensin II allows for a normal to low blood pressure (5, 44, 45). Conversely, in pre-eclampsia sensitivity to angiotensin II is increased (5, 44), and maybe counter-intuitively, levels of renin, angiotensin I and angiotensin II are decreased (44, 45).

Some investigators have found women with pre-eclampsia have auto-antibodies which stimulate the type-1 angiotensin II receptor and mediate vasoconstriction (5, 44, 45). Increased sensitivity to angiotensin II, whether or

not due to auto-antibody activation, could contribute to the vasoconstriction seen in pre-eclampsia (45, 46).

Placental ischaemia

Early in a pregnancy destined to develop pre-eclampsia, there is poor placentation (4). Placentation is the invasion of the extravillous cytotrophoblast of the placenta into the spiral (myometrial) arteries of the maternal decidua (4, 27). Poorly dilated spiral arteries, deliver less arterial blood to the placenta, as illustrated in figure 1.1 (4, 5, 27, 47). Placental flow defects can be detected as early as 12 weeks of gestation in women who subsequently develop pre-eclampsia (48). Failed remodelling and poor dilatation at the level of the myometrial spiral arteries leads to reduced utero-placental blood flow and episodes of irregular placenta perfusion (4, 5, 27, 47). These hypoxic and re-oxygenation episodes can generate reactive oxygen species (ROS), which lead to placental oxidative stress and placental dysfunction (5, 49) and contribute to maternal endothelial dysfunction (47).

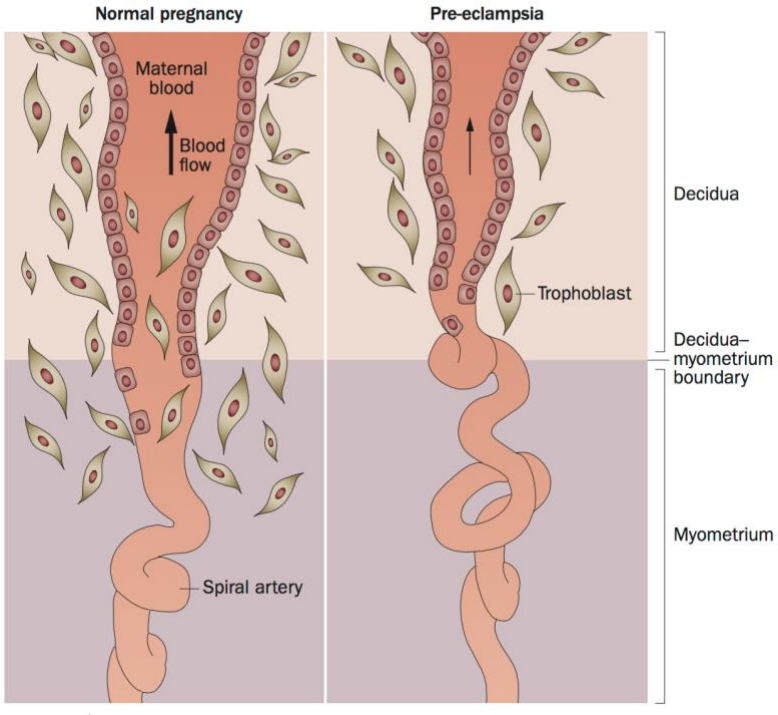


Figure 1.1: Figure showing the failure of the spiral arteries to transform into wide-calibre vessels in pre-eclampsia (5)

It is possible to assess utero-placental blood flow by carrying out uterine artery Doppler velocimetry (50). Women who have a high uterine artery PI in the first and second trimester are at higher risk of developing early onset pre-eclampsia compared to women who have uterine artery PIs within the normal range (23, 50, 51). A high uterine artery PI suggests impaired placental perfusion (52) – this corresponds with histopathological studies which found there is failure of the normal development and invasion of the spiral arteries (53).

Pre-eclampsia evolves as a consequence of poor placentation in women with pre-existing clinical, or sub-clinical, endothelial dysfunction (23, 54-57). For example, women with pre-existing hypertension, which is associated with endothelial dysfunction, have a 1:4 (25%) risk of pre-eclampsia compared with normotensive primigravid women who have a 1:20 (5%) risk (58).

Anti-angiogenic and angiogeneic factors

In women who develop pre-eclampsia there is an imbalance between angiogenic and anti-angiogenic factors, which further induces maternal endothelial damage (2, 4, 7, 27, 59).

One of the factors thought to be central to the pathogenesis of pre-eclampsia is soluble FMS-like tyrosine kinase-1 (sFlt-1), a splice variant of the VEGFR1/Flt-1 receptor (vascular endothelial growth factor receptor 1 VEGFR1) (60-64). SFlt-1 levels are increased in women before the clinical onset of pre-eclampsia by at least 5 to 6 weeks, and is higher earlier in those with earlier onset pre-eclampsia (23, 60, 61, 65-68). SFlt-1 lacks an endothelial anchoring domain so circulates freely until it binds circulating vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), blocking their interaction with membrane bound Flt-1 receptors (27, 47, 69).

SFlt-1is expressed by the syncytiotrophoblast layer of the placenta and sFlt-1 mRNA has been demonstrated to be up-regulated in pre-eclamptic placentae, compared with normotensive pregnancy (60, 70, 71). One theory is that intermittent hypoxia, caused by poor placental implantation, leads to the release of excessive anti-angiogenic proteins into the maternal circulation (4, 60, 61). Another theory is that FLT1 gene variants in the fetus and its placenta, may increase susceptibility to pre-eclampsia, by leading to an increase in sFlt-1 levels (72). A variant of the FLT1 gene has been found in fetuses born to women with pre-eclampsia (72).

SFlt-1 is more than just a biomarker of pre-eclampsia; it appears to contribute to its pathogenesis. One study found that in pregnant rats a reduction in uterine perfusion pressure led to an increase in the expression sFlt-1 (73). Another study found that an adenovirus encoding sFlt-1 injected into both pregnant and non-pregnant rats led to a pre-eclampsia like syndrome (60). This study also measured endothelial tube formation using an in-vitro model of angiogenesis, where conditions were adjusted so that HUVEC cells formed tubes only in the presence of exogenous growth factors such as PlGF or VEGF (60). It was discovered that although serum from normotensive women induced endothelial cells to form regular tube-like structures, serum from women with pre-eclampsia inhibited tube formation (60). When sFlt-1 was added to normotensive serum at concentrations found in patients with pre-eclampsia, tube formation did not occur, replicating the effects seen with serum from pre-eclamptic patients (60). Finally, when exogenous PlGF and VEGF were added to serum from pre-eclamptic patients, tube formation was restored (60). The findings from this study therefore also suggests that sFlt-1 plays a significant role in the development of pre-eclampsia.

A pre-eclampsia like syndrome in rats was also exacerbated by co-administration of soluble endoglin (sEng) (23, 60, 74). SEng is the extra-cellular domain of endoglin (75). Endoglin is highly expressed in syncytiotrophoblasts and vascular endothelial cells (75) and sEng levels increase early on in pregnancies that are then complicated by pre-eclampsia

(76). SEng promotes vascular permeability (75) and women with pre-eclampsia have higher circulating levels of sEng (23, 76).

PlGF is a glycosylated dimeric glycoprotein, which is a member of the VEGF sub-family (70). PlGF is synthesised in the villous and extravillous cytotrophoblast and is angiogenic (60-62, 68). Plasma PlGF levels increase as healthy pregnancy progresses, reaching a peak at 29-32 weeks gestation (61). PlGF levels are significantly lower in women with pre-eclampsia, compared with women who have normotensive pregnancies (61, 70).

Since higher levels of sFlt-1 and lower levels of PlGF are commonly observed prior to the onset of pre-eclampsia (23, 61), the ratio of the two provides an "index" of pre-eclampsia risk (4, 70, 77). Noori et al showed that at 10 to 17 weeks gestation women who developed pre-term pre-eclampsia had lower serum PlGF, higher sEng and a higher sFlt-1:PlGF ratio, compared with women who developed term pre-eclampsia or normotensive pregnancies, and this trend continued until 40 weeks gestation (23). The ischaemic placenta secretes an excessive amount of the anti-angiogenic factor sFLT-1 relative to the angiogenic and vasodilatory factor, PlGF (23, 61) .

VEGF is another angiogeneic peptide (4), which has several isoforms and variants, and a family of receptors; some studies have found that VEGF levels increase as a healthy pregnancy progresses (2, 7, 59) and in women with pre-eclampsia some studies have found that free VEGF levels are decreased (60, 61, 78). However the role of VEGF in pregnancy is not fully understood (5, 78, 79). VEGF is known to be necessary for endothelial stability (4, 80, 81). VEGF and PlGF induce nitric oxide synthase (NOS) and vasodilatory prostacyclins in endothelial cells (80, 81), and therefore these angiogenic factors may be involved in the fall in systemic vascular resistance that is seen in normotensive pregnancy (82, 83).

Inflammation in pre-eclampsia

Women with pre-eclampsia are reported to have a generalised hyperinflammatory state compared with normotensive pregnancy (84-86). It has been postulated that pre-eclampsia may involve hypoxia-induced upregulation of placental pro-inflammatory cytokines (27, 87, 88). The inflammatory cytokines IL-1, IL-6, IL-17 and TNF-α are elevated in pre-eclampsia, possibly due to hypoperfusion of the placenta (18, 27, 34, 89, 90). This may induce upregulation of anti-inflammatory cytokines, such as IL-10, which increases NOS and leads to vasodilatation (87). The role of inflammation, and specific cytokines in the disease mechanism, has not yet been fully elucidated (18, 27, 34).

1.3 Endothelial function and brachial artery flow-mediated dilatation

The endothelium plays a fundamental role in the regulation of vascular tone by releasing both vasodilator and vasoconstrictor substances (91). One endothelial derived vasodilator is nitric oxide (NO), which is produced by nitric oxide synthase (NOS) (92). Nitric oxide mediates relaxation of the underlying vascular smooth muscle (91, 93). Increased blood flow stimulates NOS activity and the production of NO, leading to vasodilatation. The health of endothelium can be objectively assessed by measuring the degree of vasodilatation in response to increased blood flow (91, 92). This is known as endothelium-dependent flow-mediated dilatation (FMD) (91, 94) and in a large part, mediated by NOS activity (92, 95-98).

This technique is the gold standard, non-invasive, method of assessing endothelial function in vivo (95, 98) and acts a surrogate measure of arterial health throughout the body (95, 99, 100). FMD can provide independent prognostic information on future cardiovascular events in patients with established cardiovascular disease, which has been reported to exceed the predictive value of traditional cardiovascular risk factors (95).

FMD is determined by measuring the change in brachial artery diameter in response to a blood flow stimulus (98, 101), as seen in figure 1.2. This stimulus is created by releasing an arm cuff that is inflated to a supra-systolic blood pressure level, which creates an ischaemia-induced reactive hyperaemia (95, 98, 102).

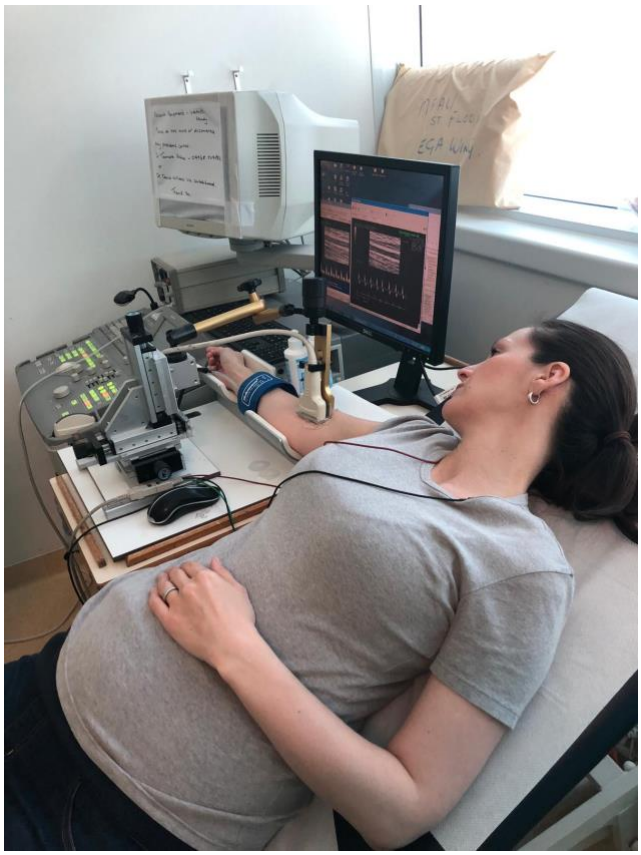


Figure 1.2: A pregnant participant having a measure of brachial artery FMD

Studies have shown a wide distribution of mean FMD values in healthy non-pregnant populations and there is a high degree of variability in what constitutes a 'healthy' FMD response. There are several studies that have found sex and age-specific differences in FMD, with being male and older age being associated with lower FMD measures (103-105).

One paper, which included 219 studies, comprising 16,680 participants, found that mean FMD values ranged from 0.2% to 19.2% in healthy populations (106); another review of 11 studies found an FMD range of 3.4% to 19.1% in

healthy populations (102). It had also been proposed that an FMD measurement of 5.0% or greater constitutes a healthy endothelial ‘response’ (107). A recent study generated sex and age-specific FMD percentile curves, by combining brachial artery FMD observations from 6 research groups (5362 individuals) (108). This study found that for healthy females, aged between 20 to 50 years old, the 50th percentile brachial artery FMD percentage ranged from 6.44% to 8.33% (108).

Impaired endothelial function, as determined by a reduced FMD measure, is associated with an increased risk of future cardiovascular events (109, 110). A meta-analysis of 14 prospective studies, which looked at populations at risk of cardiovascular events, showed that for every 1% decrease in FMD the risk of a cardiovascular event is increased by 13% (101). Endothelial dysfunction is a systemic pathological process (111, 112). It is well documented that peripheral endothelial function as assessed by FMD correlates with coronary artery endothelial function (100, 113, 114).

In healthy pregnancy, a longitudinal study found that brachial artery FMD increased non-significantly from 11 weeks until 32 weeks gestation, followed by a period of stabilisation, and then a significant decline at 36 weeks gestation (115). By 6 weeks post-partum, brachial artery FMD had returned to the non-pregnant value (115). This study did not find a significant difference between brachial artery FMD in the healthy pregnant and healthy non-pregnant controls (115). Dorup et al carried out a cross-sectional study and found that brachial artery FMD increased throughout pregnancy with the greatest response in the third trimester (32).

In women who later developed pre-eclampsia, Noori et al undertook a prospective study, which showed that, longitudinally, from 10 to 40 weeks gestation, brachial artery FMD was lower in these women compared to women who had normotensive pregnancies (23). This study recruited 163 pregnant women from a single centre. Brachial artery FMD was assessed serially in pregnancy in 159 women. All FMD studies were carried out by a single investigator, which is a strength of this study. Participants were

grouped according to pregnancy outcome and in this study 10 women developed pre-term pre-eclampsia (<37 weeks gestation) and 11 women developed term pre-eclampsia (≥37 weeks gestation). Longitudinal comparisons in FMD, mean arterial pressure, and angiogenic and anti-angiogenic factors were made across 3 or 4 time-points in pregnancy. These time points were over 7 weeks and spanned different trimesters (10 to 17 weeks; 18 to 25 weeks; 26 to 33 weeks; 34 to 40 weeks) (23), which could be considered a limitation of this study.

Similar to Noori et al, Savvidou et al carried out a study which assessed brachial artery FMD at 23 to 25 weeks gestation in 86 pregnant women(55). This study found that 10 out of 86 participants later developed pre-eclampsia and that these 10 pregnant women had a lower FMD measure at 23 to 25 weeks gestation, compared to women who did not develop pre-eclampsia (55).

Mannaerts et al carried out a small study where brachial artery FMD was carried out on 14 pregnant women, who had been admitted to hospital with pre-eclampsia (mean gestation 31 weeks) and these findings were compared with brachial artery FMD measurements in 14 normotensive pregnant women (43). It was found that FMD was reduced in women with pre-eclampsia when compared to normotensive pregnant women (FMD measurements: 4.83% +/- 3.15 vs 8.53% +/- 4.09) (43). The mean gestational at which the women with pre-eclampsia were studied in this study is very similar to the mean gestational age of the pre-eclamptic participants in my study.

These three studies, despite having small numbers, suggest that the women who go on to develop pre-eclampsia already have some impairment of their endothelial function compared to women who have normotensive pregnancies. A lower brachial artery FMD measure, reflects systemic endothelial dysfunction and therefore it can be extrapolated that in a pregnant woman with established pre-eclampsia a lower FMD level is indicative of impaired placental function.

In addition to these three studies, a systematic review and meta-analysis of 37 studies found that women who developed pre-eclampsia had an impairment in FMD before, during, and up to 3 years post-partum (116).

1.4 Ischaemic pre-conditioning

Ischaemic pre-conditioning (IPC) is a phenomenon whereby transient, brief episodes of ischaemia applied to an organ or tissue, protect that organ or tissue from a subsequent prolonged period of ischaemia or ischaemic injury (117). This phenomenon was first described in 1986 by Reimer et al (118). They showed that in dogs, brief periods of ischaemia (4 cycles of 5-minute occlusion followed by reperfusion) of the circumflex artery, which supplies arterial blood to the left atrium and left ventricle of the heart, reduced the extent of infarction induced by a subsequent prolonged occlusion of that vessel (118).

This type of IPC is also known as direct or local IPC, as the pre-conditioning stimulus is applied to the same tissue that subsequently sustains ischaemic injury (118, 119). In this thesis I will refer to local IPC.

The majority of animal studies have investigated the impact of local IPC on an organ or tissue, which has undergone ischaemia reperfusion injury. The term ischaemia reperfusion injury (IRI) describes the experimentally and clinically prevalent finding that tissue ischemia, followed by successful reperfusion, initiates a wide and complex array of inflammatory responses, which may both aggravate local ischaemic injury as well as induce impairment of local and remote organ function (120, 121). Conditions under which IRI is encountered include the different forms of acute vascular occlusions (myocardial infarction, limb ischaemia, stroke) with the respective reperfusion strategies (thrombolytic therapy, angioplasty, operative revascularization). IRI is also encountered in routine surgical procedures (organ transplantation, free-tissue-transfer, cardiopulmonary bypass, vascular surgery) and major trauma/shock (120, 121).

Understandably, due to practicalities, it is challenging to use a model of local IPC in human studies of pre-eclampsia, aside from carrying out local IPC in a limb, and then measuring local endothelial function. A small number of human studies have investigated the effect of local IPC on endothelial function without any acute IRI. Moro et al found that local IPC improved endothelial function, as measured by improved brachial artery FMD, in healthy young participants and healthy elderly participants, as well as in hypertensive elderly participants (122). However, the FMD levels reached in the hypertensive elderly group did not reach the same levels as in the heathy elderly group (122). Jones et al studied 13 healthy young men, and found that a repeated local IPC stimulus (4 cycles of 5-minute occlusion followed by reperfusion, once-a-day for seven days) improved endothelial function, as measured by improved brachial artery FMD (123). The same research group also investigated the impact of eight weeks of local IPC on endothelial function in healthy young men (124). They found that local IPC three times a week, for eight weeks, improved endothelial function, as measured by improved brachial artery FMD, in this group (124).

1.5 Remote ischaemic pre-conditioning

Remote IPC was first described in an animal model in 1993 (125). It was found that pre-conditioning the circumflex artery of the heart protected the anterior descending coronary artery territory, which had not undergone any pre-conditioning, from injury following a subsequent prolonged occlusion (125). Subsequent animal studies showed that remote IPC in a variety of vascular beds (renal, mesenteric, cerebral, aortic, femoral) afforded protection against myocardial infarction (126-131). The demonstration that remote IPC could be activated via brief periods of limb ischaemia simplified the logistics of inducing remote IPC using supra-systolic blood pressure inflations on the arm or leg (119, 132, 133). In most human studies, remote IPC is much more practical to apply than local IPC. Remote IPC is commonly administered as three or four cycles of 5-minutes of ischaemia, followed by 5-minutes of reperfusion (119, 132). This is usually carried out on the upper arm or thigh (132). Remote IPC therefore refers to the phenomenon whereby transient,

brief episodes of ischaemia applied to a distant organ or tissue, protects a distant organ or tissue from subsequent prolonged period of ischaemia or ischaemic injury (119, 134-136).

The majority of human remote IPC studies initially focused on the use of remote IPC in the field of cardioprotection. These studies typically assessed the ability of remote IPC to protect against IRI caused during coronary artery bypass surgery (during which the heart is ischaemic for a period), or caused by myocardial infarction. Small clinical studies have shown cardioprotection following remote IPC (137-141), but two major clinical outcome trials failed to show cardioprotection following remote IPC in the setting of coronary artery bypass surgery (142, 143). These two large multi-centre double-blind randomised controlled trials were ERICCA (effect of remote ischemic pre-conditioning on clinical outcomes in patients undergoing coronary artery bypass graft surgery) (142) and RIPHeart (remote ischaemic pre-conditioning for heart surgery) (143). Similarly, despite initial promising data from small clinical trials, a large international single-blind randomised controlled trial investigating remote IPC was not shown to improve outcome in patients undergoing primary PCI for ST-elevation myocardial infarction (144).

In ERICCA, patients who were undergoing coronary artery bypass graft plus/minus valve surgery were randomised to either a remote IPC group or a sham group. The remote IPC group had 4 cycles of 5-minutes of ischaemia and reperfusion of the upper arm, after anaesthesia and prior to surgical incision. Remote IPC did not significantly reduce the proportion of patients with a major cardiac event, cerebral event or acute kidney injury within 72-hours; did not reduce duration of intensive care or hospital stay and did not reduce troponin-T levels (134, 142).

In RIPHeart the patient population included all types of cardiac surgery where cardiopulmonary bypass was used (143). The remote IPC protocol in RIPHeart was similar to that used in ERICCA. In RIPHeart, remote IPC did not cause a significant reduction in the primary end-point, which was a composite of death from any cause, non-fatal myocardial infarction, new

cerebrovascular events and acute renal failure. There was a non-significant trend towards a reduction in myocardial infarction (134, 143, 145). It is of note that these studies, which failed to demonstrate a benefit from remote IPC, used propofol as an anaesthetic agent. It has been proposed that propofol may interact with the neuronal transfer of the potentially protective neural signalling pathway in remote IPC (146).

In clinical studies, remote IPC has been investigated in multiple fields, with mixed findings (119, 146). Some examples of the areas where remote IPC has been studied include: cardiac surgery, percutaneous coronary intervention for acute myocardial infarction, vascular surgery, brain and neurological injury, acute kidney injury, solid organ transplant and end organ damage from diabetes mellitus, as these are all situations where a degree of IRI takes place (119, 137, 140, 146-150). Relevant to my thesis is the impact of remote IPC on blood pressure. There is some evidence that repeated remote IPC is able to reduce blood pressure. There is one study which has shown that in a normotensive man (n=1), blood pressure (systolic, diastolic and mean arterial pressure) was reduced after repeated serial episodes of a remote IPC stimulus (151). Tong et al studied 15 newly diagnosed untreated hypertensive participants, who underwent remote IPC once-a-day for one month (152). These participants had significantly lower systolic, diastolic and 24-hour blood pressure following a month of daily remote IPC (152).

Both local and remote IPC are thought to offer protection against future ischaemia in a biphasic manner (153-155). In local IPC, the first protective window of ‘enhanced endothelial function’ occurs within 4-hours of the IPC stimulus and the second occurs at 24-hours and lasts up to 72-hours (figure 1.3) (121, 153, 155, 156). This ‘second window of protection’, in local IPC, was first described by Marber et al in 1993 (155). A study by Loukogeorgakis et al, investigated this second window of protection in remote IPC (121). It was found that remote IPC protected against IRI at 24-hours and 48-hours, but did not protect at 4-hours (121), which was contrary to the previous studies carried out in local IPC. Other studies have found that this second

window of protection or ‘late phase’ can last up to 96 hours (157). However, it is not yet known when the peak of the second window of protection occurs.

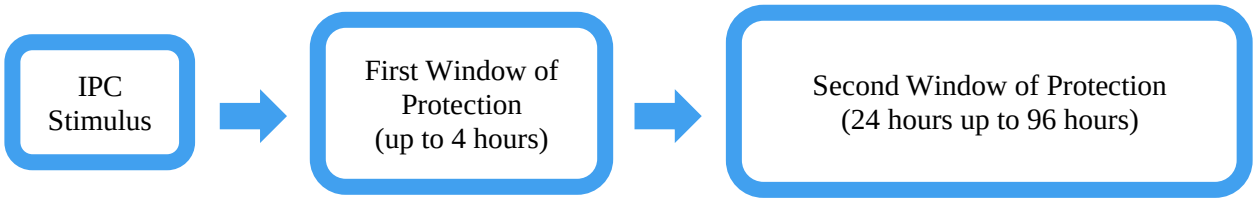


Figure 1.3: Diagram showing the two potential ‘windows of protection’ in IPC

An issue is that the results from studies investigating local and remote IPC have been variable (119, 158, 159). There has been a suggestion that some of the larger human trials may have investigated the ‘wrong’ participant group – for example in both RIPHeart and ERICCA remote IPC was applied before planned cardiac surgery, which is an environment where the heart is already well protected by conditions such as hypothermia and cardioplegia, and therefore a significant benefit may not have been conferred by remote IPC (160). Another factor may be that larger human studies investigating IPC in, may have included participants with co-morbidities such as diabetes, hypercholesterolemia or pre-existing renal impairment, or taking medications such as platelet inhibitors, which may have attenuated the efficacy of IPC (161, 162). It may also be that studies have inadvertently measured the impact of IPC between the first and second protective windows, or after the second protective window, and therefore have ‘missed’ the time of maximal effect.

1.6 Proposed mechanisms underlying ischaemic pre-conditioning

As discussed earlier in this chapter, IPC is a phenomenon whereby transient, brief episodes of ischaemia applied to an organ or tissue, protect that organ or tissue from a subsequent prolonged period of ischaemia or ischaemic injury (117). IPC can be both local (117, 118) and remote (125).

I investigated the effect of local IPC on the upper arm and measured the impact of local IPC on endothelial function using brachial artery flow-mediated

dilatation. In order to understand whether there could potentially be an impact on utero-placental blood flow I measured several potential circulating factors that have been proposed to be part of the mechanism of remote IPC.

Since the discovery of IPC, attention has focused on the incompletely understood mechanisms by which the IPC stimulus is transmitted (146, 158, 159, 163, 164). Despite over 30 years of clinical research, mainly focusing on remote IPC, the mechanism has not been elucidated (119, 132, 165, 166). However, it is agreed that the key to understanding IPC is to appreciate why the brief period of reperfusion after the period of ischaemia is so important (132, 146, 164). Some authors have postulated that all forms of ischaemic conditioning (including local IPC and remote IPC) share some common mechanistic themes (167).

With regard to local IPC, it may be that the brief episode of ischaemia followed by reperfusion causes a local increase in production of NO via NOS (168, 169). Tissue ischaemia and shear stress, which both occur with local IPC, are two stimuli for the release of endothelial progenitor cells (EPCs), which may additionally increase the local NO concentrations (170, 171).

Although the mechanism mediating remote IPC remains unclear (159, 167, 172), there are 3 potential pathways by which remote IPC could exert its mechanism of action: (a) humoral pathways, (b) systemic pathways and (c) neural pathways (135, 159, 167). There are multiple potential circulating factors/proteins that might mediate remote IPC through a humoral pathway (135, 173, 174), whereby a substance is released as a result of an IPC stimulus and is transferred to an organ or tissue to protect it (135). Plasma from animals who have undergone remote IPC has been used to transfer cardioprotection to other animals, which supports the hypothesis that there are protective factors released into the circulation following remote IPC (175-177).

The humoral pathway is the pathway that I will be investigating through measuring the circulating factors VEGF, stromal derived factor-1α (SDF-1α)

and the enzyme which cleaves the majority of active full length SDF-1α(1-67) to inactive SDF-1α(3-67), called dipeptidyl peptidase-4 (DPP-4), before and after IPC in all my study groups.

SDF-1α, is a chemokine of 10 kDa, that has been reported to be upregulated in diseases characterised by tissue hypoxia (178-180). SDF-1α, is a chemo-attractant for cells expressing its G-protein-coupled receptor, CXCR4 (178, 179). SDF-1α has been shown to have role in promoting EPCs homing and improving endothelial repair capacity (181, 182). There is evidence that proliferation of EPCs is closely linked with endothelial dysfunction in hypertensive subjects (181, 182). In pre-eclampsia, it has been found that the proliferation of EPCs is increased (183), which may be because circulating EPCs are mobilised in the peripheral blood in response to tissue ischaemia (184).

Remote IPC has been reported to increase plasma concentrations of SDF-1α (152, 179, 180). One study, mentioned previously, which investigated 15 newly diagnosed untreated hypertensive participants, who underwent remote IPC once-a-day for one month, found that plasma levels of SDF-1α increased following chronic repeated remote IPC (152). Cao et al showed that serum SDF-1α levels were higher at 24 and 48 hours in a group that had remote IPC prior to primary percutaneous coronary intervention compared to those who did not (185). However, as SDF-1α is contained in platelet granules, and can be expressed upon platelet activation, serum levels of SDF-1α may be falsely elevated in comparison with plasma levels (186). Moreover, there have been some studies that have found a reduction in SDF-1α in conditions characterised by tissue hypoxia (180, 187, 188).

SDF-1α is cleaved by several peptidases, but the majority of SDF-1α is cleaved by the peptidase dipeptidyl peptidase-4 (DPP-4). DPP4-4 cleaves active full length SDF-1α(1-67) to inactive SDF-1α(3-67) rendering it unable to bind and activate the CXCR4 receptor (179). Therefore remote IPC may lead

to a decrease in DPP-4 and therefore may cause an increase in the plasma levels of active SDF-1α(1-67) (179).

The CXCR4 receptor is also found in cytotrophoblast cells in the placenta (189-191) and therefore I am interested to discover what happens to the levels of SDF-1α and DPP-4 in both healthy pregnant women and pregnant women at risk of pre-eclampsia who undergo remote IPC.

In the pre-conditioning field, it has been reported that repeated IPC increases plasma levels of VEGF (192, 193). It may be that an increase in VEGF also contributes to increasing levels of EPCs, which results in an increase in NO availability, via NOS (192, 194).

As well as VEGF and SDF-1α, some of the other potential humoral factors involved in remote IPC include adenosine, bradykinin, calcitonin gene-related peptide, nitric oxide, opioids and HIF1-α, (119, 135, 174), however I have not investigated these other factors and they will not be discussed in this thesis.

The findings regarding VEGF, SDF-1α and DPP-4 are discussed in chapter 6.

1.7 Ischaemic pre-conditioning in pregnancy and pre-eclampsia

As discussed earlier in this chapter, it is known that women who develop pre-eclampsia have placental ischaemia and endothelial dysfunction several weeks before the clinical manifestation of the syndrome of pre-eclampsia (23, 55). This reduction in endothelial function can be measured in-vivo using brachial artery FMD.

In pre-eclampsia the placenta is relatively ischaemic, in a similar way to an ischaemic myocardium. Towards the end of a pre-eclamptic pregnancy, the placenta can become infarcted similar to a myocardial infarction (54, 195). It is not possible for a local IPC stimulus to be applied to the placenta. However, a local IPC stimulus may improve maternal endothelial function, as measured

by brachial artery FMD, and remote IPC may improve the maternal angiogeneic profile.

There are no published studies investigating local or remote IPC in pregnancy and given the relative simplicity and cost-effectiveness of IPC, and its non-invasive low-risk nature, IPC is attractive for use in pregnancy. If IPC is shown to be of benefit, its cost-effectiveness and relative simplicity make it an attractive non-pharmacological treatment to improve endothelial function in women at risk of pre-eclampsia.

1.8 Hypotheses

i. I hypothesised that an acute episode of local IPC would have a different impact on endothelial function at a time-interval of 24-hours or 48-hours in healthy non-pregnant women.

ii. I hypothesised that an acute episode of local IPC would improve endothelial function in women with pre-eclampsia.

iii. I hypothesised that an acute episode of IPC would improve the ratio between sFlt-1 and PlGF in women with pre-eclampsia.

iv. I hypothesised that VEGF, SDF-1α and DPP-4 levels would be altered following an acute episode of IPC.

1.9 Research aims and objectives

My research aims and objective were:

i. To determine if an acute episode of local IPC caused a greater enhancement of endothelial function at a time-interval of 24-hours or 48-hours in healthy non-pregnant women.

ii. To determine if an acute episode of local IPC improves endothelial function in pregnant women with pre-eclampsia.

iii. To determine if an acute episode of IPC improves the ratio between sFlt-1 and PlGF in pregnant women with pre-eclampsia.

iv. To determine if an acute episode of IPC alters VEGF levels in non-pregnant women, healthy pregnant women, women at risk of pre-eclampsia and women with pre-eclampsia.

v. To determine if SDF-1α and DPP-4 play a role in mediating changes in endothelial function following an acute episode of IPC.

CHAPTER 2

METHODOLOGY AND MATERIALS

Chapter 2 - Methodology and Materials

2.1 Introduction

In order to achieve the 4 main aims of my thesis these methodological techniques were used:

1. To identify the optimal time-interval from an acute episode local IPC to maximally enhanced endothelial function in healthy non-pregnant women I measured brachial artery FMD before and after IPC applied to the upper arm at a time-interval of 24-hours and 48-hours.

2. To identify if an acute episode of local IPC improves endothelial function in healthy pregnant women, women at risk of pre-eclampsia and with pre-eclampsia, I measured brachial artery FMD before and after an acute episode of IPC applied to the upper arm.

3. To determine whether the expression ratio of the anti-angiogenic and angiogenic factors sFlt-1 and PlGF is improved by an acute episode of IPC in women with pre-eclampsia, I analysed patient blood samples using commercial ELISA assays.

4. To determine whether VEGF, SDF-1α and DPP-4 may play a role in mediating changes in endothelial function following an acute episode of IPC, I analysed patient blood samples using commercial ELISA assays for VEGF and total SDF-1α. In addition, I used an ELISA assay developed in-house to quantify active human SDF-1α(1-67) (180). DPP-4 activity was measured using a luminescence assay.

This study was carried out in the Elizabeth Garrett Anderson (EGA) Wing of University College London Hospital (UCLH), the NIHR UCLH Clinical Research Facility (CRF) and at The Hatter Cardiovascular Institute Laboratories, UCL. The study received ethical approval entitled “An

investigation into the circulating and vascular factors that control vascular tone in pre-eclampsia and fetal growth restriction (VAMPS 1)" by the NRES Committee London - Westminster (REC reference 12/LO/1449) and the Joint UCLH/UCL Research and Development department.

2.2 Overview of study design

i. Determination of optimal study interval between local IPC and a repeat measure of endothelial function

Non-pregnant healthy women of childbearing age were studied with either a 24-hour or 48-hour study interval between an acute episode of local IPC and a repeat measure of endothelial function. This was to ascertain the optimal study interval for studying the pregnant groups. All non-pregnant women had levels of VEGF, SDF-1α and DPP-4 measured before and after IPC.

ii. Investigation of healthy pregnant women, with no risk factors for pre-eclampsia

Healthy pregnant women were studied between 24+0 and 36+6 weeks gestation to determine whether an acute episode of local IPC alters maternal endothelial function. Healthy pregnant women had levels of sFlt-1, PlGF, VEGF, SDF-1α and DPP-4 measured before and after an acute episode of IPC.

iii. Investigation of women at risk of pre-eclampsia

Women at risk of pre-eclampsia were studied between 24+0 and 36+6 weeks gestation to determine whether an acute episode of local IPC alters maternal endothelial function. Women at risk of pre-eclampsia had levels of sFlt-1, PlGF, VEGF, SDF-1α and DPP-4 measured before and after an acute episode of IPC.

iv. Investigation of women with pre-eclampsia

Women with pre-eclampsia were studied between 24+0 and 36+6 weeks gestation to determine whether an acute episode of local IPC alters maternal endothelial function. Women with pre-eclampsia had levels of sFlt-1, PlGF, VEGF, SDF-1α and DPP4 measured before and after an acute episode of IPC.

The protocols used in groups ii, iii and iv were identical.

2.3 Sample Size Calculation

Sample size calculations were carried out in collaboration with Mr Zacharias Anastasiou, a medical statistician, at the department for statistical science at University College London. These calculations were based on data I collected from 9 healthy pregnant women and 7 pregnant women at risk of pre-eclampsia who underwent the IPC protocol (described later on in this chapter). The primary outcome was brachial artery FMD, measured pre- and post- local IPC. The sample size calculation showed that, to detect a difference of 1.5% change in brachial artery FMD, 35 participants per group would need to be included in the study, with a statistical power (α) of 0.8, and 5% chance of type I error (table 2.1).

Difference in means between pregnant cases and controls (% change in brachial artery FMD)	Standard deviation	Type 1 error	Power	Correlation	Sample size in each group
0.98	3.1	0.05	0.8	0.7	81
1.0	3.1	0.05	0.8	0.7	77
1.2	3.1	0.05	0.8	0.7	54
1.5	3.1	0.05	0.8	0.7	35

Table 2.1: Sample size calculations based on pilot data

Recruiting for this study was challenging, due to the visits required on two consecutive days for participants, who needed to be fasted for at least 4 hours prior to the study and may already have had several other appointments regarding their pregnancy. Therefore, I recruited a slightly higher number of healthy pregnant control women (n = 42) as I was aware it may not be possible to recruit 35 women at risk of pre-eclampsia and with pre-eclampsia within the study time-frame.

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7 and Stata version 16.

Simple descriptive statistics were used to compare the study participants. Characteristics of study participants, such as age and BMI, have been summarised using means and standard deviations.

For the brachial artery FMD, statistical analysis was carried out in collaboration with Dr Aviva Petrie, Honorary Associate Professor in Biostatistics, based at the UCL Eastman Dental Institute. Initially, a multi-level mixed model regression analysis was carried out. This analysis was examined for variance and normality; these assumptions were not satisfied, and in some cases there was a significant interaction between the groups. Therefore, this was not the appropriate analysis to use for these samples. As such, and to ensure consistency, all statistical comparisons tests were carried out using non-parametric analyses, due to normality and variance assumptions not being satisfied in all data sets.

When more than two groups were being analysed, a 1-way ANOVA was carried out, followed by the Kruskal-Wallis Test and Dunn's multiple comparisons test was done to look at the differences between the groups. The 1-way ANOVA approach used did not account for repeated measures. When comparisons were needed between two groups, without pairing, the Mann-Whitney Test was used. For paired comparisons within groups,

Wilcoxon matched-pairs signed rank test was used. No adjustment for multiple testing was applied to either the Mann-Whitney Test or the Wilcoxon matched-pairs signed rank test. Correlation analysis was carried out using Pearson Correlation Coefficient. Data were presented as mean and standard deviation (SD) where appropriate.

Data from the assays of sFlt-1, PlGF, VEGF, SDF-1α and DPP-4 were analysed using GraphPad Prism version 7. Where two groups were compared, a paired t-test with Bonferroni correction was used. More than two groups were compared using a 1-way ANOVA, followed by the Kruskal-Wallis Test, when looking at differences between the groups. If a difference was found, then a Dunn's multiple comparison test was carried out to determine significance. The 1-way ANOVA approach used did not account for repeated measures. Data were presented as mean and standard deviation (SD) where appropriate.

A statistically significant result was determined with a p-value ≤ 0.05.

2.5 Recruitment Criteria and Participants

Four groups of women were recruited to the study, as follows:

- Non-pregnant healthy women of childbearing age (n = 24)
- Healthy pregnant women, with no risk factors for pre-eclampsia (n = 42)
- Pregnant women, with risk factors for pre-eclampsia (n = 20)
- Pregnant women, with pre-eclampsia (n = 10)

Approximately 60% of pregnant women approached declined to participate. The common reasons being: unable to commit to the two visits on

consecutive days; concern about ‘discomfort’ from the IPC stimulus; declining additional venesection.

Inclusion and exclusion criteria for study groups were defined. There were slightly different criteria for groups iii and iv, due to women at risk of pre-eclampsia and with pre-eclampsia having a range of phenotypes, including higher BMI and pregnancies at more advanced maternal age.

Inclusion Criteria

i. Non-pregnant healthy women of childbearing age (n = 24)
   - Non-pregnant women
   - Age range of 18-45 years old
   - Non-smokers
   - No previous pregnancy related complications (such as pre-eclampsia, pregnancy induced hypertension, gestational diabetes)
   - No significant medical problems
   - BMI range from 19.5-30 kg/m^2

ii. Healthy pregnant women, with no risk factors for pre-eclampsia (n = 42)
   - Healthy pregnant women with no significant medical problems
   - Age range of 18-45 years old
   - Non-smokers
   - Singleton pregnancy
   - BMI in first trimester range from 19.5-30 kg/m^2
   - Gestation at time of study 24+0 to 36+6 weeks gestation

iii. Pregnant women, with risk factors for pre-eclampsia (n = 20)
   - Age range of 18-49 years old
   - Non-smokers
   - Singleton pregnancy

- Previous history of pre-eclampsia and/or pre-existing hypertension
- BMI in first trimester range from 19.5-35 kg/m^2
- Gestation at time of study 24+0 to 36+6 weeks gestation
- Taking Aspirin for the prevention of pre-eclampsia

iv. Pregnant women, with pre-eclampsia (n = 10)

- Age range of 18-49 years old
- Non-smokers
- Singleton pregnancy
- BMI at in the first trimester range from 19.5-35 kg/m^2
- Gestation at time of study 24+0 to 36+6 weeks gestation
- Not previously studied in any other group

Exclusion criteria

- Multiple pregnancy
- Diabetes/Gestational diabetes
- Severe anaemia
- Known thrombophilia
- Thrombosis
- Polycystic ovarian syndrome
- Poorly controlled thyroid disease
- Pre-existing cardiac disease

2.6 Experimental Design and Study Protocol

This was a clinical study, which investigated the impact of IPC in non-pregnant women, healthy pregnant women, pregnant women at risk of pre-eclampsia and pregnant women with pre-eclampsia.

2.6.1 Study Participants

Non-pregnant healthy women of childbearing age were recruited through personal contacts and expressions of interest from members of the wider clinical research network at UCLH. All participants were given a participant information sheet (see appendix 1) and had the opportunity to ask questions about the study before giving informed consent (see appendix 3).

Pregnant women were recruited from midwifery clinics, doctor-led antenatal clinics or the antenatal ward at the EGA Wing of UCLH. I also advertised the study with posters located in the EGA Wing of UCLH.

I reviewed the medical records of interested women to ensure they met the criteria for the study. I provided all potential participants with a participant information sheet (see appendices 1 and 2) and answered questions about the study in a face to face meeting. Some women also emailed me about the study, and I answered questions about the study and provided a participation information sheet via email.

Women agreeable to participation in the study provided written informed consent (see appendix 4) prior to taking part in the study and has the opportunity to ask questions prior to the study on each day they attended. There were five women who only consented to the assessment of their endothelial function pre and post IPC, as they were ‘needle phobic’ and did not wish to have venesection carried out. Three of these women were in the healthy pregnant group, one in the at risk of pre-eclampsia group and one in the pre-eclampsia group.

2.6.2 Ischaemic pre-conditioning protocol

IPC in non-pregnant healthy women of childbearing age (n = 24)

Participants were allocated to either a 24-hour study interval group (n = 14) or a 48-hour study interval group (n = 10). There were no significant differences between the groups in age and BMI (see chapter 3).

It is known that stimulants, caffeine, alcohol and exercise can all impact FMD (95). Therefore, all participants had fasted for at least 4 hours, had not ingested any caffeine or alcohol for at least 12 hours, and had not carried out any strenuous exercise in the previous 24 hours in order to minimise the effect of these confounding factors.

All studies were performed in a temperature controlled room.

The protocol carried out was as follows:

1. **10 ml mid-stream urine sample**

2. **20 ml venesection:**
   2 x serum separating tube samples
   2 x K2 ethylenediaminetetraacetic acid (EDTA) tube samples
   2 x sodium citrate tube samples

3. **Measure of blood pressure, mean arterial pressure and heart rate (using an aneroid sphygmomanometer, with the participant in a sitting positon)**

4. **An assessment of brachial artery FMD**

Measurements of Endothelial Function using FMD

I carried out all FMD assessments, using previously reported established protocols, which have previously been used by members of my research group, who trained me in the technique of brachial artery FMD (196, 197).

The right arm was rested in an arm holder. A 6cm wide pneumatic blood pressure cuff (Hokanson SC5 tourniquet cuff, PMS Instruments Ltd, Maidenhead, UK) was placed around the upper forearm, 2 cm distal to the medial epicondyle. Cuff inflation was controlled by an automatic cuff inflator (Hokanson Cuff Inflator, PMS Instruments Ltd, Maidenhead, UK) (see figures 2.1, 2.2 and 2.3).

Using an Aloka SSD 5000 ultrasound machine (Aloka Holding Europe, AG Switzerland) with a 13-MHx linear array transducer probe, a segment of the brachial artery was identified in the longitudinal plane, and studied before and after 5 minutes of compressive arterial occlusion.

The image was acquired in B-mode and the probe was fixed so that a 5-10 cm segment of the brachial artery, proximal to the antecubital fossa was in view.

Longitudinal end-diastolic images, in conjunction with electrocardiogram tracing, were acquired every 3 seconds during the 11-minute recording. During an 11-minute recording there was 1 minute of baseline measurements, 5 minutes with the cuff inflated to 300 mmHg and 5 minutes with the cuff deflated, resulting in a brief episode of reactive hyperaemia (see figures 2.4 and 2.5).

Blood flow velocity was monitored continuously during the examination by switching to a B/D (Doppler) mode for the duration of the test. Images were acquired and analysed using automated software (Brachial Tools Medical Imaging Applications, IA, USA). With the initial segment recorded, a smaller region with maximum clarity was selected for analysis.

FMD was calculated as a percentage change from baseline brachial artery diameter (mm) to maximum dilation (mm) after reperfusion, using semi-automated continuous capture software. The Brachial Tools Medical Imaging Applications software also provided data on the time to peak dilatation for each study.

FMD is negatively correlated with baseline arterial diameter (98, 198). In order to adjust for artery diameter, a measure of FMD can be allometrically scaled (199-201). Allometric scaling uses statistical models based on principles from Albrecht at al (202). Such an adjustment will avoid for example, exaggerating the measure of FMD in a small compared with large artery, as it has been suggested that the peak diameter does not always increase as a constant proportion of baseline diameter (199).

One study investigated whether allometric scaling was necessary (203). This study assessed FMD in 18 young adults and 17 older adults (205). They showed that FMD remained similarly greater in a group of young compared with older adults despite being allometrically scaled (203).

In the brachial artery FMD studies I carried out, the brachial artery baseline diameter was measured in the studies before and after the acute IPC stimulus. As can been seen in chapters 3 and 4 there was no statistically significant difference between the baseline brachial artery diameter before and after an acute episode of local IPC. In this work the main comparisons made, with regard to brachial artery FMD, are within each group. Additionally, allometric scaling has not been used in any of the published work on FMD in pregnancy. Therefore, in this thesis, I am not including allometrically scaled FMD data.

Figure 2.1: A study participant having a brachial artery FMD study. This was her post-IPC visit, as one can see the image from the pre-IPC FMD study is to the left of the screen.

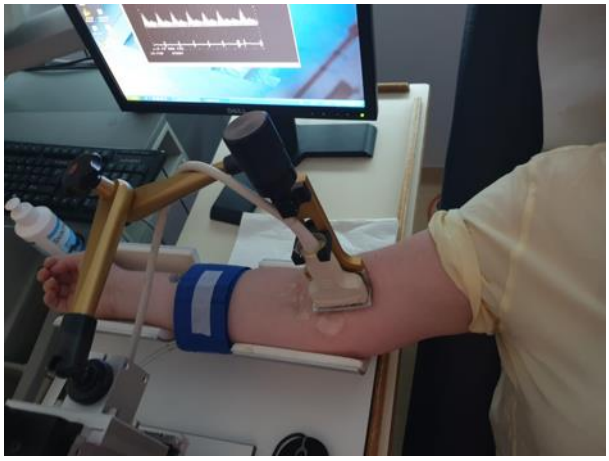


Figure 2.2: Photograph showing the probe and cuff placement for a brachial artery FMD study

Figure 2.3: Photograph showing TK undertaking a brachial artery FMD study

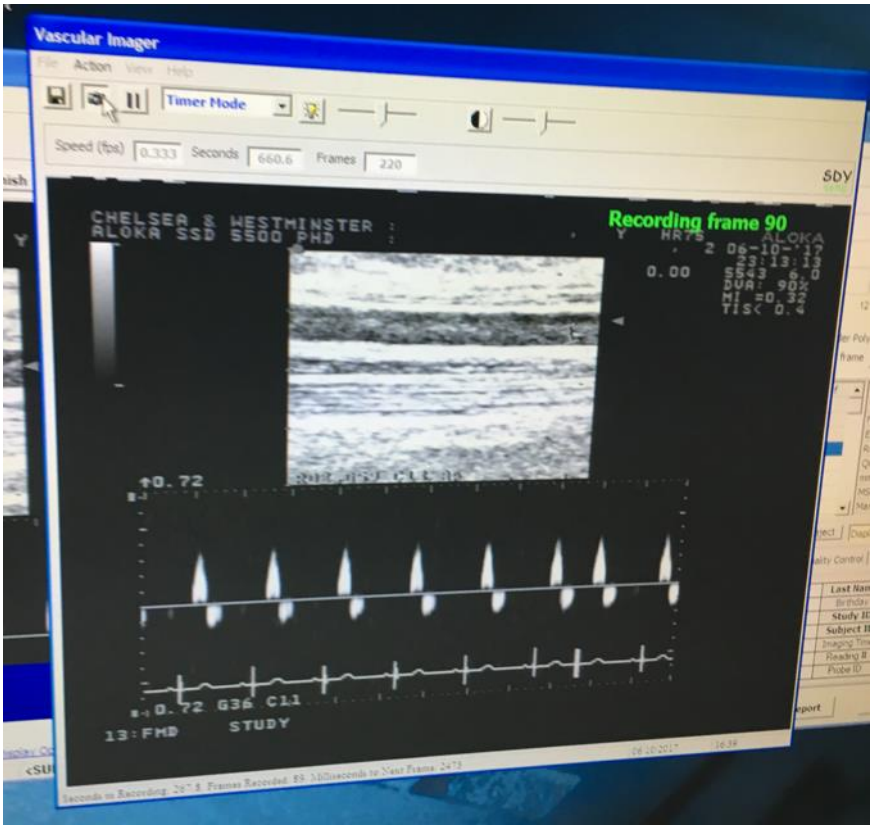


Figure 2.4: An image of a brachial artery during cuff inflation – prior to blood flow stimulus

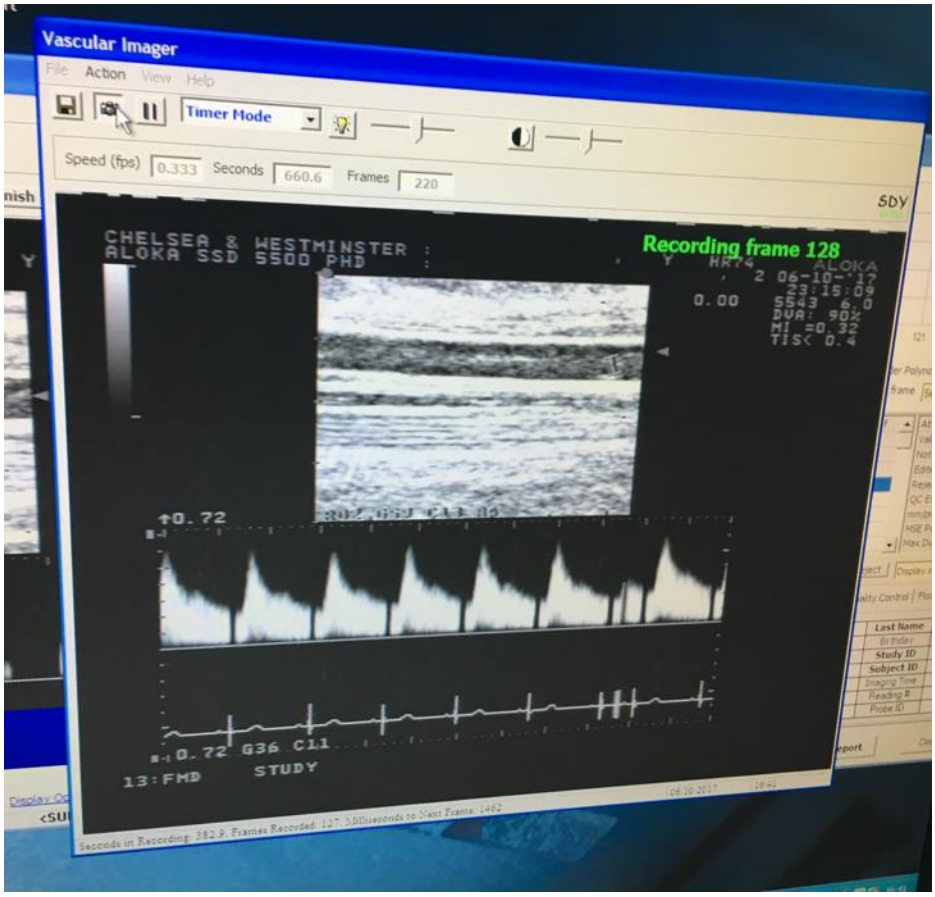


Figure 2.5: An image of the same brachial artery as in figure 2.4, following cuff deflation. The brachial artery has dilated, following the blood flow stimulus.

I was trained to carry out brachial artery FMD during my Academic Clinical Fellowship (2011-2014) at the University College London Institute for Women's Health. I was trained at the UCLH CRF, which was (at that time) located on the ground floor of the EGA Wing of UCLH. I was trained by Mr William Jenner a MB PhD candidate, Dr Kristin Veighey a nephrology registrar and PhD candidate and Dr Sara Hillman an obstetrics and gynaecology registrar and PhD candidate - all were subsequently awarded their PhDs, which involved the technique of brachial artery FMD. I had a blinded assessment to confirm that my brachial artery FMD was valid and reproducible and later carried out some brachial artery FMD studies to assist Dr Sara Hillman with her maternal medicine research.

5. An acute episode of IPC

IPC was induced by inflating a 13 cm wide blood pressure cuff (Hokanson SC12 straight segmental, PMS Instruments Ltd, Maidenhead, UK) on the

upper part of the right arm (see figure 2.6). The cuff was inflated to 200 mmHg using a Hokanson E20 rapid cuff inflator (PMS Instruments Ltd, Maidenhead, UK) for 5 minutes, and then followed by 5 minutes of deflation. The inflation/deflation cycle was performed three times.

Figure 2.6: A study participant undergoing an episode of local IPC

6. **A questionnaire was completed**

Information on menstrual cycle, previous pregnancy history, past medical and family history, current medication, most recent food, caffeine and exercise were gathered (see appendix 5).

7. **A measure of weight and height was taken and BMI calculated**

Twenty-four or forty-eight hours later steps 1 to 4 were repeated.

IPC in healthy pregnant women with no risk factors for pre-eclampsia (n = 42)

IPC in pregnant women at risk of pre-eclampsia (n = 20)

IPC in pregnant women with pre-eclampsia (n = 10)

The protocol used for the other groups (specified above) was identical to that of the non-pregnant healthy women of childbearing age, apart from all participants were studied with a 24-hour study interval between the first and repeat measure of FMD.

In the pregnant study groups, a questionnaire was completed with questions asked about previous pregnancy history, past medical and family history, current medication, most recent food, caffeine and exercise. Maternal antenatal paper notes and/or electronic records were reviewed to provide pertinent data. This included BMI at the beginning of pregnancy, estimated date of delivery from 11 to 13-week ultrasound scan, information about previous pregnancies and previous pregnancy complications (see appendix 6).

2.6.3 Biological Samples

Urine samples were aliquoted into 4 x 2ml Eppendorf tubes and stored in a -80°C freezer.

The serum and EDTA samples were centrifuged at 3000 rpm, for 10 minutes, at 4°C and then the serum and plasma samples aliquoted into 2ml Eppendorf tubes and stored in a -80°C freezer.

The sodium citrate tube samples were centrifuged at 1600 g for 20 minutes at room temperature. Then the plasma supernatant was transferred to 1.5 ml Eppendorf tubes and centrifuged at 10,000 g for 30 minutes, at room temperature. The platelet poor plasma supernatant was then transferred into 2ml Eppendorf tubes and stored in a -80 °C freezer.

At the CRF and The Hatter Cardiovascular Institute I processed, stored and disposed of blood and urine in accordance with all applicable legal and regulatory requirements, including the Human Tissue Act 2004 and any subsequent amendments.

sFlt-1, PlGF, VEGF and SDF-1α

Human sFlt-1, human PlGF, human VEGF and total human SDF-1α levels were measured using commercially available quantitative sandwich ELISA kits from R+D systems. All ELISA studies were carried out in duplicate according to manufacturer's instructions (204-207).

Human VEGF and total human SDF-1α levels were measured in the plasma of the participants in all the groups studied. Human sFlt-1 and human PlGF levels were measured in the plasma of the participants in the pregnant groups.

The R+D Systems Quantikine ELISA kit protocols were similar for measuring human sFlt-1, human PlGF, human VEGF and total human SDF-1α levels (204-207).

R+D ELISA Protocol

All reagents were brought to room temperature. The following were prepared, as per assay instructions: Wash Buffer, Substrate Solution, Calibrator Diluent, Human [VEGF R1/PlGF/VEGF/SDF-1α] Standard. Then 100 μL of Assay Diluent was added to each well. Then 100 μL of standard or sample was added to each well and each plate was covered with an adhesive strip. Plates were then incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm*. Each well was then aspirated and washed and this step was repeat three times, for a total of four washes. After the last wash the plates were inverted and blotted against clean paper towels. 200 μL of Human [VEGF R1/PlGF/VEGF/SDF-1α] Conjugate was then added to each well and each plate was covered with a new adhesive strip. Plates were then incubated for 2 hours at room temperature on the microplate shaker*. The aspiration/wash steps described above were then repeated. 200 μL of Substrate Solution was then added to each well and the plates were

incubated for 30 minutes at room temperature on the bench-top, protected from light. 50 μL of Stop Solution was then added to each well and the colour in the wells then changed from blue to yellow. The optical density of each well was then determined within 30 minutes, using a microplate reader set to 450 nm.

(*For VEGF and PlGF – both 2-hour incubation periods occurred on the benchtop, not on the microplate shaker)

These ELISAs were analysed using a BMG Labtech Fluostar plate reader to measure the absorbance at 450 nm. For each ELISA assay, the absorbance of a 'blank' sample was subtracted from all the readings, and the samples were compared against a standard curve of purified recombinant protein to obtain the actual concentration in the sample. Each sample was assayed in duplicate and the mean value obtained and used as the final value.

Active SDF-1α(1-67)

Active human SDF-1α(1-67) was measured in the platelet poor plasma of the participants in all the groups studied. This ELISA is not commercially available (180). This ELISA was characterised using rh-SDF-1α taken from the R&D Systems Human CXCL12/SDF-1 alpha Quantikine ELISA Kit.

The protocol is used to assay active SDF-1α(1-67) using HCI.SDF-1α primary antibody.

Active human SDF-1α(1-67) Protocol

Wells were coated with 100 µl 5 µg/ml HCI.SDF-1α in 0.2M anhydrous sodium carbonate-sodium bicarbonate buffer at 4ºC. The wells were then washed three times for 5 minutes with 0.05% PBS-T and then blocked with 5% BSA/PBS-T and left for 1-hour at room temperature. The wells were then washed three times, for 5 minutes, using 0.05% PBS-T. 100 µl of sample was

then added to each well. Standard samples were then prepared using known concentrations of rh-SDF-1α, starting at 10,000 pg/ml with two-fold dilution steps (range 156-10,000 pg/ml) in BUF037A. The plates were then incubated for 2-hours at room temperature. The wells were washed three times, for 5 minutes, with 0.05% PBS-T. 0.4 µg/ml BAF310, diluted in Hispec buffer, was then added to each well. The plates were then incubated for a further 1-hour at room temperature. Then the plates were washed three times, for 5 minutes, with 0.05% PBS-T. The plates were then incubated with 1:200 streptavidin-HRP in Hispec buffer for 20 minutes, in the dark, at room temperature. The wells were washed five times, for 5 minutes, with 0.05% PBS-T. Then 100 µl of Substrate Solution was added to each well. The plates were then incubated for a further 20 minutes, in the dark, at room temperature. Without further washes, 50 µl of 2N sulfuric acid was then added to each well. The plates were then read immediately, using a BMG Labtech Fluostar plate reader microplate reader set to an absorbance of 450 nm and 20 flashes per well. All standards and samples are measured in triplicate, and the mean value was obtained and used as the final value. Standards and sample values were baseline corrected. Averaging and baseline correcting was done in Excel, and analysis performed in GraphPad Prism.

DPP-4

DPP-4 activity was measured in the serum of participants in all groups using a commercially available protease luminescence assay from Promega (DPPIV-Glo Protease Assay)(208). After initial pilot work on the standard curve for this assay the optimal dilution of blood was found to be 1:200. This dilution was used to assay blood samples in duplicate.

DPPIV-Glo Protease Assay Protocol

50 ml of Buffer was prepared, following the assay instructions, and then the DPPIV-Glo Buffer was thawed to room temperature. The Luciferin Detection Reagent was then equilibrated to room temperature. The DPPIV-Glo Substrate was then resuspended by adding 110 µl of ultrapure water to

substrate vial and this was mixed by vortexing briefly.10 mM substrate stock was then made. The Luciferin Detection Reagent was then reconstituted by adding the DPPIV-Glo Buffer. The DPPIV-Glo Reagent was then prepared, following the assay instructions. The DPPIV-Glo Substrate was then added to the resuspended Luciferin Detection Reagent. 100 μl of the DPPIV-Glo Substrate was then added to 50 ml of the Luciferin Detection Reagent. The DPPIV-Glo Reagent was then kept at room temperature for 30-60 minutes (to allow removal of any contaminating free aminoluciferin). 50 μl of the DPPIV-Glo Reagent was then added to each well, and then 50 μl of standard or serum sample was added to each well. The contents of the wells were gently mixed using a plate shaker set to 300-500 rpm for 30 seconds. The plates were then covered with foil and incubated at room temperature for 30 minutes. Each sample was assayed in duplicate and the mean value obtained and used as the final value. Luminescence was then recorded using a BMG Labtech Fluostar plate reader.

The following experimental chapters will investigate if IPC improves endothelial function in healthy pregnant women, pregnant women at risk of pre-eclampsia and women with pre-eclampsia. They will also investigate whether IPC alters sFlt-1 and PlGF levels in these three groups of pregnant women, and whether IPC alters the levels of VEGF, SDF-1α and DPP-4 in these three groups, as well as in healthy non-pregnant women of childbearing age.

CHAPTER 3

DETERMINING THE OPTIMAL TIME-INTERVAL FROM LOCAL ISCHAEMIC PRE-CONDITIONING TO MAXIMALLY ENHANCED ENDOTHELIAL FUNCTION IN HEALTHY NON-PREGNANT WOMEN

Chapter 3 – Determining the optimal time-interval from local ischaemic pre-conditioning to maximally enhanced endothelial function in healthy non-pregnant women

3.1. Background

In healthy non-pregnant individuals and those with hypertension, local IPC has been found to improve endothelial function, as measured by brachial artery FMD (122-124, 209). The main aim of my thesis is to investigate whether local IPC improves endothelial function in pregnant women with pre-eclampsia.

No studies have previously investigated the effect of local IPC in pregnancy, or in healthy young women, it was therefore important to discover the optimal time-interval from local IPC to maximally enhanced brachial artery FMD. It is already known that there are sex-specific differences in FMD, with females having higher FMD levels than males (104, 105, 108), which is thought to be related to differences in sex-hormones (210-212) . Therefore, extrapolating from this, there may be different vascular responses following local IPC in females when compared to males. Additionally, an optimal study time-interval needed to be decided upon, as I was aware that pregnant women, especially those with pre-eclampsia, may be unable to commit to multiple studies within a short time-frame.

In this study, I investigated the impact of local IPC on brachial artery FMD. This involved assessing the change in FMD in the same arm that had the IPC stimulus. I planned to apply the time-interval following IPC that produced the greatest increase in FMD in non-pregnant women to my later studies of pregnant women.

Unlike most published studies, my study of healthy non-pregnant women did not include a cohort with pathological ischaemia, such as myocardial

ischaemia. Healthy women do however have the potential for ‘enhanced endothelial function’ following IPC (122, 124).

As discussed in chapter 1, both local and remote IPC are thought to offer protection against future ischaemia in a biphasic manner (153-155). In local IPC, the first protective window of ‘enhanced endothelial function’ occurs within 4-hours of the IPC stimulus and the second occurs at 24-hours and lasts up to 96-hours (121, 153, 155, 157). The second window of protection in IPC shares many common signalling pathways with the first window of protection (213, 214), however it is thought that the second window requires the synthesis of new proteins, including inducible NOS and cyclooxygenase-2 (213, 215). The synthesis of new proteins requires a ‘trigger’ and it is thought that endogenous NO and ROS are important triggers, as they then ‘recruit’ mediators, such as protein kinase C, which activate transcription factors resulting in the synthesis of proteins such as inducible NOS and cyclooxygenase-2 (213, 215). Despite the fact that the second window of protection was first described in 1993 (155), the precise timing of the ‘peak’ of the second window of ‘enhanced endothelial function’ in humans is unknown, and may differ depending on the method and duration of the IPC stimulus.

In human studies, where IPC has been administered to protect against IRI, IPC has been administered as either three or four cycles of 5-minutes of ischaemia, followed by 5-minutes of reperfusion (119, 132, 216). However, there is currently no evidence that one protocol is superior to another. In this study I used three cycles of 5-minutes of ischaemia, followed by 5-minutes of reperfusion. Since the women being studied were having brachial artery FMD carried out to assess their endothelial function prior to the acute episode of IPC it was felt that three cycles (30 minutes total application time) would be more acceptable to the participants than four cycles (40 minutes total) and help with recruitment.

In this experimental chapter I investigate the hypothesis that local IPC would have a different impact on endothelial function at a time interval of 24-hours or 48-hours in healthy non-pregnant women. I was interested in investigating this

as the precise timing of the ‘peak’ of the second window of ‘enhanced endothelial function’ in humans is unknown. I did not choose a time interval of less than 24-hours as it was felt this would be too much for pregnant women to endure in one day.

I hypothesised that an acute episode of local IPC would have a different impact on endothelial function at a time-interval of 24-hours or 48-hours in healthy non-pregnant women.

3.2 Study Design

The study started with a baseline measure of brachial artery FMD followed by a further measure of FMD either 24 hours or 48 hours after an acute episode of local IPC. This study took place in the Fetal Medicine Unit, in the EGA Wing of UCLH between January and July 2017.

3.3 Study Participants

All study participants were non-pregnant healthy women of childbearing age (n = 24). Participants were recruited through personal contacts and expressions of interest from members of the wider clinical research network at UCLH. They were allocated to either a 24-hour study interval group (n = 14) or a 48-hour study interval group (n = 10). Inclusion and exclusion criteria can be found in chapter 2.

3.4 Study Protocol

All participants had fasted for at least 4 hours prior to the study, had not ingested caffeine or alcohol for at least 12 hours, and had not carried out any strenuous exercise in the previous 24 hours. All studies were performed in a temperature-controlled room.

Once the participant had arrived in the clinical study room, a consent form was completed (see appendix 3). A 10 ml mid-stream urine sample was taken

followed by a 20 ml blood sample from the left antecubital fossa. A measure of blood pressure, mean arterial pressure and heart rate was then taken, followed by a measurement of endothelial function using brachial artery FMD of the right arm. Then the participant underwent an acute episode of IPC of the right arm, by inflating the arm cuff to 200 mmHg for 5-minutes followed by deflation for 5-minutes, repeated 3 times. A questionnaire was completed with questions on: menstrual cycle history, previous pregnancy history, past medical and family history, current medication, most recent food, caffeine and exercise (see appendix 5). The participant's BMI was calculated from measures of weight and height. Twenty-four or 48-hours later, a further blood sample was taken and blood pressure and FMD were measured again. IPC was not repeated.

A detailed study protocol is in chapter 2.

3.5 Statistical Analysis

Statistical analysis was carried out using Stata 16 and GraphPad Prism 7.

For consistency, statistical comparisons were carried out using non-parametric analyses, due to normality and variance assumptions not being satisfied in all data sets. Wilcoxon matched-pairs signed rank test for paired comparisons within the two groups. When comparisons were needed between the two groups, without pairing, the Mann-Whitney Test was used. Data are presented as mean and standard deviation (SD) where appropriate.

3.6 Results

Participant characteristics in the 24-hour interval group and 48-hour interval group are shown in table 3.1 and baseline blood pressure measurements and those following IPC are shown in table 3.2.

	24-hour (n = 14) Mean (SD)	48-hour (n = 10) Mean (SD)	p value
Age (years)	33 (4.5)	32 (3.3)	0.51
BMI (kg/m^2)	24.8 (3.7)	23.8 (3.6)	0.33

Table 3.1: Age and BMI of participants in 24-hour and 48-hour interval group. Data are presented as mean and SD. Statistical comparisons were carried out using the Mann-Whitney Test. There was no significant difference between the groups.

	Blood pressure (mmHg) Mean (SD)			
	24-hour (n = 14)		48-hour (n = 10)	
	Pre IPC	Post IPC	Pre IPC	Post IPC
Systolic	114 (12)	118 (10)	113 (14)	112 (13)
p value	0.19		0.94	
Diastolic	70 (9)	70 (10)	68 (10)	66 (9)
p value	0.73		0.08	
MAP	84 (13)	86 (9)	82 (11)	80 (8)
p value	0.22		0.39	

Table 3.2: Comparison of mean systolic blood pressure, mean diastolic blood pressure and mean arterial pressure pre and post IPC in non-pregnant women studied at 24 and 48-hour study intervals. Data are presented as mean and SD. Statistical comparisons were carried out using Wilcoxon matched-pairs signed rank test. There were no significant differences in any blood pressure domain before and after IPC in either group.

Brachial Artery FMD

Figure 3.1 shows that in the 24-hour group (n=14) mean brachial artery FMD was 8.5 % (SD 3.3) before local IPC and 10.7% (SD 4.1) after local IPC (p =

0.035*). In the 48-hour group (n=10) mean brachial artery FMD was 8.0 % (SD 2.2) before local IPC and 8.4% (SD 4.5) after local IPC (p = 0.92).

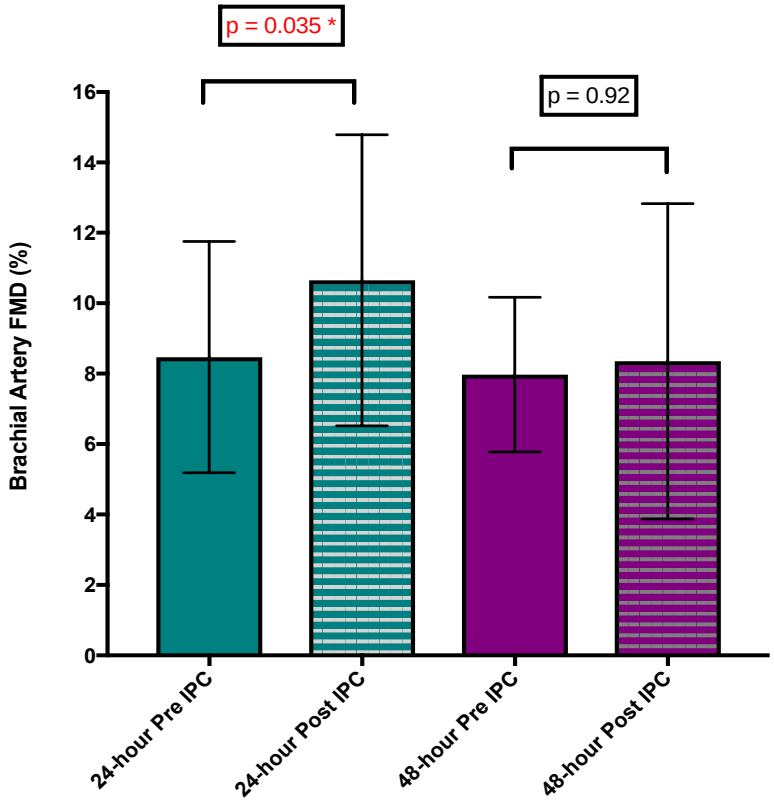


Figure 3.1: Mean brachial artery FMD (%) measured in participants before and either 24 or 48-hours after local IPC. Data are presented as mean and SD. Statistical comparisons were carried out using Wilcoxon matched-pairs signed rank test. Brachial artery FMD increased significantly 24-hours after local IPC but was unchanged after 48-hours.

Brachial Artery Baseline Diameter

As shown in table 3.3, there was no significant difference in mean brachial artery baseline diameter before and after local IPC in either group.

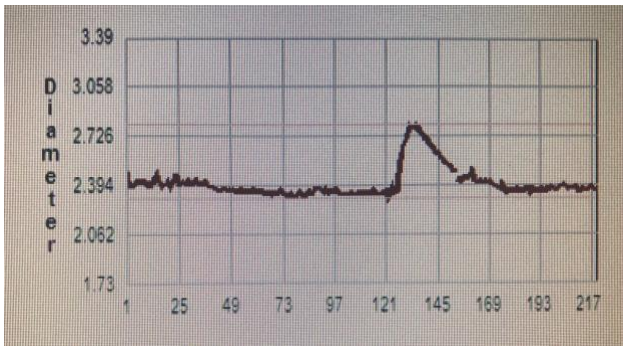
Brachial artery baseline diameter (mm) Mean (SD)			
24-hour group (n=14)		48-hour group (n=10)	
Pre IPC	Post IPC	Pre IPC	Post IPC
2.30 (0.30)	2.30 (0.29)	2.10 (0.26)	2.15 (0.24)
p = 0.33		p = 0.89	

Table 3.3**:** A comparison of mean baseline brachial artery diameter (mm) in the 24-hour group and the 48-hour group. Data are presented as mean and SD. Statistical comparisons were carried out using Wilcoxon matched-pairs signed rank test. There was no significant difference in mean brachial artery baseline diameter before and after local IPC in either group.

Time to peak brachial artery dilatation

Figure 3.2 shows the analysis of brachial artery dilatation during a brachial artery FMD study.

Figure 3.2: Analysis of brachial artery dilatation during an FMD study. The **'time to peak'** is the time from the start of the FMD study to peak dilatation.



When the time to peak brachial artery dilatation was analysed in the 2 non-pregnant groups, there was no significant difference in either the 24-hour or 48-hour group (table 3.4).

Time to peak brachial artery dilatation (s) Mean (SD)			
24-hour group (n=14)		48-hour group (n=10)	
Pre IPC	Post IPC	Pre IPC	Post IPC
424 (31)	426 (35)	425 (38)	423 (32)
p = 0.67		p = 0.34	

Table 3.4: Time to peak brachial artery dilatation (s) in the 24-hour group and the 48-hour group. Data are presented as mean and SD. Statistical comparisons were carried out using Wilcoxon matched-pairs signed rank test. There was no significant difference in time to peak before and after local IPC in either group.

Phase of Menstrual Cycle

I analysed brachial artery FMD before and after local IPC, in all participants according to the phase of their menstrual cycle (table 3.5).

Difference in brachial artery FMD before and after IPC (%) Mean (SD)			
Follicular Phase	Luteal Phase	Follicular Phase	Luteal Phase
24-hour group (n=6)	24-hour group (n=8)	48-hour group (n=4)	48-hour group (n=6)
2.6 (3.9)	1.8 (3.4)	1.3 (5.8)	1.0 (2.2)
p = 0.75		p = 0.76	

Table 3.5: The difference in brachial artery FMD before and after IPC, according to their menstrual cycle phase (follicular phase or luteal phase). Data are presented as mean and SD. Statistical comparisons were carried out using the Mann Whitney Test. There was no significant change in the mean difference in FMD before and after local IPC in any of the groups when analysed according to the phase of their menstrual cycle.

3.7 Discussion

In this chapter, I showed that local IPC significantly increased brachial artery FMD after 24-hours but not after 48-hours.

The second window of protection in IPC shares many common signalling pathways with the first window of protection (213, 214), however as discussed earlier, it is thought that the second window requires the synthesis of new proteins, including inducible NOS and cyclooxygenase-2 (213, 215).

It is possible that 24-hours is the peak of the second window of endothelial enhancement. Interestingly some of the early animal studies which investigated the second window of protection used 24-hours as their time interval of interest rather than 48-hours (155, 156, 217). It is possible that the de novo protein synthesis of mediators involved in this second window have a peak at 24-hours, and then some of these proteins gradually decay during the following 24 to 48 hours (214, 218)

It is interesting that in this group of healthy women with healthy endothelium, there was an improvement in endothelial function with only one acute episode of local IPC. Other studies that have shown an improvement in endothelial function when investigating groups of healthy young participants, have only shown this following repeated episodes of local and remote IPC (123, 124). There are a few studies where local and remote IPC has improved endothelial function, as measured by improved brachial artery FMD, in healthy young men and women, as well as in people with underlying endothelial dysfunction (122, 219), but never exclusively in a group of healthy young women. Based on my results, I chose to study pregnant women 24 hours, not 48 hours after local IPC.

Changes in FMD following local IPC were not due to differences in participant characteristics (table 3.1). The mean systolic blood pressure, diastolic blood pressure and mean arterial pressure did not change, in either group, following IPC (table 3.2). It could be that in this instance the single acute episode of IPC

may only be causing changes to the conduit artery in the arm undergoing IPC, rather than the entire systemic vasculature. However, it is worth noting that this study was not powered to detect differences in blood pressure following IPC, only differences in brachial artery FMD.

There was also no statistically significant difference between the baseline brachial artery diameter before and after local IPC. This is what would be expected for a valid brachial artery FMD study. FMD is determined by measuring the percentage change in brachial artery diameter in response to a blood flow stimulus (95, 98). A stable brachial artery diameter before each FMD measure validates the comparison before and after local IPC.

I also did not find any change in the difference in FMD following local IPC when the groups were analysed according to the phase of menstrual cycle the participants were in when they were part of the study. I had postulated that there may be a difference between the follicular and luteal phase of the menstrual cycle because of the higher levels of progesterone in the luteal phase. Progesterone has been found to cause relaxation in both arteries and veins in an ex-vivo study (220). It has also been found that mean arterial pressure is reduced, along with a decrease in systemic vascular resistance, in the mid-luteal phase, compared to the mid-follicular phase (221). However, a study of 17 women showed no difference in FMD between the follicular and luteal phases of the menstrual cycle (222).

3.8 Limitations

It would have been preferable to study the same participants at 24-hour and 48-hour study intervals, but unfortunately due to participant availability this was not possible.

In terms of IPC, having a protocol where both local IPC and remote IPC were assessed, with both a 24-hour and 48-hour study time interval would have been interesting. If brachial artery FMD was assessed in both the arm that had IPC stimulus, and the contralateral arm, or leg, this would have provided

more information regarding the impact of IPC on local and systemic endothelial function.

3.9 Key Findings

In this study of healthy non-pregnant women, I observed that brachial artery FMD increased 24-hours after an acute episode of local IPC, but not after 48 hours.

In the following experimental chapters, I therefore measured brachial artery FMD 24-hours after local IPC in healthy pregnant women, pregnant women at risk of pre-eclampsia and pregnant women with pre-eclampsia. I also investigated the pathways that may explain the protective effect of interval ischaemia on endothelial function in non-pregnant and pregnant women.

CHAPTER 4

LOCAL ISCHAEMIC PRE-CONDITIONING IN PREGNANCY

Chapter 4 – Local ischaemic pre-conditioning in pregnancy

4.1. Background

Pregnancy, pre-eclampsia and endothelial function

Pre-eclampsia is a multi-system syndrome of pregnancy, classically defined as the presence of de novo hypertension after 20 weeks gestation accompanied by proteinuria and/or evidence of maternal acute kidney injury, liver dysfunction, neurological features, haemolysis or thrombocytopenia, and/or fetal growth restriction (1).

Pre-eclampsia is associated with significant maternal and neonatal morbidity and mortality, due to the sequelae of iatrogenic pre-term delivery and fetal growth restriction (3, 6). Women who have had pre-eclampsia have an increased risk of developing and dying from cardiovascular disease (12-15). Additionally, growth restricted babies, born to women with pre-eclampsia, have themselves an increased likelihood of cardiovascular disease in adulthood (17). Preventing the development of pre-eclampsia or ameliorating the severity of disease could be beneficial to both mother and child in the short and long-term.

At present, low dose aspirin is the only effective prophylaxis against pre-term pre-eclampsia (19, 22, 223). Many other drugs have been and are being investigated to determine if they can provide additional risk reduction of pre-eclampsia. These include anti-oxidant vitamins, vitamin D, folic acid, calcium and statins (224-228). A simple, effective prophylaxis against pre-eclampsia would be invaluable to low-resource nations where maternal and perinatal morbidity is highest. Furthermore, the only cure for pre-eclampsia is delivery of the placenta (5, 9). Therefore, I investigated the role of a novel non-invasive intervention to improve maternal endothelial function, and hopefully lower maternal blood pressure, in women at risk of pre-eclampsia and with pre-eclampsia.

Measuring endothelial function: a sub-clinical measure of pre-eclampsia risk

Endothelial function can be measured in vivo using a measure of brachial artery FMD (95, 98), which is largely mediated by NOS activity (92, 95-97). Impaired endothelial function, as determined by a reduced FMD measure, is associated with an increased risk of future cardiovascular events (109, 110). In chapter 1 I discussed three key studies, which have investigated endothelial function in pregnant women who develop pre-eclampsia (23, 43, 55). These studies demonstrated that women with pre-eclampsia have reduced endothelial function, as measured by brachial artery FMD, which is evident before the clinical onset of pre-eclampsia (23, 43, 55). As discussed in chapter 1, endothelial dysfunction is a systemic pathological process (111, 112). Therefore, a lower brachial artery FMD measure, reflects systemic endothelial dysfunction, which in a pregnant woman with established pre-eclampsia or at risk of pre-eclampsia is indicative of impaired placental function.

Ischaemic pre-conditioning

IPC is a phenomenon whereby transient, brief episodes of ischaemia applied to an organ or tissue, protect that organ or tissue from a subsequent prolonged period of ischaemia or ischaemic injury (117). This type of IPC is known as local IPC, as the pre-conditioning stimulus is applied to the same tissue that subsequently sustains ischaemic injury (118, 119). In human studies remote IPC is much more practical than local IPC and is commonly administered as three or four cycles of 5-minutes of ischaemia, followed by 5-minutes of reperfusion (119, 132).

In pre-eclampsia the placenta is relatively ischaemic, in a similar way to an ischaemic myocardium, and towards the end of a pre-eclamptic pregnancy, the placenta can become infarcted similar to a myocardial infarction (54, 195). It is not possible for a local IPC stimulus to be applied to the placenta. However, a local IPC stimulus may improve maternal endothelial function, as

measured by brachial artery FMD, and remote IPC may improve the maternal angiogeneic profile.

There are no published studies investigating local or remote IPC in pregnancy. Given the relative simplicity and cost-effectiveness of IPC, and its non-invasive low-risk nature, IPC is attractive for use in pregnancy. If IPC is shown to be of benefit, it is an attractive non-pharmacological treatment to improve endothelial function in pregnant women with pre-eclampsia and at risk of pre-eclampsia.

The aim of the experiments described in this chapter were to investigate if local IPC improves maternal endothelial function in women with pre-eclampsia. I hypothesised that an acute episode of local IPC would improve endothelial function in women with pre-eclampsia.

4.2 Study Design

Three groups of pregnant women were studied:

- Healthy pregnant women, with no risk factors for pre-eclampsia (n= 42)
- Pregnant women at risk of pre-eclampsia (n=20)
- Pregnant women with pre-eclampsia (n=10)

Pre-eclampsia was defined as new onset hypertension (BP >140/90 mmHg on 2 separate occasions at least 4 hours apart) and a urinary protein to creatinine ratio (PCR) >30 mg/mmol.

These studies took place in the Fetal Medicine Unit in the EGA Wing of UCLH.

4.3 Study Participants

Pregnant women suitable for one of the study groups were identified in midwifery and doctor-led antenatal clinics, the antenatal ward and in response to posters located in the EGA Wing, UCLH. Women agreeable to participation

provided written informed consent prior to taking part in the study. Five women only consented to assessment of their endothelial function pre and post IPC, as they were ‘needle phobic’ and did not wish to have venesection carried out. Three of these women were in the ‘healthy pregnant’ group, one in the ‘at risk’ group and one in the ‘pre-eclampsia’ group.

4.4 Study Protocol

All pregnant women were studied between 24+0 and 36+6 weeks gestation. All participants had fasted for at least 4 hours, had not ingested any caffeine or alcohol for at least 12 hours, and had not carried out any strenuous exercise in the previous 24 hours. All studies were performed in a temperature-controlled room.

Once the study participant arrived, a consent form was completed (see appendix 4), with the participant having the opportunity to ask questions. A 10 ml mid-stream urine sample was taken, followed by a 20 ml venesection from the left antecubital fossa. A baseline measure of blood pressure, mean arterial pressure and heart rate was then taken, followed by a measurement of endothelial function using brachial artery FMD of the right arm. The participant then underwent an acute episode of IPC of the right arm, which included 3 episodes of ischaemia created by a cuff inflated to 200mmHg for 5 minutes. A questionnaire was also completed with questions on: previous pregnancy history, past medical and family history, current medication, most recent food, caffeine and exercise (see appendix 6).

Twenty-four hours later, measures of maternal BP, heart rate and endothelial function using brachial artery FMD were repeated. IPC was not repeated.

The detailed study protocol is described in chapter 2.

4.5 Statistical Analysis

Statistical analysis was carried out using Stata version 16 and GraphPad Prism version 7. Initially a multi-level mixed model regression analysis was carried out. This analysis was checked for variance and normality; these assumptions were not satisfied, and in some cases there was a significant interaction between the groups. Therefore, this was not the appropriate analysis to use. For consistency, all statistical comparisons tests were carried out using non-parametric analyses, due to normality and variance assumptions not being satisfied in all data sets.

When more than two groups were being analysed, a 1-way ANOVA was carried out, followed by the Kruskal-Wallis Test and Dunn's multiple comparisons test was done to look at the differences between the groups. When comparisons were needed between two groups, without pairing, the Mann-Whitney Test was used. For paired comparisons within groups, Wilcoxon matched-pairs signed rank test was used. Correlation analysis was carried out using Pearson Correlation Coefficient. Data are presented as mean and standard deviation (SD) where appropriate.

4.6 Results

Characteristics of the pregnant women studied

The characteristics of the pregnant women studied are shown in table 4.1. Compared with healthy pregnant women, pregnant women at risk of pre-eclampsia and those with pre-eclampsia had a higher BMI ($p = 0.0018$ and $p = 0.0002$). There was no significant difference between the ages of the participants (healthy vs at risk $p = 0.20$; healthy vs pre-eclampsia $p = 0.59$; at risk vs pre-eclampsia $p = 0.73$). All pregnant groups were studied at a comparable gestation (healthy vs at risk $p = 0.80$; healthy vs pre-eclampsia $p = 0.14$; at risk vs pre-eclampsia $p = 0.06$). For parity and ethnicity percentages are shown.

Characteristic	Healthy (n = 42)	At risk (n = 20)	Pre-eclampsia (n = 10)
Age (years)	35 (3)	37(6)	36 (8)
BMI (kg/m^2)	23.0 (2.1)	26.7 (4.3) *****	27.8 (2.9) *****
Gestation studied at (weeks and days)	30+2 (4.1)	29+6 (3.1)	32+2 (2.1)
Primigravida	29 (69%)	8 (40%)	6 (60%)
Parous	13 (31%)	12 (60%)	4 (40%)
Ethnicity - White	33 (79%)	12 (60%)	3 (30%)
Ethnicity - Black	1 (2%)	5 (25%)	5 (50%)
Ethnicity - Asian	8 (19%)	3 (15%)	2 (20%)

Table 4.1: Characteristics of the pregnant women studied. Comparison of baseline phenotype data for the 3 groups of pregnant women studied. Data are presented as mean and standard deviation for age, BMI and gestation. Statistical comparisons were carried out using a 1-way ANOVA, followed by a Kruskal-Wallis Test and Dunn's multiple comparisons test.

Women at risk of pre-eclampsia: Phenotypic characteristics

Twenty women at risk of pre-eclampsia were studied. Their risks were due to previous pre-eclampsia (n=11), pre-existing hypertension (n=7) and both previous pre-eclampsia and pre-existing hypertension (n= 2). All of these women took aspirin once a day as pre-eclampsia prophylaxis:

- 14/20 women were taking 75 mg aspirin
- 6/20 women were taking 150 mg aspirin

Six women were also taking anti-hypertensive medication. All of these women had pre-existing hypertension. Three women took labetalol alone, 1 took labetalol and nifedipine, 1 took nifedipine alone and 1 took methyldopa alone.

Of the 20 women at risk of pre-eclampsia, 6 went on to develop pre-eclampsia. Of these 6 women, 3 had pre-existing hypertension requiring anti-hypertensive medication prior to developing pre-eclampsia.

Women with pre-eclampsia: Phenotypic characteristics

I studied 10 women with pre-eclampsia. Of these women, three of these women had previous pre-eclampsia, 2 had pre-existing hypertension and 2 had both previous pre-eclampsia and pre-existing hypertension. The other 3 were primigravid.

Medications

All women with pre-eclampsia were on anti-hypertensive medication. They were taking different doses and combinations of labetalol, nifedipine or methyldopa. At the time of being studied, 7/10 were taking aspirin and low molecular weight heparin. The other 3 women were considered to be within 24-48 hours of needing childbirth and therefore were not taking aspirin or low molecular weight heparin.

Brachial artery FMD

Brachial artery FMD was measured before and after local IPC in healthy pregnant women (healthy), women at risk of pre-eclampsia (at risk) and women with pre-eclampsia (pre-eclampsia) before and 24-hours after local IPC (table 4.2 and figure 4.1). In healthy women there was no change in FMD following IPC. In pregnant women at risk of pre-eclampsia or with pre-eclampsia there was a significant improvement in endothelial function, as measured by FMD, following local IPC.

Brachial artery FMD (%) (mean and SD)					
Healthy (n = 42)		At risk (n = 20)		Pre-eclampsia (n = 10)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
10.3 (4.5)	10.5 (4.5)	7.6 (3.7)	8.9 (3.3)	3.5 (1.7)	5.8 (2.4)
p = 0.33		p = 0.016*		p = 0.006*	

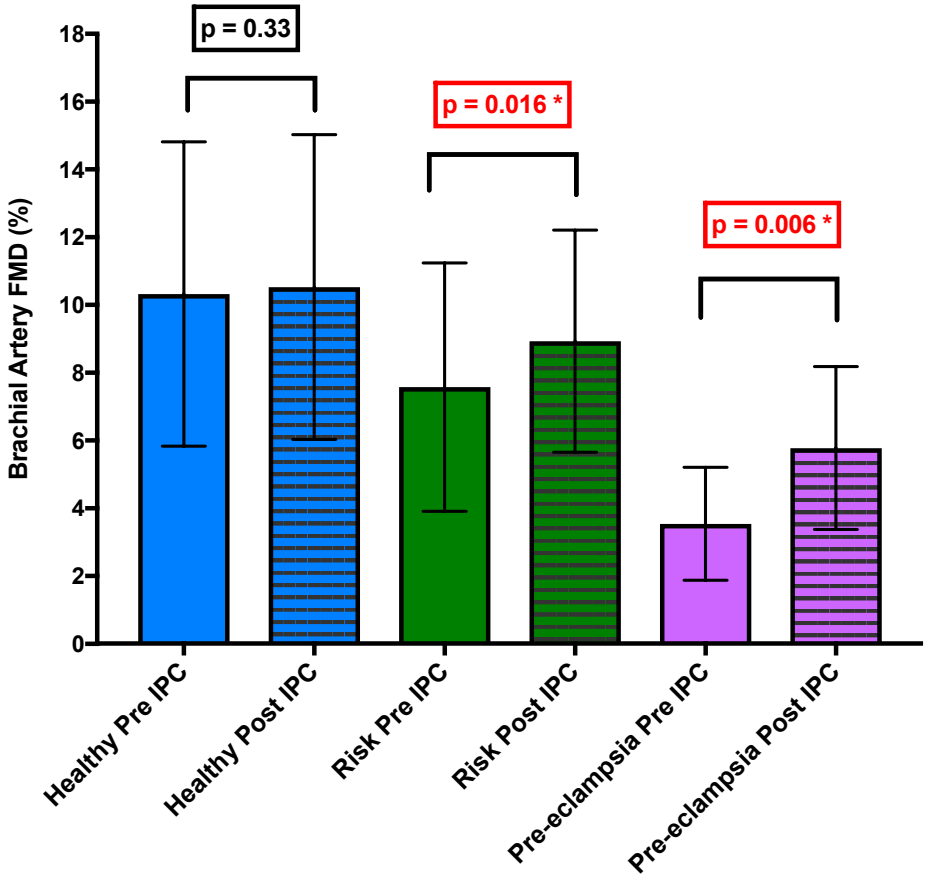


Table 4.2 and Figure 4.1: Brachial artery FMD (%) before and after local IPC in each group of pregnant women**.** Data are presented as mean and SD. Statistical comparisons were carried out using a 1-way ANOVA, followed by a Kruskal-Wallis Test and Dunn's multiple comparisons test. IPC caused a significant increase in FMD in the at risk and pre-eclampsia women.

Women at risk of pre-eclampsia; Brachial artery FMD

The women at risk of pre-eclampsia were analysed in sub-groups of women at risk who did not develop pre-eclampsia and the women at risk who did develop pre-eclampsia. Figure 4.2 shows the brachial artery FMD measurements before and 24-hours after local IPC. Women at risk who went on to develop pre-eclampsia had a lower baseline FMD compared with women who did not develop pre-eclampsia, although this did not reach statistical significance.

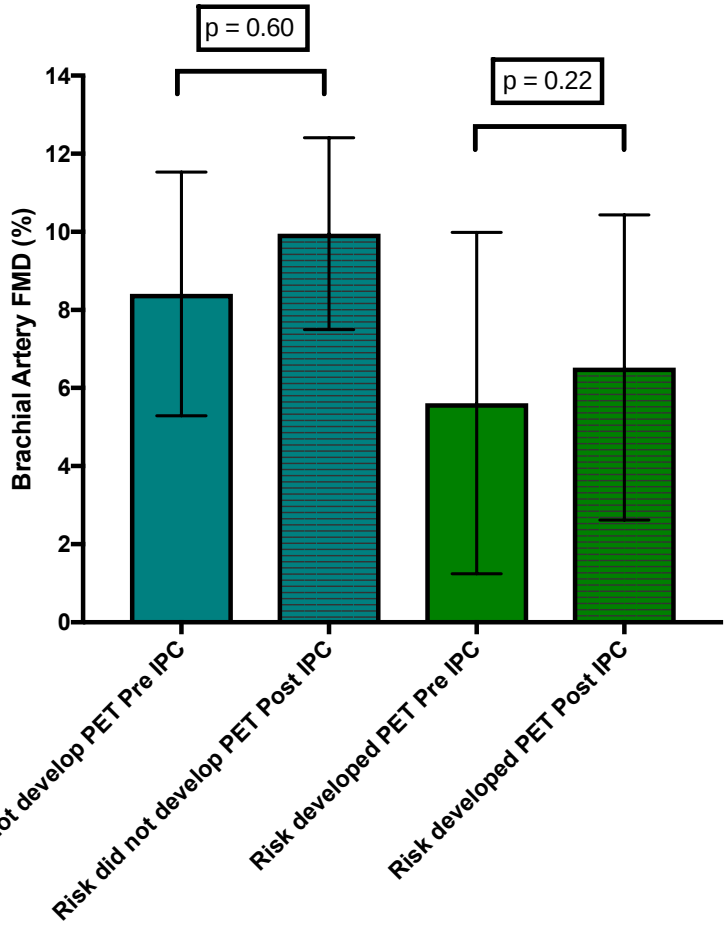


Figure 4.2: Brachial artery FMD (%) before and after local IPC in women at risk of pre-eclampsia who did not develop pre-eclampsia and women at risk of pre-eclampsia who did develop pre-eclampsia**.** Data are presented as mean and SD. Statistical comparisons were made within each group using Wilcoxon matched-pairs signed rank test. Women at risk who went on to develop pre-

eclampsia had a lower baseline FMD compared with women who did not develop pre-eclampsia, although this did not reach statistical significance.

As shown in table 4.3, women at risk of pre-eclampsia who were taking anti-hypertensive medication had a lower baseline FMD compared with women at risk of pre-eclampsia who were not taking anti-hypertensive medication. There was a statistically significant improvement, following local IPC, in the FMD measure in the group of women taking anti-hypertensive medication.

Brachial artery FMD (%) Mean (SD)			
At risk - no anti-hypertensive medication (n = 14)		At risk - taking anti-hypertensive medication (n = 6)	
Pre IPC	Post IPC	Pre IPC	Post IPC
8.5 (3.4)	9.9 (2.4)	5.4 (3.6)	6.5 (4.0)
p = 0.14		p = 0.03*	

Table 4.3: Brachial artery FMD (%) in the at risk group, using sub-groups of development of pre-eclampsia, and if they were on anti-hypertensive medication at the time of the study. Data are presented as mean and SD. Statistical comparisons were made within each group using Wilcoxon matched-pairs signed rank test. Following local IPC, there was a significant improvement in FMD in the group of at risk women taking anti-hypertensive medication.

Brachial artery baseline diameter

As shown in table 4.4, women with pre-eclampsia had the largest mean brachial artery baseline diameter and women at risk had the smallest mean brachial artery baseline diameter.

Brachial artery baseline diameter (mm) Mean (SD)					
Healthy (n = 42)		At risk (n = 20)		Pre-eclampsia (n = 10)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
2.54 (0.42)	2.54 (0.38)	2.37 (0.45)	2.37 (0.50)	3.10 (0.54)	3.15 (0.55)
p = 0.78		p = 0.88		p = 0.35	

Table 4.4**:** A comparison of mean baseline brachial artery diameter (mm) in the 3 groups. Data are presented as mean and SD. Comparisons were made within each group using Wilcoxon matched-pairs signed rank test. There was no difference in brachial artery baseline diameter before or after local IPC within each group studied.

Correlation between mean brachial artery baseline diameter (mm) and brachial artery FMD (%)

Figure 4.3 demonstrates the finding that a larger mean brachial artery baseline diameter is correlated with a low brachial artery FMD result.

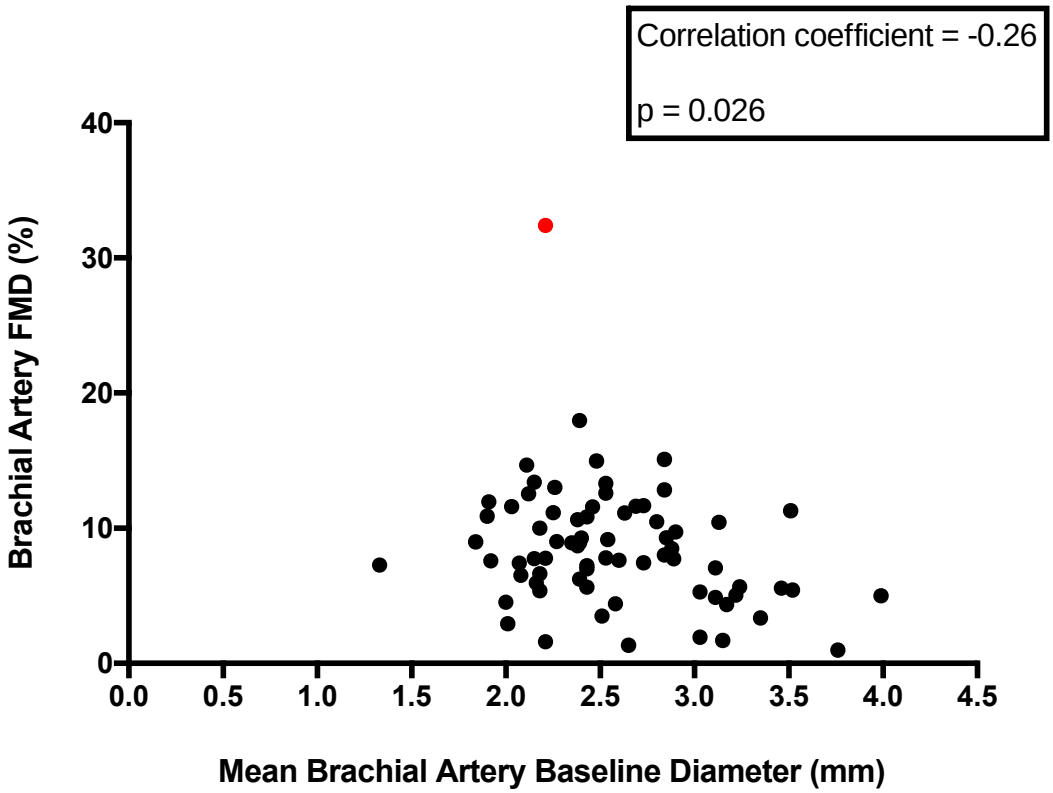


Figure 4.3: Graph showing a negative correlation between mean brachial artery baseline diameter and brachial artery FMD. Non-parametric Spearman correlation analyses used. Outlier data point in red.

Time to peak brachial artery dilatation

When the time to peak brachial artery dilatation was analysed in the 3 pregnant groups studied (see table 4.5), there was a significant improvement in the time it took to reach the peak brachial artery FMD following local IPC in the healthy group - 425s vs 414s (p = 0.013).

Time to peak brachial artery dilatation (s) Mean (SD)					
Healthy (n = 42)		At risk (n = 20)		Pre-eclampsia (n = 10)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
425 (27)	414 (19)	422 (33)	424 (42)	424 (26)	427 (52)
p = 0.013*		p = 0.50		p = 0.66	

Table 4.5: Time to peak brachial artery dilatation in the 3 pregnant groups. Data are presented as mean and SD. Comparisons were made within each group using Wilcoxon matched-pairs signed rank test. There was a significant improvement in the time it took to reach the peak brachial artery FMD following local IPC in the healthy group. In the other two groups there was no significant change.

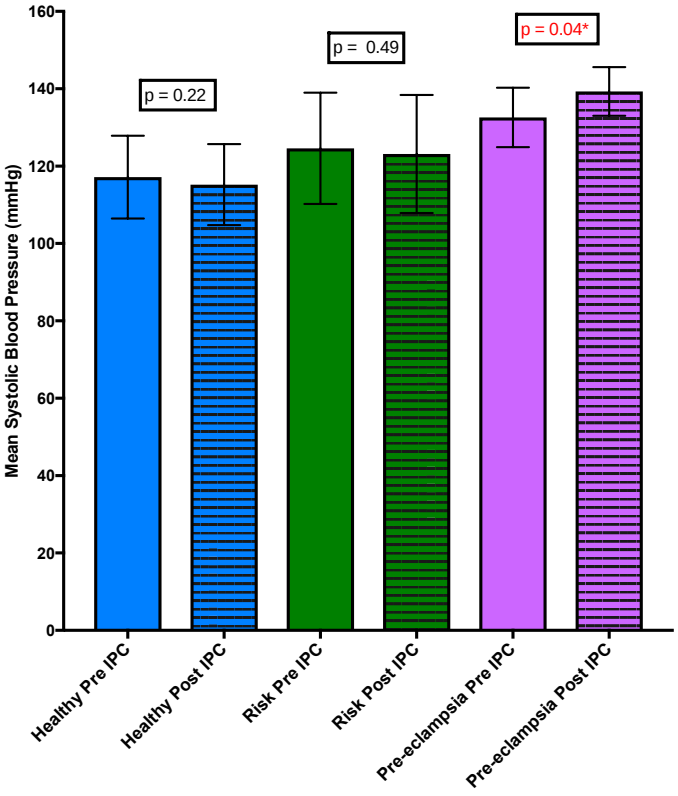
Blood pressure

Table 4.6 and figures 4.4, 4.5 and 4.6 illustrate the systolic blood pressure, diastolic blood pressure and mean arterial pressure in the 3 pregnant groups before and after IPC.

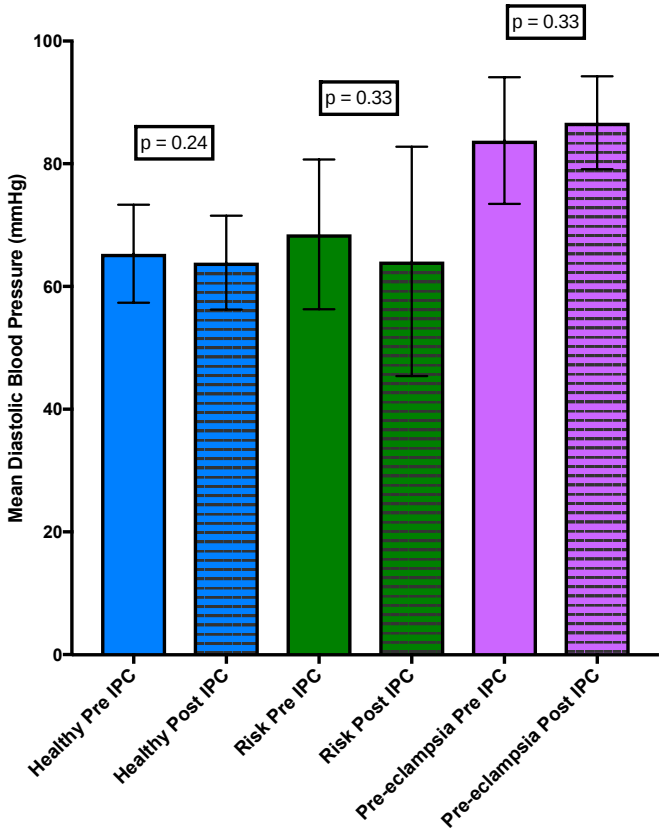
Blood pressure (mmHg) Mean (SD)						
	Healthy (n = 42)		At risk (n = 20)		Pre-eclampsia (n = 10)	
	Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
Systolic	117 (11)	115 (10)	125 (14)	123 (15)	133 (8)	139 (6)
p value	0.22		0.49		0.04*	
Diastolic	65 (8)	64 (8)	69 (12)	64 (19)	84 (10)	87 (8)
p value	0.24		0.33		0.33	
MAP	84 (8)	83 (9)	90 (12)	86 (21)	103 (9)	107 (10)
p value	0.04*		0.26		0.26	

Table 4.6: Systolic blood pressure, diastolic blood pressure and mean arterial pressure in the 3 groups pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using Wilcoxon matched-pairs signed rank test. Women with pre-eclampsia had a significant increase in their systolic blood pressure following IPC; in this group there was no significant increase in diastolic blood pressure or mean arterial pressure. The healthy group had a significant decrease in their mean arterial pressure following IPC; in this group there was no significant decrease in systolic or diastolic blood pressure.

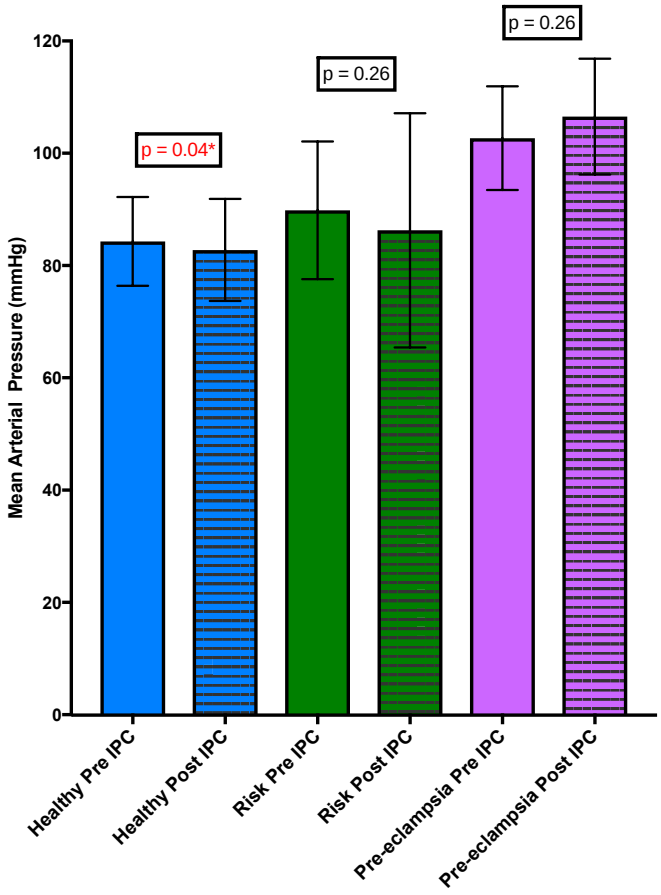
Systolic Blood Pressure



Diastolic Blood Pressure



Mean Arterial Pressure



Figures 4.4, 4.5 and 4.6: Graphical representation of systolic blood pressure, diastolic blood pressure and mean arterial pressure in the 3 pregnant groups pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using Wilcoxon matched-pairs signed rank test. Women with pre-eclampsia had a significant increase in their systolic blood pressure following IPC. The healthy group had a significant decrease in their mean arterial pressure following IPC.

Birth weight centile

The birth weight centiles were compared in 4 different groups – healthy pregnant women (n = 42), women at risk of pre-eclampsia who did not develop pre-eclampsia (n = 14), women at risk of pre-eclampsia who

developed pre-eclampsia (n = 6) and women with pre-eclampsia (n = 10) (see figure 4.7). There was a significant difference when the healthy group were compared to both the pre-eclampsia group and the at risk of pre-eclampsia group who developed pre-eclampsia; there was also a significant difference when the at risk group who did not develop pre-eclampsia were compared to the pre-eclampsia group.

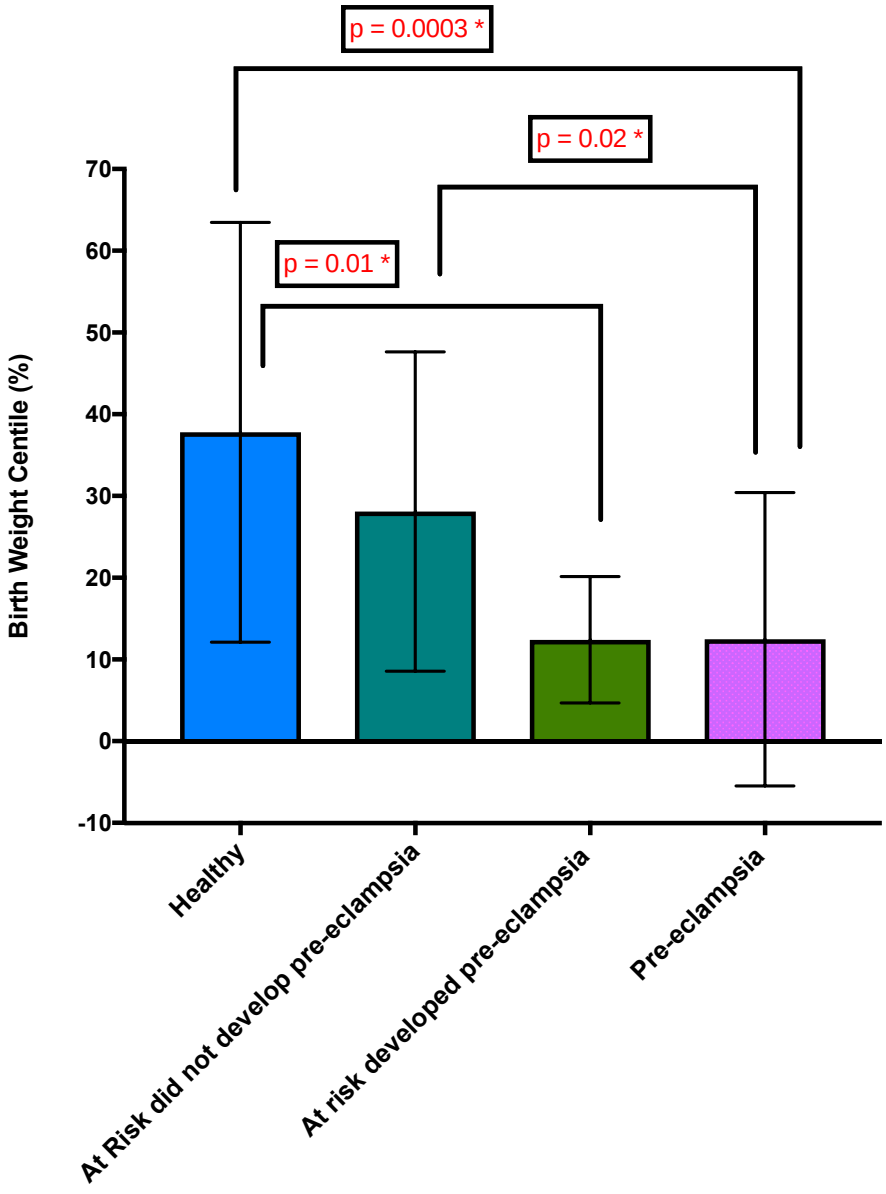


Figure 4.7: Comparisons are made across 4 groups (healthy, at risk did not develop pre-eclampsia, at risk developed pre-eclampsia, pre-eclampsia) for birth weight centile, corrected for gestation. Birth weight centiles were calculated using the Intergrowth-21st birth weight centile calculation software.

Data are presented as mean and SD. All comparisons between the groups were carried out using a 1-way ANOVA, followed by the Kruskal-Wallis Test and Dunn's multiple comparisons test was done to look at the differences between the groups. Women with pre-eclampsia (n=10) and women at risk who developed pre-eclampsia (n=6) had babies born on the lowest birth weight centiles. There was a significant difference when the healthy group were compared to both the pre-eclampsia group and the at risk group who developed pre-eclampsia; there was also a significant difference when the at risk group who did not develop pre-eclampsia were compared to the pre-eclampsia group. Unless stated there was no significant difference between the groups.

Gestation at delivery

Gestation at delivery centiles were compared in 4 different groups – healthy pregnant women, women at risk of pre-eclampsia who did not develop pre-eclampsia, women at risk of pre-eclampsia who developed pre-eclampsia and women with pre-eclampsia and are shown in table 4.7.

Gestation at Delivery (weeks and days) Mean (SD)			
Healthy (n = 42)	At risk did not develop pre-eclampsia (n = 14)	At risk developed pre-eclampsia (n = 6)	Pre-eclampsia (n = 10)
39+6 (1.4)	38+4 (1.0)	36+3 (1.7)	34+2 (1.9)

Table 4.7: Comparisons were made across 4 groups (healthy, at risk did not develop pre-eclampsia, at risk developed pre-eclampsia, pre-eclampsia) for gestation at delivery (weeks and days). Data are presented as mean and SD. All comparisons between the groups were carried out using a 1-way ANOVA, followed by the Kruskal-Wallis Test and Dunn's multiple comparisons test.

There was a significant difference between the gestation at delivery in the following groups:

- Healthy (39+6 weeks) vs at risk did not develop pre-eclampsia (38+4 weeks): p = 0.007

- Healthy (39+6 weeks) vs at risk developed pre-eclampsia (36+3 weeks): p = 0.0002

- Healthy (39+6 weeks) vs pre-eclampsia (34+2 weeks): p = < 0.0001

- At risk did not develop pre-eclampsia (38+4 weeks) vs pre-eclampsia (34+2 weeks): p = 0.02

All healthy pregnant women delivered their babies at term (> 37+0 weeks). Five women in this group had babies < 10th centile. These 5 women had a mean FMD of 8.9% (SD 3.2), compared with 37 healthy women who had appropriate for gestational age babies, mean FMD 10.5% (SD 4.6) (p = 0.46).

Four women at risk of pre-eclampsia had pre-term pre-eclampsia (at 34+3, 34+5, 35+1 and 36+6 weeks) with babies < 10th centile, corrected for gestation. Two other women at risk of pre-eclampsia developed term pre-eclampsia at 37+0 weeks and 39+1 weeks gestation. These babies had birth weights on the 19.5% and 3.0% centile respectively, corrected for gestation. All 10 women with pre-eclampsia delivered their babies between 31+5 and 37+2 weeks gestation. Seven women had babies <10th centile birthweight, when corrected for gestation.

4.7 Discussion

This is the first study to investigate the effect of local IPC in pregnant women. My results support my main hypothesis that in women with pre-eclampsia, maternal endothelial function is improved by an acute episode of local IPC.

The underlying mechanism by which local IPC improves endothelial function remains unknown. One possibility is that the increased shear stress resulting from the reactive hyperaemia due to local IPC stimulates a local increase in endothelial NOS activity (168, 169, 229, 230). A second possibility is that the transient tissue ischaemia due to local IPC causes may also stimulate the release of EPCs, which may additionally increase local NO concentrations (170, 171). Both possible mechanisms are not mutually exclusive. There may also be a role in dysregulation of prostacyclin and other endothelium-derived relaxing factors (231), however I did not investigate these pathways.

Similar to previously published studies, I found that women with pre-eclampsia and those at-risk of pre-eclampsia, had a lower baseline FMD than normotensive healthy pregnant women (23, 55, 232).

Healthy pregnant women did not demonstrate a change in their brachial artery FMD following local IPC. This may be due to healthy pregnancy endothelium reaching a maximum vasodilatory capacity and unable to vasodilate further in response to IPC. (33, 122, 233, 234). I showed in chapter 3, that following IPC, healthy non-pregnant women improved their FMD to levels similar to the baseline FMD of healthy pregnant women. This increase in FMD may reflect a maximal reserve of endothelial function that is utilised during pregnancy.

Following local IPC however, healthy pregnant women had a significant shortening in the ‘time to peak’ brachial artery FMD. This may reflect a physiological improvement in endothelial function, once FMD has reached maximum vasodilatation. The mechanistic pathway to faster peak FMD is uncertain and has not been thoroughly investigated. Previous studies have

found that younger subjects have a faster time to peak FMD when compared with older subjects (235-237).

One theory relates to the fact that older people have increased arterial stiffness, which impacts the compliance of the arterial wall (235, 237). We know that an increase in arterial stiffness leads to an increase in pulse-wave velocity, as the pressure wave in the arterial tree travels faster (238, 239), and as the arterial system ages, the arterial lumen dilates and the walls thicken (238, 240). Therefore, it may be that the time to reach peak brachial artery FMD is influenced by arterial diameter, as baseline brachial artery diameters appear to be larger in older participants (236).

Conversely, women at risk of pre-eclampsia and women with pre-eclampsia had no shortening in time to reach peak brachial artery FMD following local IPC. Similar to older people, women with pre-eclampsia had the largest mean brachial artery baseline diameter compared with the other two groups. Indeed, their FMD was inversely correlated with baseline brachial artery diameter, which has been reported in previous studies (98, 241, 242). It is worth noting that the work on allometrically scaled FMD data found that with a traditional non-allometrically scaled FMD percentage calculation there can be a statistical bias towards the baseline diameter (199, 200).

It is possible that women with pre-eclampsia have larger brachial arteries upstream from increased peripheral vascular resistance similar to patients with hypertension outside of pregnancy (43, 243, 244). Larger mean brachial artery baseline diameters, may also result from anti-hypertensive medication. Patients taking calcium channel blockers (nifedipine) and beta-blockers (labetalol) can increase brachial artery diameter, although most studies showed this effect after one month of treatment (245-248).

One surprising result was that although women with pre-eclampsia had improved FMD following local IPC, their systolic blood pressure increased. However, their diastolic and mean arterial pressure did not significantly change following IPC. It may be that local IPC improved local endothelial

function but did not have a discernible systemic effect. Several of the women with pre-eclampsia had their babies delivered by emergency caesarean section within days of my study, as their syndrome of pre-eclampsia was continuing to progress and worsen. The rise in systolic blood pressure in this group may therefore not be associated with the IPC stimulus.

The only other blood pressure change observed was in the healthy pregnant group, who had a decrease in their mean arterial pressure following IPC. This finding may relate to the same mechanism by which the 'time to peak' FMD was significantly shortened, which may reflect a physiological improvement in endothelial function and perhaps also an impact on systemic vasculature. There is one study which has shown that in a normotensive man (n=1), blood pressure (systolic, diastolic and mean arterial pressure) was reduced after repeated serial episodes of an IPC stimulus (151). Another study found a reduction in blood pressure in 15 newly diagnosed untreated hypertensive participants, who underwent remote IPC once-a-day for one month (152).

Therefore, it may be that in women with pre-eclampsia repeated episodes of IPC could not only improve endothelial function, but also make blood pressure control easier and allow the pregnancy to last longer.

Women who go on to develop pre-eclampsia have utero-placental ischaemia for several weeks before its clinical onset (249). As discussed earlier, before the onset of pre-eclampsia, women who go on to develop pre-eclampsia have a lower brachial artery FMD (23). Endothelial dysfunction is a central component of the pathophysiology of pre-eclampsia and contributes to the pathogenesis of hypertension and cardiovascular sequelae (231, 250). Pre-eclampsia is a disease that can be understood in terms of both placental and maternal endothelial dysfunction, coupled with genetic and immunological factors (4, 8).

Women in the at risk of pre-eclampsia group who went on to develop pre-eclampsia had a lower baseline FMD measure than those who did not develop pre-eclampsia. However, I found that when analysed separately,

neither group increased their FMD following local IPC. This may reflect small numbers in each group. However, the FMD level in the women at risk of pre-eclampsia, who did not develop pre-eclampsia, did reach the levels of the healthy pregnant group following local IPC. This may be due to less impaired baseline FMD, and an ability to mount a good response to local IPC stimulus.

Hypertensive women taking anti-hypertensive medication had a greater improvement in FMD than those not taking anti-hypertensive treatment. Following an acute episode of local IPC, brachial artery FMD increased the most in women with the worst endothelial function, but the level of FMD was not restored to that of healthy pregnant women.

Six hypertensive women in the group at risk of pre-eclampsia and all women with pre-eclampsia were taking anti-hypertensive medication. These women were taking combinations of labetalol, nifedipine or methyldopa, which are commonly prescribed in pregnancy. There are mixed findings from studies investigating the effect of anti-hypertensive medication on endothelial function. Some studies found no acute effect, but that longer term use, varying from one month of use, to 48 weeks of use, improved endothelial function (248). However, other studies found no impact of anti-hypertensive medication on brachial artery FMD (246, 247). It is important to note that the majority of these studies included mostly male participants, therefore their relevance to females and pregnancy in particular is uncertain (245-248).

It remains to be tested whether repeated local IPC would have a cumulative beneficial effect on endothelial function and pregnancy outcome in women with pre-eclampsia or at risk of pre-eclampsia. Two studies in healthy adults and one in adults with coronary artery disease showed that repeated IPC improved FMD (123, 124, 209).

4.8 Strengths

Most studies that have measured brachial artery FMD longitudinally throughout pregnancy have shown a decline in the third trimester. In my study

I ensured the mean gestation at which participants were studied was similar across all groups.

Another strength of my study was that I ensured baseline brachial artery diameter did not differ within each group before and after the local IPC stimulus. A stable brachial artery diameter before each FMD measure validates the comparison before and after local IPC.

As anticipated, I found that women at risk of pre-eclampsia and those who had pre-eclampsia had a higher BMI than healthy pregnant women. This observation combined with associated endothelial dysfunction may explain the higher risk of long-term cardiovascular diseases following pre-eclampsia (6, 12, 58, 251).

4.9 Limitations

This study would likely have benefitted from a less heterogeneous pre-eclampsia group. All 10 women with pre-eclampsia delivered their babies between 31+5 and 37+2 weeks gestation, however only 7 women had babies <10th centile birthweight. A group of women with earlier onset pre-eclampsia (before 34 weeks) would have been associated with a low birthweight for all the babies. There was also some heterogeneity in the anti-hypertensive medication women in this group were taking: some women were taking multiple anti-hypertensive agents, whereas others were only taking one anti-hypertensive medication. Finally, 6 women in this group were primigravid. In future work, studying only primigravid women with pre-eclampsia, or a larger number of primigravid women with pre-eclampsia, may have led to less heterogeneity and a more severe pre-eclampsia phenotype.
Having shown that an acute episode of local IPC improves brachial artery FMD, it would be interesting to investigate whether IPC in the arm improves utero-placental blood flow remote from the ischaemic stimulus. This would have provided fascinating information as to whether remote IPC had been able to condition the placenta and ultimately decrease placental ischaemia. An improvement in utero-placental perfusion may improve fetal growth and

reduce perinatal morbidity associated with pre-eclampsia. If I had also measured brachial artery FMD in the contralateral arm following IPC, I would have learned more about the possibility of an effect on remote endothelial function.

4.10 Key Findings

- I have shown, for the first time, that an acute episode of local IPC improves endothelial function in women with pre-eclampsia and pregnant women at risk of pre-eclampsia.

- I have shown that in healthy normotensive pregnant women, an acute episode of local IPC shortened the time taken to reach peak brachial artery dilatation.

These results provide promising pilot data for a further investigation of local and remote IPC in preventing the development of pre-eclampsia or ameliorating the severity of disease. In future work it may be that instead of a single acute episode of local IPC I could investigate repeated local or remote IPC. This may not only further improve FMD in a group of women with pre-eclampsia, but may also improve utero-placental blood flow and lower maternal blood pressure. This may therefore prolong pregnancy and improve maternal and fetal outcomes.

In the next chapter I will examine the effect of IPC on sFlt-1 and PlGF, both factors known to be involved in the pathogenies of pre-eclampsia.

Chapter 5

Does ischaemic pre-conditioning affect levels of sFlt-1 and PlGF in women with pre-eclampsia

Chapter 5 – Does ischaemic pre-conditioning affect levels of sFlt-1 and PlGF in women with pre-eclampsia

5.1 Background

In this chapter I investigate whether IPC alters sFlt-1 and PlGF plasma levels in 3 groups of women; (i) healthy pregnant women, (ii) women at risk of pre-eclampsia and (iii) women with pre-eclampsia. These angiogeneic factors appear to be largely derived from the placenta, downstream of the utero-placental circulation (4, 60-62, 68). In this regard, any effect of IPC in the arm could be considered as remote IPC, rather than local IPC. As discussed in chapter 1, remote IPC refers to the phenomenon whereby transient, brief episodes of ischaemia applied to a distant organ or tissue, protects another distant organ or tissue from subsequent ischaemic injury (119, 125). I therefore postulated that remote IPC of the arm alleviates utero-placental ischaemia associated with pre-eclampsia, which in turn rebalances maternal sFlt-1 and PlGF plasma levels to those seen in healthy pregnancy.

As discussed in chapter 1, sFlt-1 levels, increase at least 5 to 6 weeks before the clinical onset of pre-eclampsia (23, 60, 61, 65-68) and PlGF levels are significantly lower in women with pre-eclampsia, compared with women who have healthy normotensive pregnancies (61, 70).

Since higher levels of sFlt-1 and lower levels of PlGF are commonly observed prior to the onset of pre-eclampsia (23, 61), the ratio of the two provides an ‘index’ of pre-eclampsia risk (4, 70, 77). In clinical practice, one-time PlGF testing, between 20 and 34+6 weeks gestation (26), is used to aid the diagnosis of pre-eclampsia and inform the level of risk of pre-term delivery fetal or maternal complications within 14 days, rather than sFlt-1 alone. In some units, the ratio of sFlt-1:PlGF is also used to support the diagnosis of pre-eclampsia.

I hypothesised that an acute episode of IPC would improve the ratio between sFlt-1 and PlGF in women with pre-eclampsia.

5.2 Study Design

The same three pregnant groups were studied as in previous sections, i.e.:

- Healthy pregnant women, with no risk factors for pre-eclampsia (n= 39)
- Pregnant women at risk of pre-eclampsia (n=17)
- Pregnant women with pre-eclampsia (n=7)

A blood sample was taken from each participant in the Fetal Medicine Unit in the EGA wing of UCLH. The blood samples were centrifuged at the NIHR UCLH CRF, and stored in a -80°C freezer. The ELISA studies were carried out at The Hatter Cardiovascular Institute, UCL.

5.3 Study Participants

The pregnant participants were the same as in previous sections.

Measures of sFlt-1 and PlGF were carried out towards the end of the study period, but before all clinical IPC studies in the at risk and pre-eclampsia groups had occurred. The number of study samples for these tests were therefore as stated in 5.2.

5.4 Study Protocol

Human sFlt-1 and PlGF levels were measured in the plasma of all study participants from the pregnant groups. EDTA venesection samples were centrifuged at 3000 rpm, for 10 minutes, at 4°C and then the plasma samples aliquoted into 2ml Eppendorf tubes and stored in a -80°C freezer.

Human sFlt-1 and human PlGF levels were measured using commercially available quantitative sandwich ELISA kits from R+D systems (204, 205). All ELISA studies were carried out in duplicate according to manufacturer's instructions, as detailed in chapter 2. These ELISAs were analysed using a BMG Labtech Fluostar plate reader.

At the CRF and The Hatter Cardiovascular Institute I processed, stored and disposed of blood samples in accordance with all legal and regulatory requirements, including the Human Tissue Act 2004 and subsequent amendments.

5.5 Statistical Analysis

All statistical analysis was carried out using GraphPad Prism version 7 software. Data are presented as mean and standard deviation (SD) where appropriate. Statistical comparisons between the groups were carried out using a 1-way ANOVA, followed by the Kruskal-Wallis Test. If a difference was found, then a Dunn's multiple comparison test was carried out to determine significance. Statistical comparisons were carried out using a paired t-test with Bonferroni correction for comparisons within groups.

5.6 Results

Baseline levels of sFlt-1, PlGF and calculated sFlt-1:PlGF ratio

SFlt-1 levels were measured in all women at baseline (tables 5.1a, 5.1b and 5.1c). SFlt-1 levels were lowest in the at risk group and were significantly higher in the pre-eclampsia group (figure 5.1a). PlGF levels were highest in the healthy group and significantly lower in the pre-eclampsia group (figure 5.1b). As expected, the ratio of sFlt-1:PlGF was highest in the pre-eclampsia group (figure 5.1c).

	Healthy (n = 39) Mean (SD)	Pre-eclampsia (n = 7) Mean (SD)	p value
sFlt-1 (pg/ml)	2974 (651)	3653 (177)	<0.05
PlGF (pg/ml)	344 (157)	60 (31)	<0.001
sFlt-1:PlGF (pg/ml)	11.4 (7.3)	94.7 (93.8)	<0.001

Table 5.1a: SFlt-1, PlGF and sFlt-1:PlGF levels at baseline in the healthy and pre-eclampsia groups. Data are presented as mean and SD. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

	Healthy (n = 39) Mean (SD)	At risk (n = 17) Mean (SD)	p value
sFlt-1 (pg/ml)	2974 (651)	2359 (385)	<0.001
PlGF (pg/ml)	344 (157)	317 (214)	<0.01
sFlt-1:PlGF (pg/ml)	11.4 (7.3)	9.6 (4.3)	<0.001

Table 5.1b: SFlt-1, PlGF and sFlt-1:PlGF levels at baseline in the healthy and at risk groups. Data are presented as mean and SD. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

	At risk (n = 17) Mean (SD)	Pre-eclampsia (n = 7) Mean (SD)	p value
sFlt-1 (pg/ml)	2359 (385)	3653 (177)	<0.001
PlGF (pg/ml)	317 (214)	60 (31)	ns
sFlt-1:PlGF (pg/ml)	9.6 (4.3)	94.7 (93.8)	ns

Table 5.1c: SFlt-1, PlGF and sFlt-1:PlGF levels at baseline in the at risk and pre-eclampsia groups. Data are presented as mean and SD. Significant

differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

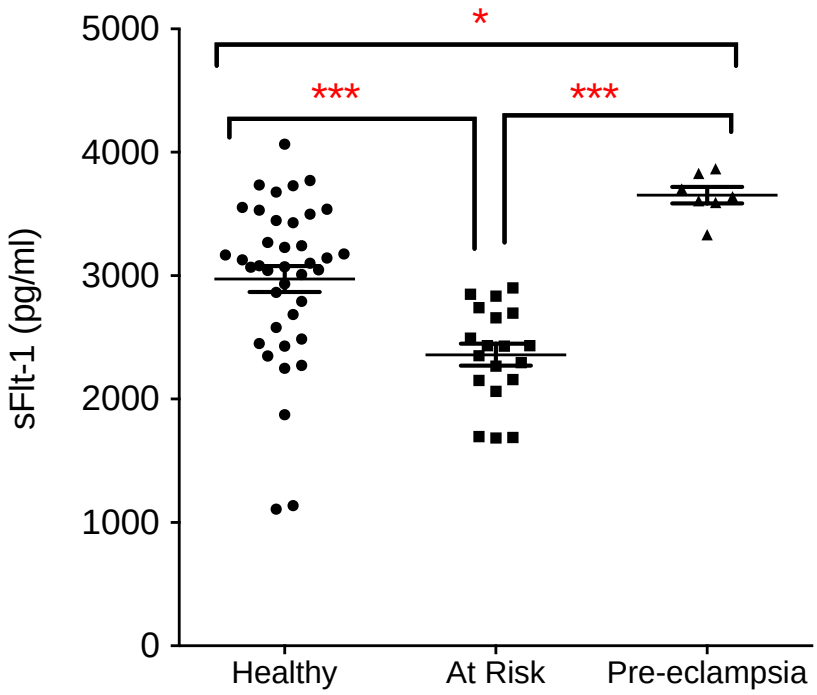


Figure 5.1a: SFlt-1 levels at baseline in all 3 pregnant groups. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test in all groups - * $p < 0.05$; *** $p < 0.001$.

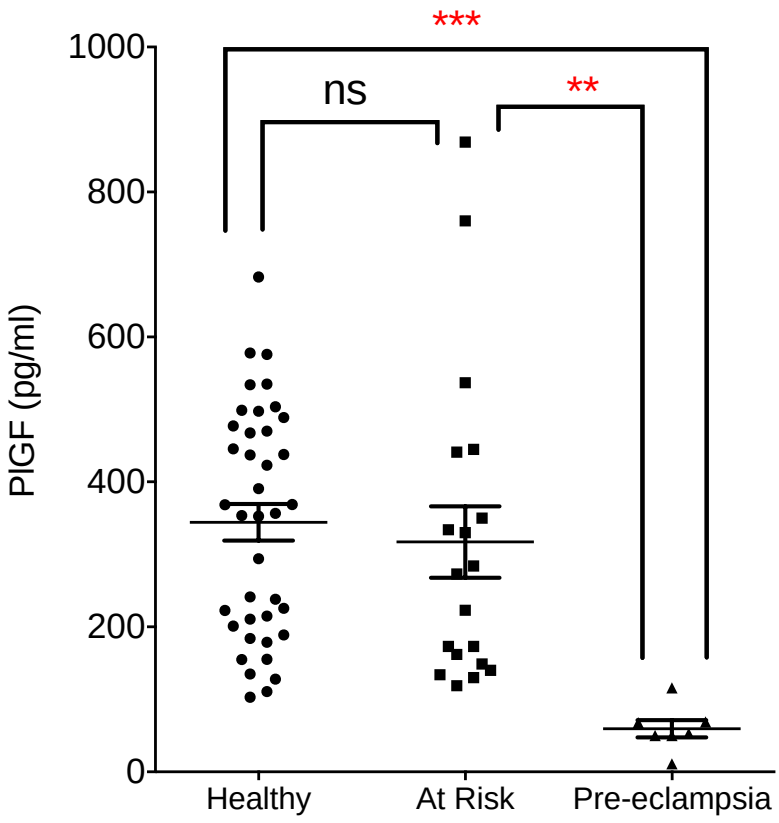


Figure 5.1b: PlGF levels at baseline in all 3 pregnant groups. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test between two groups - ** $p < 0.01$; *** $p < 0.001$. PlGF levels were lowest in the pre-eclampsia group, with significant differences when the healthy and at risk groups were compared to the pre-eclampsia group.

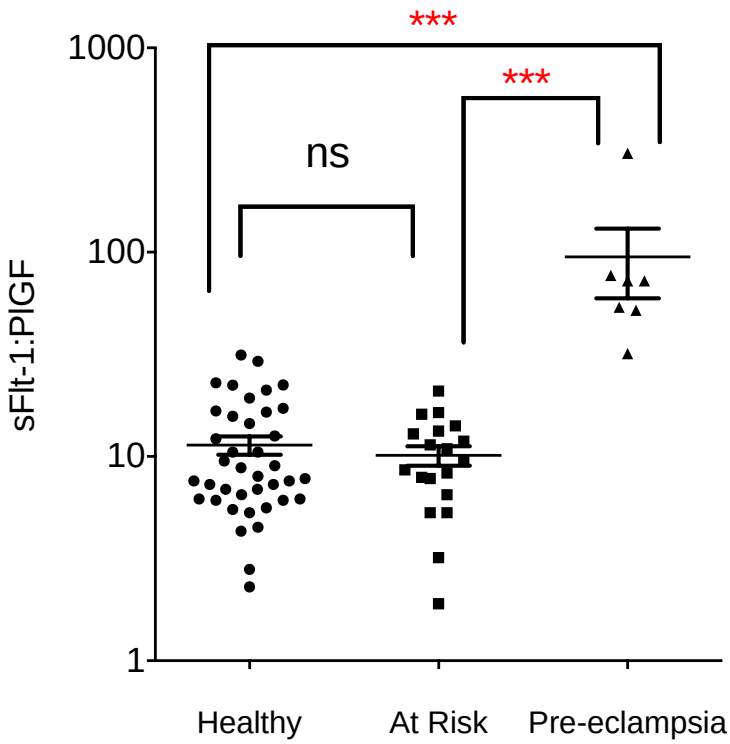


Figure 5.1c: SFlt-1:PlGF ratio at baseline in all 3 pregnant groups – using a log scale. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test when the healthy and at risk groups were compared to the pre-eclampsia group - *** p <0.001. The sFlt-1:PlGF ratio was highest in the pre-eclampsia group. There was a significant difference when the healthy and at risk groups were compared to the pre-eclampsia group.

SFlt-1 levels pre and post IPC in all 3 groups are shown in table 5.2, and then the levels pre and post IPC in the women at risk of pre-eclampsia separated into those that did not develop and developed pre-eclampsia are shown in table 5.3.

sFlt-1 (pg/ml)					
Healthy (n = 39) Mean (SD)		At risk (n = 17) Mean (SD)		Pre-eclampsia (n = 7) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
2974 (651)	3021 (683)	2359 (385)	2431 (585)	3653 (177)	3446 (457)
p = 0.31		p = 0.34		p = 0.15	

Table 5.2: SFlt-1 levels pre and post IPC in all 3 groups. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was no significant difference in any of the groups pre and post IPC.

sFlt-1 (pg/ml)			
At risk of pre-eclampsia Did not develop pre-eclampsia (n = 11) Mean (SD)		At risk of pre-eclampsia Developed pre-eclampsia (n = 6) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC
2324 (365)	2481 (494)	2436 (451)	2723 (315)
p = 0.40		p = 0.12	

Table 5.3: SFlt-1 levels in the at risk group separated into those that did not develop and developed pre-eclampsia pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction There was no significant difference in sFlt-1 levels in either group following IPC.

PlGF levels pre and post IPC are shown in table 5.4, and then the PlGF levels pre and post IPC in the women at risk of pre-eclampsia separated into those that did not develop and developed pre-eclampsia are shown in table 5.5.

PlGF (pg/ml)					
Healthy (n = 39) Mean (SD)		At risk (n = 17) Mean (SD)		Pre-eclampsia (n = 7) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
344 (157)	352 (170)	317 (214)	291 (152)	60 (31)	71 (53)
p = 0.61		p = 0.35		p = 0.30	

Table 5.4: PlGF levels pre and post IPC in all 3 groups. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was no significant difference in any of the groups pre and post IPC.

PlGF (pg/ml)			
At risk of pre-eclampsia Did not develop pre-eclampsia (n = 11) Mean (SD)		At risk of pre-eclampsia Developed pre-eclampsia (n = 6) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC
369 (255)	308 (164)	253 (129)	259 (136)
p = 0.32		p = 0.74	

Table 5.5: PlGF levels in the at risk group separated into those that did not develop and developed pre-eclampsia pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was no significant difference pre and post IPC.

The ratio of sFlt-1:PlGF levels pre and post IPC are shown in table 5.6, and then the ratio of sFlt-1:PlGF pre and post IPC in the women at risk of pre-eclampsia separated into those that did not develop and developed pre-eclampsia are shown in table 5.7.

sFlt-1:PlGF (pg/ml)					
Healthy (n = 39) Mean (SD)		At risk (n = 17) Mean (SD)		Pre-eclampsia (n = 7) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC	Pre IPC	Post IPC
11.4 (7.3)	11.5 (8.2)	9.6 (4.3)	10.0 (5.1)	94.7 (93.8)	75.8 (50.5)
p = 0.79		p = 0.72		p = 0.44	

Table 5.6: SFlt-1:PlGF ratio pre and post IPC in all 3 groups. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was no significant difference in any of the groups pre and post IPC.

sFlt-1:PlGF			
At risk of pre-eclampsia Did not develop pre-eclampsia (n = 11) Mean (SD)		At risk of pre-eclampsia Developed pre-eclampsia (n = 6) Mean (SD)	
Pre IPC	Post IPC	Pre IPC	Post IPC
9.3 (4.1)	9.6 (3.0)	10.1(5.0)	10.7 (8.1)
p = 0.79		p = 0.82	

Table 5.7: SFlt-1:PlGF ratio in the at risk group separated into those that did not develop and developed pre-eclampsia pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was no significant difference pre and post IPC.

5.7 Discussion

In this study, I confirmed that women with pre-eclampsia had higher sFlt-1 levels, and lower PlGF levels than women without pre-eclampsia. However, following an acute episode of IPC there was no significant difference in sFlt-1 levels, PlGF levels or the ratio of sFlt-1:PlGF.

It is worth noting from my results that the standard deviations are very large in the group of women with pre-eclampsia, so despite the appearance of a trend towards an improvement in levels of sFlt-1, PlGF and the ratio of sFlt-1:PlGF these results were not statistically significant. If I had studied a larger number of women with pre-eclampsia, especially those with a more severe disease phenotype with early onset disease (<34 weeks gestation), then this may not have been the case.

Therefore, my results do not support my hypothesis that an acute episode of IPC would result in lower sFlt-1 levels, higher PlGF levels and a lower sFlt-1:PlGF ratio in the pre-eclamptic group, by alleviating utero-placental ischaemia. This is a disappointing finding, considering that in chapter 4 I demonstrated that an acute episode of local IPC improved endothelial function, as measured by brachial artery FMD, in women with pre-eclampsia. I was likely underpowered in the pre-eclampsia group. Measures of sFlt-1 and PlGF were only carried out in 7 women with pre-eclampsia, as I carried out these assays the end of the study period, but before all IPC studies in the pre-eclampsia group had occurred.

In pregnancies complicated by pre-eclampsia, sFlt-1 levels may be elevated because the intermittently hypoxic placenta releases excessive anti-angiogeneic proteins into the maternal circulation (4, 60, 61, 68). However, we now also know that there is possibly a genetic component, as it has been found that the FLT1 genotype is associated with late-onset pre-eclampsia (72). This may mean that there are patterns of inheritance implicating both maternal and paternal factors in the development of pre-eclampsia (72), which is a field of research that requires further investigation.

Unexpectedly, sFlt-1 levels were lower in the at risk group than the other 2 groups. In the at risk group, the 6 women who went on to develop pre-eclampsia were studied at a mean time of 9 weeks and 3 days (SD 3.6 days) prior to the clinical onset of their pre-eclampsia. Maternal levels of sFlt-1 are elevated as early as 6 to 10 weeks prior to the clinical onset of pre-eclampsia

(23, 60, 61, 65-68), therefore, some of the women I studied may have been on the cusp of exhibiting elevated sFlt-1 at the time they were studied.

Lower levels of PlGF have also been observed prior to the onset of pre-eclampsia (4, 70, 77). Noori et al showed that at 10 to 17 weeks women who developed pre-term pre-eclampsia (defined as prior to 37 weeks gestation) had lower serum PlGF levels, compared with women who had term pre-eclampsia or normotensive pregnancies (23). A higher sFlt-1:PlGF ratio has also been found in women who have pre-term pre-eclampsia, when compared with women who had term pre-eclampsia or normotensive pregnancies (23). This is in keeping with the clinical application of a recent trial, which involves one-time PlGF testing, between 20 and 34+6 weeks gestation, to support the diagnosis of pre-eclampsia and inform the level of risk of pre-term delivery, fetal or maternal complications within 14 days (26).

As mentioned earlier, the 6 at risk women who went on to develop pre-eclampsia were studied at a mean time of 9 weeks and 3 days (SD 3.6 days) prior to the clinical onset of their pre-eclampsia. It may have been that had these women been studied with a slightly shorter time interval then they may have had lower PlGF levels and higher sFlt-1 levels.

There are some possible explanations for this apparent lack of association found in this chapter. If remote IPC transiently alleviates ischaemia in the utero-placental arteries, it is possible that this was insufficient to alter angiogenic factors in the time-frame of my study. Ideally, utero-placental blood flow would have been directly measured, to assess placental ischaemia. Measuring uterine artery Doppler velocimetry would have given an insight into the impact of remote IPC on placental function and whether both the baseline findings, or a change in uterine artery Doppler velocimetry findings, correlated with sFlt-1 and PlGF levels. We know that women who have abnormal uterine artery Doppler velocimetry - an increased PI and/or the presence of diastolic notching in the waveform are more likely to develop early onset pre-eclampsia compared to women who have uterine artery PIs within the normal range (23, 50, 252, 253). We also know that women with

pre-eclampsia have had placental ischaemia for several weeks before its clinical onset (249).
It also remains to be tested whether repeated IPC, applied daily for several weeks, would improve utero-placental blood flow and the sFlt-1:PlGF ratio.

There are also sources of sFlt-1 and PlGF, which are not placental in origin or pregnancy related. We know that sFlt-1 is expressed in many cell types including endothelial cells and renal mesangial cells (254-256). There has also been a recent study which found that high sFlt-1 levels were associated with a severe COVID-19 phenotype and a possible association between sFlt-1 upregulation and endothelial dysfunction and organ failure (257). PlGF is also expressed in other tissues, such as the heart and lung (258-261). Therefore, it may be that both local and remote IPC have a role in altering the levels of sFlt-1 and PlGF.

5.8 Limitations

As discussed, this section of the study was almost certainly underpowered, partly as the measures of sFlt-1 and PlGF were carried out towards the end of the study period, but before all clinical IPC studies in women with pre-eclampsia and the pregnant women at risk of pre-eclampsia had occurred.

It is also likely that even if I had studied 10 women with pre-eclampsia, that these numbers may still have lacked statistical power. The very large standard deviations in the pre-eclampsia group meant that despite the appearance of a trend towards an improvement in levels of sFlt-1 and PlGF these results were not statistically significant.

It would have also been interesting to have included additional women with early-onset pre-eclampsia (<34 weeks), with a more severe disease phenotype, as a single acute episode of IPC may have led to significant changes in sFlt-1 and PlGF levels in this cohort of women.

It would have also been interesting to carry out venesection at additional time-points, not only 24-hours after an acute episode of IPC. However, as discussed in the earlier chapters, given the time commitment this study already required from pregnant women, it was decided it was not appropriate to ask participants to attend multiple times.

5.9 Key Findings

- I confirmed higher plasma sFlt-1, lower plasma PlGF and higher sFlt1:PlGF ratios in women with pre-eclampsia when compared to healthy pregnant women and women at risk of pre-eclampsia.

- 24-hours after an acute episode of IPC, plasma levels of sFlt-1 and PlGF remained unchanged in all groups.

In the next chapter I will investigate the effect of an acute episode of IPC on VEGF, SDF-1α and DPP-4, all factors which have been implicated in the mechanistic pathways underlying remote IPC.

Chapter 6

Does ischaemic pre-conditioning alter circulating levels of VEGF, SDF-1α and DPP-4?

Chapter 6 - Does ischaemic pre-conditioning alter circulating levels of VEGF, SDF-1α and DPP-4?

6.1 Background

In this chapter I investigate whether factors that have been implicated in remote IPC in other studies are altered in my 4 groups of women: non-pregnant women, healthy pregnant women, pregnant women at risk of pre-eclampsia and pregnant women with pre-eclampsia. Specifically, I measured circulating levels of VEGF, SDF-1α and DPP-4 before and after an acute episode of IPC.

As discussed in chapter 1, women with pre-eclampsia have excess production of sFlt-1 that binds VEGF in the circulation and is thought to prevent its membrane-bound vasodilatory activity (4, 60, 61). In women with pre-eclampsia sFlt-1 levels are increased (60, 61, 78), however there have been mixed findings when measuring VEGF levels in normotensive pregnancy and pre-eclampsia (2, 61, 262-264). Interestingly, it has been reported that repeated application of remote IPC over at least 4 weeks increases plasma levels of VEGF (192, 193). It has been suggested that an increase in VEGF contributes to increasing levels of EPCs, which then increases NO availability (192, 194). I therefore it may be that an increase in VEGF would be detected following remote IPC in my study.

Remote IPC has been reported to increase plasma concentrations of SDF-1α (152, 179, 180). Cao et al showed that serum SDF-1α levels were higher at 24 and 48 hours in a group of patients that had remote IPC prior to primary percutaneous coronary intervention compared to those who did not (185). However, an important consideration is that as there are also high levels of SDF-1α within platelet granules, which can be expressed upon platelet activation. Therefore, serum levels of SDF-1α may be falsely elevated in comparison with plasma levels (186). Moreover, there have been some

studies that have found a reduction in plasma SDF-1α in conditions characterised by tissue hypoxia (180, 187, 188).

One complicating issue is that all commercially available antibodies have been raised against interna peptides within the protein sequence for SDF-1α, and therefore cannot distinguish between full length active SDF-1α(1-67) and cleaved inactive SDF-1α(3-67) which lacks only the N terminal 2 amino acids. Consequently, it has been postulated that commercial assays may provide mixed results due to the poor differentiation between full-length and cleaved SDF-1α (180). Therefore, results are presented in this chapter using both a commercial ELISA for total SDF-1α and the ELISA for active SDF-1α(1-67) for measurements of both total plasma SDF-1α and plasma active SDF-1α(1-67). Most published papers refer to total SDF-1α as 'SDF-1α'.

SDF-1α is cleaved by several peptidases, but the majority of SDF-1α is cleaved by the peptidase DPP-4. DPP-4 cleaves full length SDF-1α(1-67) to cleaved inactive SDF-1α(3-67) meaning it is unable to bind and activate the CXCR4 receptor (179). Therefore if remote IPC leads to a decrease in DPP-4, it may cause an increase in the levels of active SDF-1α(1-67).

I hypothesised that VEGF, SDF-1α and DPP-4 levels would be altered following an acute episode of IPC.

6.2 Study Design

The same groups were studied as in previous sections. The number of samples and sample type for each group and experimental test carried out are shown in table 6.1:

	Number of samples in each group studied (n)			
	Non-pregnant	Healthy Pregnant	At Risk	Pre-eclampsia
VEGF (plasma)	24	38	15	10
Total SDF-1α (plasma)	24	39	17	7
Active SDF(1-67) (platelet poor plasma)	9	38	11	9
DPP-4 (serum)	22	33	16	9

Table 6.1: Numbers of samples and sample type used for each experimental assay in all 4 groups

A blood sample was taken from each participant in the Fetal Medicine Unit in the EGA Wing of UCLH. The blood samples were centrifuged at the NIHR UCLH CRF, and stored in a -80°C freezer. The assays were carried out at The Hatter Cardiovascular Institute, UCL.

6.3 Study Participants

The participants were the same as in previous sections.

Measures of VEGF, total SDF-1α, active SDF(1-67) and DPP-4 were carried out at different times towards the end of the study period. Biological samples were also not available for all participants. The number of samples for these tests varied and were therefore as stated in table 6.1.

6.4 Study Protocol

Human VEGF and total SDF-1α levels were measured in plasma. Active SDF-1α(1-67) levels were measured in platelet poor plasma. Due to limited sample availability, DPP-4 levels were measured in serum, but results were expected to be the same as plasma DPP-4 measurements. All comparisons of proteins between groups were made from samples frozen stored in the same way.

The EDTA and serum venesection samples were centrifuged at 3000 rpm, for 10 minutes, at 4°C and then the serum and plasma samples aliquoted into 2ml Eppendorf tubes and stored in a -80°C freezer.

The sodium citrate tube samples were centrifuged at 1600 g for 20 minutes at room temperature. Then the plasma supernatant was transferred to 1.5 ml Eppendorf tubes and centrifuged at 10,000 g for 30 minutes, at room temperature. The platelet poor plasma supernatant was then transferred into 2ml Eppendorf tubes and stored in a -80 °C freezer.

Human VEGF and total SDF-1α levels were measured using commercially available quantitative sandwich ELISA kits from R+D systems (206, 207). All commercial ELISA studies were carried out in duplicate and quantified relative to a standard curve, according to manufacturer's instructions, as detailed in chapter 2.

Active human SDF-1α(1-67) was measured using an ELISA developed by Sean Davidson and Daniel Bromage, The Hatter Cardiovascular Institute, UCL (180). This ELISA was characterised using rh-SDF-1α taken from the R&D Systems Human CXCL12/SDF-1 alpha Quantikine ELISA Kit. This ELISA study was carried out in triplicate, as detailed in chapter 2.

DPP-4 activity was measured using a commercially available protease luminescence assay from Promega (DPPIV-Glo Protease Assay) (208).

All assays were analysed using a BMG Labtech Fluostar plate reader.

At the CRF and The Hatter Cardiovascular Institute I processed, stored and disposed of blood samples in accordance with all legal and regulatory requirements, including the Human Tissue Act 2004 and subsequent amendments.

6.5 Statistical Analysis

All statistical analysis was carried out using GraphPad Prism version 7 software. Data are presented as mean and standard deviation (SD) where appropriate. Statistical comparisons between the groups were carried out using a 1-way ANOVA, followed by the Kruskal-Wallis Test. If a difference was found, then a Dunn's multiple comparison test was carried out to determine significance. Statistical comparisons were carried out using a paired t-test with Bonferroni correction for comparisons within groups.

6.6 Results

For the purposes of clarity, when the results in this chapter are displayed as graphs, they will be as bar charts, rather than a scatter plot of individual data points (as in chapter 5).

VEGF

Baseline measures of VEGF are shown in table 6.2. Non-pregnant women had the highest levels of VEGF, when compared to the 3 other groups. Women with pre-eclampsia had the lowest levels of VEGF, which were significantly lower than the non-pregnant and healthy pregnant group.

VEGF (pg/ml) Mean (SD)			
Non-pregnant (n=24)	Healthy (n=38)	At risk (n=15)	Pre-eclampsia (n=10)
94.7(67.3)	12.0 (7.4)	10.5 (3.2)	7.7 (1.0)

Dunn's multiple comparison test showed significant differences in the following groups:

Non-pregnant vs Healthy $p < 0.001$

Non-pregnant vs At risk $p < 0.001$

Non- pregnant vs Pre-eclampsia $p < 0.001$

Healthy vs Pre-eclampsia p = 0.009

Table 6.2: VEGF levels at baseline in all 4 groups. Data are presented as mean and SD. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

VEGF levels pre and post remote IPC are shown in figure 6.1. There was a decrease in the non-pregnant group following IPC with a p value equal to 0.05. However, there are very wide standard deviations and no allowance was made for multiple testing, so this decrease may have been a chance variation.

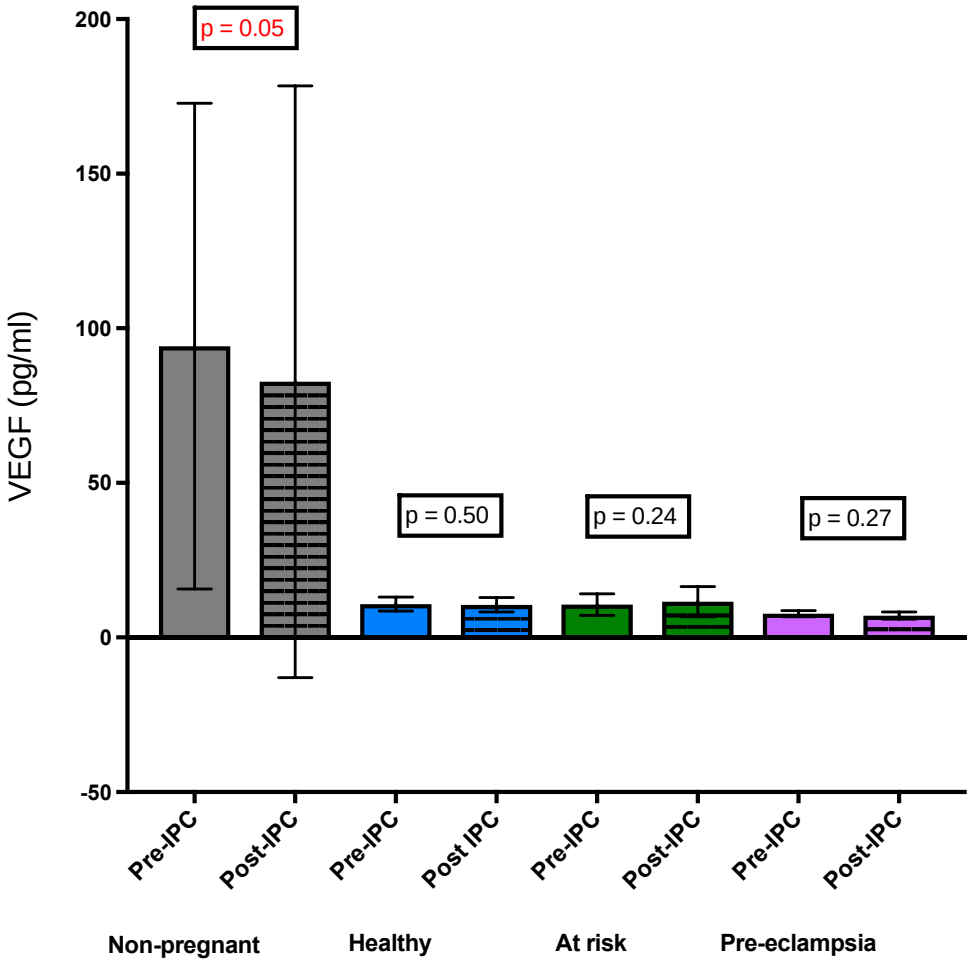


Figure 6.1: VEGF levels in all 4 groups pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction.

Total SDF-1α

Baseline measures of total SDF-1α are shown in table 6.3. Non-pregnant women and women with pre-eclampsia had the highest levels of total SDF-1α. There were significant differences when non-pregnant women were compared to the healthy and at risk groups; and when the pre-eclampsia groups were compared to the healthy and at risk groups.

Total SDF-1α (pg/ml) Mean (SD)			
Non-pregnant (n=24)	Healthy (n=39)	At risk (n=17)	Pre-eclampsia (n=7)
1604 (347)	891 (247)	917 (164)	1631 (409)

Dunn's multiple comparison test showed significant differences in the following groups:

Non-pregnant vs Healthy	$p < 0.001$
Non-pregnant vs At risk	$p < 0.001$
Healthy vs Pre-eclampsia	$p = 0.0005$
At risk vs Pre-eclampsia	$p = 0.01$

Table 6.3: Total SDF-1α levels at baseline in all 4 groups. Data are presented as mean and SD. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

Total SDF-1α levels pre and post IPC are shown in figure 6.2. There was a significant decrease in SDF-1α levels in healthy pregnant women and those with pre-eclampsia following IPC.

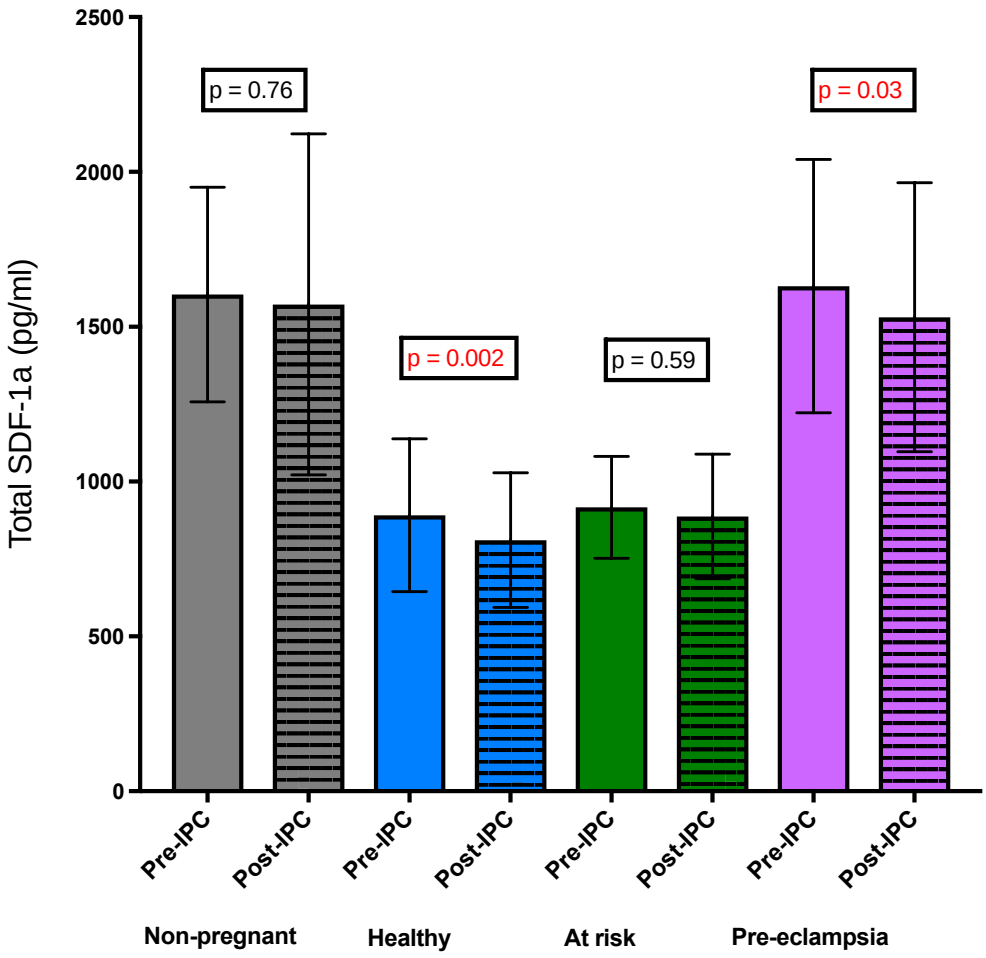


Figure 6.2: Total SDF-1α levels in non-pregnant, healthy, at risk and pre-eclampsia groups pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was a significant decrease in total SDF-1α levels in healthy pregnant women and those with pre-eclampsia following IPC.

Active SDF-1α(1-67)

Baseline measures of active SDF-1α(1-67) are shown in table 6.4. Non-pregnant women had the highest levels of active SDF-1α(1-67). There were no significant differences between any of the groups.

Active SDF-1α(1-67) (pg/ml) Mean (SD)			
Non-pregnant (n=9)	Healthy (n=33)	At risk (n=11)	Pre-eclampsia (n=9)
62 (36)	29 (20)	26 (16)	24 (11)

A 1-way ANOVA and the Kruskal-Wallis test did not detect any significant differences between any of the groups.

Table 6.4: Active SDF-1α(1-67) levels at baseline in all 4 groups. Data are presented as mean and SD. There were no significant differences between any of the groups.

Active SDF-1α(1-67) levels pre and post IPC are shown in figure 6.3. There were no significant differences between any of the groups.

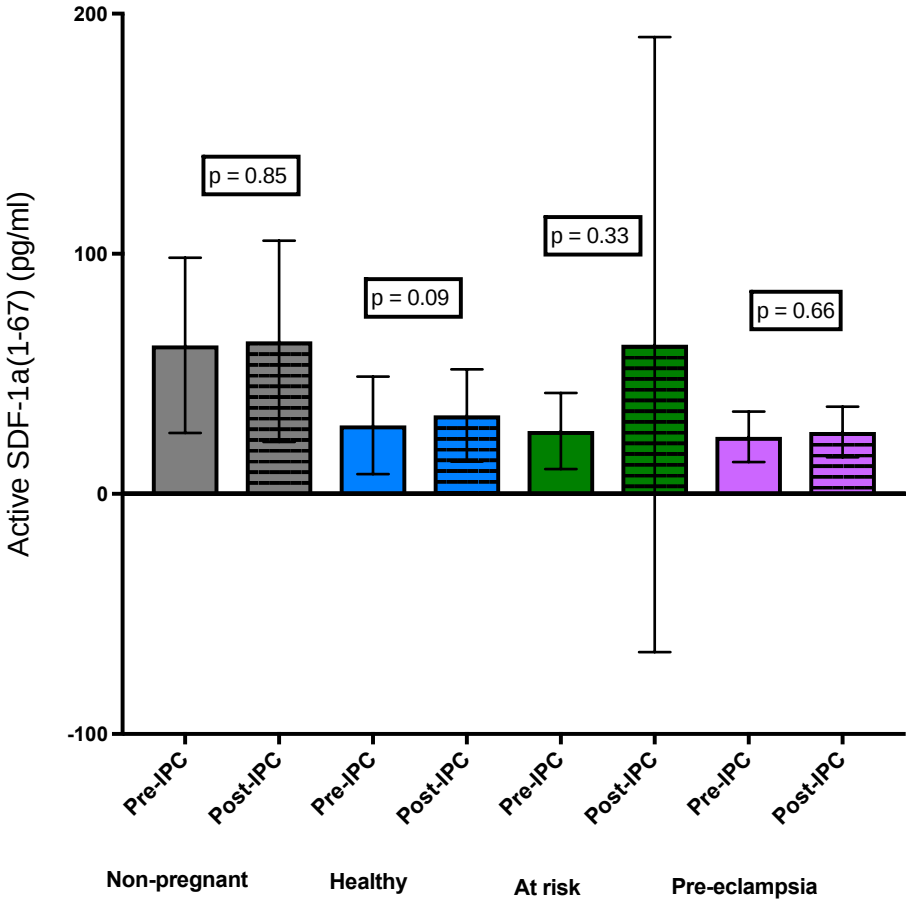


Figure 6.3: Active SDF-1α(1-67) levels in non-pregnant, healthy, at risk and pre-eclampsia groups pre and post IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There were no significant differences between any of the groups.

DPP-4

Baseline measures of DPP-4 are shown in table 6.5. Non-pregnant women had the highest levels of DPP-4, which were significantly higher when compared to the healthy, at risk and pre-eclampsia groups.

DPP-4 (pg/ml) Mean (SD)			
Non-pregnant (n=22)	Healthy (n=38)	At risk (n=16)	Pre-eclampsia (n=9)
1714 (261)	1116 (381)	1016 (306)	1242 (310)

Dunn's multiple comparison test showed significant differences in the following groups:

Non-pregnant vs Healthy	$p < 0.001$
Non-pregnant vs At risk	$p < 0.001$
Non-pregnant vs Pre-eclampsia	$p = 0.025$

Table 6.5: DPP-4 levels at baseline in all 4 groups. Data are presented as mean and SD. Significant differences detected by a 1-way ANOVA, the Kruskal-Wallis test and Dunn's multiple comparison test.

DPP-4 levels pre and post remote IPC are shown in figure 6.4. There was a significant decrease in the non-pregnant group, and a significant increase in the pre-eclampsia group following IPC.

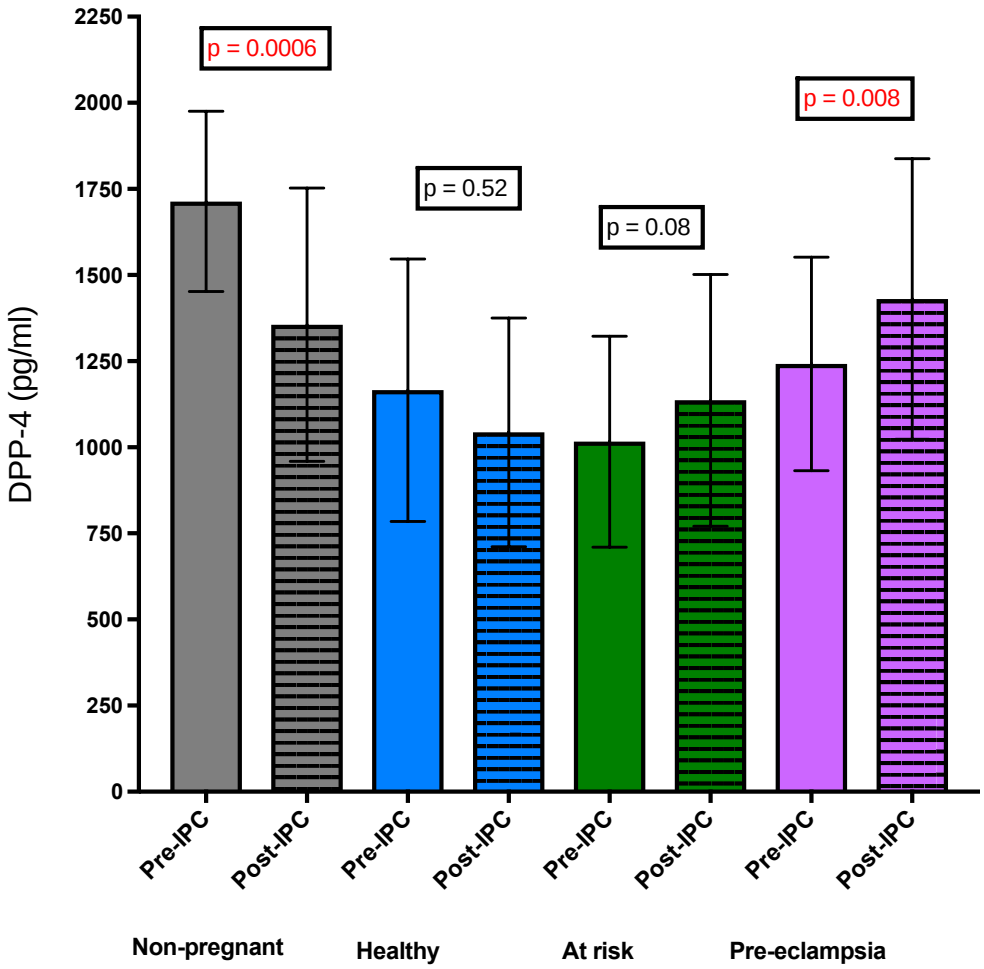


Figure 6.4: DPP-4 levels in all 4 groups pre and post remote IPC. Data are presented as mean and SD. Comparisons were made within each group using a paired t-test with Bonferroni correction. There was a significant decrease in the non-pregnant group, and a significant increase in the pre-eclampsia group following IPC.

6.7 Discussion

Very few previous studies have investigated SDF-1α levels in pre-eclampsia and normotensive pregnancies. One study found that the placental bed expression of SDF-1α was lower in pregnancies complicated by pre-eclampsia, when compared to normotensive pregnancies (265). Another study found that SDF-1α levels were higher in the serum of women with pre-eclampsia compared to healthy pregnant controls (266).

In this study non-pregnant women and women with pre-eclampsia had the highest baseline levels of total SDF-1α (table 6.3). It is interesting that the levels of total SDF-1α were very similar in the non-pregnant and pre-eclamptic groups. This may be a reflection of the fact that women with pre-eclampsia do not exhibit the cardiovascular system adaptions that occur in early pregnancy in normotensive healthy women (29, 31, 33) and therefore do not have the same significant vasodilated state when compared to normotensive women. Pregnant women with pre-eclampsia may therefore have a state of vasodilation similar to those of non-pregnant women, and this could be reflected in the similarity in total SDF-1α levels in these two groups.

There were no significant differences in baseline active SDF-1α(1-67) levels (table 6.4). However, for the non-pregnant group (n = 9) the analysis may have been underpowered, as the active SDF-1α(1-67) levels were much higher in the non-pregnant group compared to the other groups, but did not reach significance.

I found total SDF-1α levels decreased following one acute episode of IPC in healthy pregnant women and women with established pre-eclampsia (figure 6.3). However, I found no significant difference in active SDF-1α(1-67) levels in any groups following one acute episode of IPC (figure 6.3). Active SDF-1α(1-67) levels did increase non-significantly in all groups following an episode of acute IPC, and it is possible that, with a larger number of samples and an increase in statistical power, these results may have reached significance. As discussed earlier in this chapter, previous studies have found differing results with regard to the impact of remote IPC on total SDF-1α levels. Some studies have demonstrated an increase in levels of total SDF-1α following remote IPC (152, 179, 185) and others have shown a decrease in levels of total SDF-1α following remote IPC (180, 187, 188). Furthermore, a proteomic analysis of human plasma samples, following remote IPC, did not find SDF-1α as one of the candidates being upregulated (267).

I found that non-pregnant women had the highest baseline levels of DPP-4 (table 6.5), which were significantly higher when compared to the 3 pregnant groups. I found that DPP-4 levels decreased following remote IPC in non-pregnant women, but increased in women with pre-eclampsia following remote IPC (figure 6.4).

For the non-pregnant group the decrease in DPP-4 was not correlated with an increase in total SDF-1α or active SDF-1α(1-67). This was surprising given that the majority of active SDF-1α(1-67) is cleaved by DPP-4 (179) and therefore one would expect a decrease in DPP-4 to lead to an increase in active SDF-1α(1-67) as well as total SDF-1α.

However, in the women with pre-eclampsia, following remote IPC, the increase in DPP-4 was correlated with a fall in total SDF-1α, which can be explained by DPP-4's mechanism in cleaving full length SDF-1α(1-67) to inactive SDF-1α(3-67). This finding is also supported by previous studies that found a reduction in SDF-1α levels in conditions characterised by tissue hypoxia (180, 187, 188).

I did not calculate a ratio of active SDF-1α(1-67) to total SDF-1α, as the ELISAs were carried out on different sample types (platelet poor plasma versus plasma), with some differences in the participants used for each ELISA. This would have been a useful analysis to have carried out, as it may have been that an acute episode of IPC altered the ratio of active SDF-1α(1-67) to total SDF-1α.

Baseline levels of VEGF were highest in non-pregnant women; and were significantly higher when this group was compared to all pregnant groups (table 6.2). This is in keeping with two previous studies (268, 269), which found VEGF levels were highest in non-pregnant women, and lower in normotensive pregnant women and women with pre-eclampsia. The authors postulated that this may be explained by pregnant women having enhanced

production of the soluble VEGF receptor, which acts as a VEGF antagonist by binding VEGF (268).

In my study, baseline levels of VEGF were significantly higher in healthy pregnant women when compared to baseline VEGF levels in women with pre-eclampsia (table 6.2). However, as previously mentioned there have been mixed findings when measuring VEGF levels in normotensive pregnancy and pre-eclampsia (2, 61, 262-264). One study, with similar findings to my study but with a larger number of participants, found that VEGF levels were lower in the serum of women who had pre-eclampsia, compared to normotensive pregnant women, when samples were taken at 37 to 41 weeks gestation (61). This study also found that for samples taken at 21 to 32 weeks gestation, the VEGF levels were lower in a group of women who developed pre-eclampsia within 5 weeks of the specimen being taken (61). However, another of these studies, which had comparable participant numbers to my study, found that prior to the onset of pre-eclampsia, serum VEGF levels were significantly elevated in the group of pregnant women who later went on to develop pre-eclampsia compared to normotensive pregnant women and women with gestational hypertension (264). This study also found that VEGF samples were significantly elevated in women with pre-eclampsia who had samples taken 24-hours prior to delivery, compared to normotensive pregnant women and women with gestational hypertension (264). Another study looked at VEGF expression in placentae and found that significantly higher numbers of pre-eclamptic placentae expressed VEGF when compared to placentas from normotensive pregnancies (2). One reason for the differences in VEGF levels in these studies may be related to the different VEGF isoforms that exist (65, 270) and as previously discussed we are yet to fully understand the role VEGF plays in normotensive pregnancy and pre-eclampsia.

Following an acute episode of IPC, there was no convincing change in VEGF levels in the non-pregnant group (figure 6.1). The standard deviations of the estimates were very wide and no allowance was made for multiple testing, so the decrease that can be seen could well be due to chance. VEGF levels did not change in any of the pregnant groups following IPC (figure 6.1). The

human studies that have reported an increase in VEGF levels following IPC used repeated remote IPC as their stimulus (192, 193), therefore it may be necessary to carry out repeated episodes of remote IPC to obtain a detectable increase in VEGF levels. One of these studies found that an increase in serum VEGF levels correlated with an improvement in EPCs (192). An increase in EPCs has been postulated to lead to an increase in systemic NO concentrations, which may be partly responsible for an improvement in endothelial function following local and remote IPC (192, 194). It is also known that in response to hypoxia, hypoxia-inducible factor (HIF) accumulates (159, 271). HIF-1 and its subunit HIF-1α regulate the transcription of genes leading to protective cellular responses, including an increase in the growth factor VEGF (135, 271, 272). This may be a pathway by which IPC increases VEGF levels.

6.8 Limitations

It would have been preferable to be able to study samples from a greater number of individuals in all groups. Larger numbers would have added statistical power to all the analyses, and especially that of active SDF-1α(1-67) where I only had 7 samples from women with pre-eclampsia and 9 samples from healthy pregnant women.

It would have also been interesting to carry out venesection at additional time-points, not only 24-hours after an acute episode of IPC. However, as discussed in the earlier chapters, given the time commitment this study already required from pregnant women, it was decided it was not appropriate to ask participants to attend multiple times.

It would have been interesting to calculate a ratio of active SDF-1α(1-67) to total SDF-1α for all study participants. This would require all participants to have platelet poor plasma samples taken. Some of the participants were challenging to carry out venesection on, and taking plasma and serum samples was prioritised over platelet poor plasma samples.

6.9 Key Findings

The key findings in this study were:

- Total SDF-1α levels decreased 24-hours after an acute episode of IPC in the healthy pregnant women with established pre-eclampsia

- There was no difference in active SDF-1α (1-67) levels in any groups 24-hours after an acute episode of IPC

- DPP-4 activity was decreased 24-hours after an acute episode of IPC in non-pregnant women and increased in women with pre-eclampsia

In the next chapter I will summarise the key findings, conclusions and discuss potential further work.

CHAPTER 7

DISCUSSION AND FUTURE RESEARCH

Chapter 7 – Discussion and future research

7.1 Key Findings and Conclusions

Local IPC and endothelial function

During my research, I made 2 significant discoveries with regards the effect of an acute episode of local IPC on maternal endothelial function.

A. Women with pre-eclampsia and those at risk of pre-eclampsia had reduced endothelial function, as measured by brachial artery FMD, which improved 24-hours after an acute episode of local IPC.

This is the first reported study of IPC in pre-eclampsia. It is exciting that I have demonstrated that one acute episode of local IPC is able to improve local endothelial function, when measured 24-hours after the acute IPC stimulus, in women with pre-eclampsia and at risk of pre-eclampsia, although this finding requires replication. As IPC is low-risk, cost-effective and relatively simple, it warrants further investigation as a non-pharmacological prophylaxis or treatment to improve endothelial function and pregnancy outcome for women with pre-eclampsia or pregnant women at risk of pre-eclampsia. If effective, repeated IPC could be used in low-resource settings where maternal and perinatal morbidity from pre-eclampsia is highest and where a simple, effective and cheap prophylaxis against pre-eclampsia would be invaluable.

B. In healthy normotensive pregnant women, an acute episode of local IPC shortened the time taken to reach peak brachial artery dilatation.

This is the first reported study of IPC in healthy normotensive pregnancy. The significant shortening in the ‘time to peak’ brachial artery dilatation, rather than an increase in peak brachial arterial dilatation itself, may reflect a

physiological improvement in endothelial function, once FMD has reached its maximum due to gestational vasodilatation.

The impact of IPC on factors involved in pre-eclampsia

I had hypothesised that an acute episode of IPC may improve the maternal angiogeneic profile in women with pre-eclampsia, but elevated sFlt-1 levels and low PlGF levels did not change 24-hours after an acute episode of IPC. One acute episode of IPC may have transiently alleviated ischaemia in the utero-placental arteries, however it is possible that this was insufficient to alter angiogenic factors in the time-scale of my study. Also worth noting is that there was wide variability in the levels of these angiogenic factors in women with pre-eclampsia, so despite the appearance of a trend towards an improvement in levels of sFlt-1, PlGF and the ratio of sFlt-1:PlGF these results were not statistically significant. In future work it would be interesting to investigate the impact of repeated IPC on sFlt-1 levels, PlGF levels and the ratio between plasma sFlt-1 and PlGF.

Mechanism underlying IPC

With regard to the possible mechanism underlying remote IPC, I investigated whether there would be changes, after 24 hours, in the levels of total SDF-1α, active SDF-1α(1-67), DPP-4 and VEGF following one acute episode of IPC.

Levels of active SDF-1α(1-67) increased non-significantly in all groups following remote IPC. It may be with a larger number of samples and an increase in statistical power, these results may reach significance and would provide some evidence to support the role of active SDF-1α(1-67) in the mechanism underlying remote IPC.

In the group of women with pre-eclampsia there was a decrease in total SDF-1α levels and an increase in DPP-4 activity following remote IPC. This finding

in isolation does not provide any conclusive evidence for the role of SDF-1α in IPC however, I feel that it would be interesting to further investigate the candidates SDF-1α and DPP-4. It would be prudent to repeat these measures with not only a larger sample size, but also to calculate a ratio of active SDF-1α(1-67) to total SDF-1α to investigate this mechanistic pathway further.

In non-pregnant women there was a decrease in VEGF levels following IPC, but no change in the levels of VEGF following IPC in any of the pregnant groups. Human studies that have reported an increase in VEGF levels following IPC used repeated remote IPC as their stimulus (192, 193), and therefore it may be necessary to carry out repeated remote IPC in order to obtain a detectable change in VEGF levels in healthy pregnancy and pre-eclampsia.

7.2 Future Research

This thesis has generated several results that deserve further investigation. Most importantly is the potential for local IPC to improve outcomes for pregnant women with hypertensive disorders in pregnancy.

1. It would be valuable to explore whether endothelial function in women with pre-eclampsia improves following remote rather than local IPC. This could be done by measuring brachial artery FMD in both the arm that had the local IPC stimulus and the contralateral arm. It would also be prudent, in future work, to include both traditional and allometrically scaled FMD data. If remote, as well as local, IPC improves endothelial function in women with pre-eclampsia then IPC may have a role in improving pregnancy outcomes for these women, as well as potentially reducing their life-long risk of future cardiovascular events.

2. Similarly, it would be exciting to discover whether remote IPC improves uterine artery Doppler velocimetry in women with pre-eclampsia or at risk of pre-eclampsia. If remote IPC is able to condition the placenta,

and ultimately decrease placental ischaemia, then it would be a fantastic discovery in terms of managing a condition that has no cure, aside from childbirth.

3. Additionally, it would be valuable to carry out an investigation into the use of repeated episodes of local and remote IPC – perhaps applied daily for several weeks – and to see whether this further improves not only endothelial function and utero-placental blood flow, but also maternal blood pressure. Repeated remote IPC may be necessary to improve pregnancy outcomes for women with pre-eclampsia and at risk of pre-eclampsia.

4. It may have been that measuring levels of factors involved in pre-eclampsia and potential candidates involved in the mechanism underlying IPC as a one-off 24-hours after an IPC stimulus meant that significant changes were missed. Therefore, in future work, measuring levels of sFlt-1, PlGF, total SDF-1α, active SDF-1α(1-67), DPP-4 and VEGF at multiple time-points following an IPC stimulus may yield significant results. For example, measuring levels prior to the IPC stimulus, immediately after the IPC stimulus, at 4-hours, 24-hours and 48-hours would give further information of the levels of these factors during both the first and the second window of protection following IPC.

5. Of the other proposed pathways involved in the mechanism underlying IPC it would be valuable to investigate the systemic inflammatory response following remote IPC. In this pathway - the systemic pathway - it is postulated that remote IPC has an effect on the systemic inflammatory response, which then prevents the exacerbation of ischaemic damage (159). There is evidence that pre-eclampsia is associated with a pro-inflammatory systemic environment (84, 86), including the upregulation of some pro-inflammatory cytokines (27, 87, 88). Therefore, if remote IPC can modulate systemic inflammation and potentially increase the levels of anti-inflammatory cytokines (271-275)

then this could provide further evidence to support the use of remote IPC in pre-eclampsia.

7.3 Summary

This thesis provides compelling preliminary evidence that local IPC improves maternal endothelial function, in women with pre-eclampsia and pregnant women at risk of pre-eclampsia.

Whether IPC improves remote endothelial function in women with pre-eclampsia, and whether repeated IPC can improve pregnancy outcomes in women with pre-eclampsia remains to be tested.

The non-invasive low-risk nature of IPC, coupled with its relative simplicity and cost-effectiveness, make it an attractive intervention worthy of further investigation as a non-pharmacological prophylaxis or treatment of pre-eclampsia. If IPC could prevent pre-eclampsia, or safely prolong pregnancy in women with pre-term pre-eclampsia, it would not only improve pregnancy outcomes for these women, but may also reduce their life-long risk of future cardiovascular disease. A simple, low-risk intervention such as IPC would also be invaluable to low resource settings, where maternal and perinatal morbidity from pre-eclampsia is highest.

My results support the need for further investigation of local IPC, remote IPC and repeated IPC in preventing the development of pre-eclampsia and ameliorating the severity of disease.

APPENDICES

Appendix 1 – Participant information sheet for non-pregnant women, healthy pregnant women and pregnant women at risk of pre-eclampsia

University College London Hospitals NHS
NHS Foundation Trust

Participant Information Sheet

The VAMPS study

(Vasoactivity of Maternal Pregnancy Study)

('PIS; RIPC' v20; 29.05.2018)

Researchers:

Dr David Williams, Consultant Obstetric Physician
Dr Tamara Kubba, Clinical Research Fellow

A Study to Prevent Pre-eclampsia

Pre-eclampsia is a serious condition of pregnancy that affects both mother and baby. It is recognised by the new onset of high blood pressure (hypertension) and protein in the urine (proteinuria) after 20 weeks' gestation, but can cause serious complications for baby and mother. Currently, there is no reliable method of preventing pre-eclampsia in women who are at high risk of the condition. Once pre-eclampsia has developed, the only cure is childbirth. Premature childbirth can cause complications for the new born baby.

Pregnant women who have certain risk factors are at increased risk of developing pre-eclampsia. **This study aims to discover whether we can reduce the risk of pre-eclampsia in women at high risk of the condition.**

Remote ischaemia pre-conditioning (RIPC)

Men and non-pregnant women who have heart or kidney disease can be protected from the effects of reduced blood flow by a technique called 'remote ischemia pre-conditioning' (RIPC). This simple and safe technique involves inflating and deflating a blood pressure cuff around the upper arm for 5 minutes for 3 times. We wish to investigate whether RIPC can reduce the risk of pre-eclampsia in women at high risk of the condition.

If we find that remote ischemia pre-conditioning improves the blood flow and reduces blood pressure then we may have discovered an effective treatment to delay or prevent the onset of pre-eclampsia and improve the outcome for both mother and baby.

Why have I been invited?

We would like to include you in our study because you fit into one of the following groups:

Group 1: Pregnant, at risk of pre-eclampsia.

Group 2: Pregnant, with a normal grown baby and normal blood pressure.

Group 3: Not pregnant.

Do I have to take part?

No. You are completely free to decide if you want to take part or not. If you decide to take part you will be asked to sign a consent form prior to your participation. You are free to drop out or withdraw from the study at any time and without giving a reason. A decision to withdraw will not affect the standard of care you receive in anyway, now or in the future.

What will happen to me if I take part?

This study requires 2 visits to our clinical research unit.

First visit

1. **Upper Arm Flow Mediated Dilatation**

   Using an ultrasound probe similar to that used to look at your baby, we will measure blood flow in the arm (brachial) artery. We will inflate a cuff (like that to measure blood pressure) for 5 minutes and measure blood flow before, during and after this time. The whole study will take about 30-40 minutes. Our team have carried out this test many times before, during and after pregnancy.

2. **A 20mL blood test (2 tablespoons)**

3. **A urine sample**

4. **A check of your blood pressure and heart rate**

5. **Remote Ischemic Pre-conditioning (RIPC).** This involves an upper arm cuff inflated (up) for 5 minutes and then deflated (down) for 5 minutes on 3 occasions (i.e. a total of 30 minutes of your time).

Second visit – 24 hours later

1. **Upper Arm Flow Mediated Dilatation (as described above)**

2. **A 20mL blood test (2 tablespoons)**

3. **A urine test**

4. **A check of your blood pressure and heart rate**

What are the possible disadvantages or risks of taking part?

1. **Taking blood**

Minor discomfort may be experienced with taking blood and a small bruise may occur afterwards.

2. **Measurement of blood flow in upper arm and remote ischemic pre-conditioning**

The cuff that is inflated above normal blood pressure for 5 minutes may cause discomfort, but has been well tolerated by children and pregnant women as well as men, who have taken part in similar studies.

What are the possible benefits of taking part?

We expect this study to increase our understanding of how pre-eclampsia develops. Furthermore, we will discover whether we can prevent or delay the onset of pre-eclampsia in women at high risk of developing the condition. If successful, we will extend this investigation to determine if regular RIPC can help improve the outcome for mother and baby.

Travel costs to and from University College Hospital to participate in the study and baby-care whilst I am involved in the study

We will assist your participation in the study by providing travel costs for you to come to UCLH and return home. With prior arrangement, we can also provide baby care while you are participating in the study.

How will my confidentiality be protected?

Only the researchers involved in this study will have access to the data collected in the course of this study. Any information you give us will only be used in the course of the study. No data will be published that allows for any individual to be identified in any way.

Will my GP know that I am in this study?

With your consent we will inform your GP of your participation in this study. In the unlikely event that our tests reveal something about you, which we feel your GP should be made aware, we will discuss this with you and notify your GP so that appropriate advice and treatment may be given to you.

What will happen to the results of the research study?

They will be stored in an encrypted way on hospital and university based computers, which will only be accessible with a personal password owned by the researchers. All data will be coded for recognition by clinical members of the study group.

If you are interested, we will send you an easy-to-understand summary of the results when they are published.

Who has reviewed the study?

This study has been independently approved by The NRES Committee - Westminster. The Research Ethics reference number for this study is 12/LO/1449.

Complaints

If you have any comments or concerns you may discuss these with the investigators. If you wish to go further and complain about any aspect of the way in which you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL Hospitals. Please ask Dr Kubba or Dr Williams if you would like more information on this. Details can also be obtained from the Department of Health website: http://www.dh.gov.uk.

What if there is a problem?

Every care will be taken in the course of this study. However, in the unlikely event that you are injured by taking part, compensation may be available.

University College London holds insurance against claims from participants for injury caused by their participation in this clinical study. Participants may be able to claim compensation if UCL has been negligent. However, as this clinical study is being carried out on the UCL Hospital site, the hospital continues to have a duty of care to the participant of this study. University College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. University College London Hospital has clinical negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary can be provided on request. If you suspect that the injury is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. You can discuss any issues with Dr Kubba or Dr Williams, or the complaints manager at UCL Hospital. A claim can be made in writing to Dr Williams, the Chief Investigator.

Contact for further information:

Dr Tamara Kubba - Clinical Research Fellow
Email: t.kubba@doctors.org.uk mobile: 07968 507882
or
Dr David Williams - Consultant Obstetric Physician
Email: d.j.williams@ucl.ac.uk

Version 20: 29.05.2018 REC reference: 12/LO/1449

Appendix 2 – Participant information sheet for women with pre-eclampsia

University College London Hospitals NHS
NHS Foundation Trust

Participant Information Sheet

The VAMPS study

(Vasoactivity of Maternal Pregnancy Study)

('PIS; RIPC' v20; 29.05.2018)

Researchers:

Dr David Williams, Consultant Obstetric Physician
Dr Tamara Kubba, Clinical Research Fellow

A Study to Prevent Pre-eclampsia

Pre-eclampsia is a serious condition of pregnancy that affects both mother and baby. It is recognised by the new onset of high blood pressure (hypertension) and protein in the urine (proteinuria) after 20 weeks' gestation, but can cause serious complications for baby and mother. Currently, there is no reliable method of preventing or treating pre-eclampsia. Once pre-eclampsia has developed, the only cure is childbirth. Premature childbirth can cause complications for the new born baby.

Remote ischaemia pre-conditioning (RIPC)

Men and non-pregnant women who have heart or kidney disease can be protected from the effects of reduced blood flow by a technique called 'remote ischemia pre-conditioning' (RIPC). This simple and safe technique involves inflating and deflating a blood pressure cuff around the upper arm for 5 minutes for 3 times. We wish to investigate whether RIPC can improve blood vessel health in women who have developed pre-eclampsia.

If we find that remote ischemia pre-conditioning improves the blood flow to the womb and reduces blood pressure then we may have discovered an effective treatment of pre-eclampsia, which will improve the outcome for both mother and baby.

Why have I been invited?

We would like to include you in our study because you have been admitted to the antenatal ward with pre-eclampsia.

Do I have to take part?

No. You are completely free to decide if you want to take part or not. If you decide to take part you will be asked to sign a consent form prior to your participation. You are free to drop out or withdraw from the study at any time and without giving a reason. A decision to withdraw will not affect the standard of care you receive in anyway, now or in the future.

What will happen to me if I take part?

This study requires 2 visits to our clinical research unit.

First visit

1. **Upper Arm Flow Mediated Dilatation**

Using an ultrasound probe similar to that used to look at your baby, we will measure blood flow in the arm (brachial) artery. We will inflate a cuff (like that to measure blood pressure) for 5 minutes and measure blood flow before, during and after this time. The whole study will take about 30-40 minutes. Our team have carried out this test many times before, during and after pregnancy.

2. **A 20mL blood test (2 tablespoons)**

3. **A urine sample**

4. **A check of your blood pressure and heart rate**

5. **Remote Ischemic Pre-conditioning (RIPC).** This involves an upper arm cuff inflated (up) for 5 minutes and then deflated (down) for 5 minutes on 3 occasions (i.e. a total of 30 minutes of your time).

Second visit – 24 hours later

1. **Upper Arm Flow Mediated Dilatation (as described above)**

2. **A 20mL blood test (2 tablespoons)**

3. **A urine test**

4. **A check of your blood pressure and heart rate**

What are the possible disadvantages or risks of taking part?

1. **Taking blood**

Minor discomfort may be experienced with taking blood and a small bruise may occur afterwards.

2. Measurement of blood flow in upper arm and remote ischemic pre-conditioning

The cuff that is inflated above normal blood pressure for 5 minutes may cause discomfort, but has been well tolerated by children and pregnant women as well as men, who have taken part in similar studies.

What are the possible benefits of taking part?

We expect this study to increase our understanding of the pathology of pre-eclampsia. Furthermore, we will discover whether we can treat pre-eclampsia. If successful, we will extend this investigation to determine if regular RIPC can help improve the outcome for mother and baby.

How will my confidentiality be protected?

Only the researchers involved in this study will have access to the data collected in the course of this study. Any information you give us will only be used in the course of the study. No data will be published that allows for any individual to be identified in any way.

Will my GP know that I am in this study?

With your consent we will inform your GP of your participation in this study. In the unlikely event that our tests reveal something about you, which we feel your GP should be made aware, we will discuss this with you and notify your GP so that appropriate advice and treatment may be given to you.

What will happen to the results of the research study?

They will be stored in an encrypted way on hospital and university based computers, which will only be accessible with a personal password owned by the researchers. All data will be coded for recognition by clinical members of the study group.

If you are interested, we will send you an easy-to-understand summary of the results when they are published.

Who has reviewed the study?

This study has been independently approved by The NRES Committee - Westminster. The Research Ethics reference number for this study is 12/LO/1449.

Complaints

If you have any comments or concerns you may discuss these with the investigators. If you wish to go further and complain about any aspect of the way in which you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL Hospitals. Please ask Dr Kubba or Dr Williams if you would like more information on this. Details can also be obtained from the Department of Health website: http://www.dh.gov.uk.

What if there is a problem?

Every care will be taken in the course of this study. However, in the unlikely event that you are injured by taking part, compensation may be available.

University College London holds insurance against claims from participants for injury caused by their participation in this clinical study. Participants may be able to claim compensation if UCL has been negligent. However, as this clinical study is being carried out on the UCL Hospital site, the hospital continues to have a duty of care to the participant of this study. University College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. University College London Hospital has clinical negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary can be provided on request. If you suspect that the injury is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. You can discuss any issues with Dr Kubba or Dr Williams, or the complaints manager at UCL Hospital. A claim can be made in writing to Dr Williams, the Chief Investigator.

Contact for further information:

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Email: t.kubba@doctors.org.uk mobile: 07968 507882
or
Dr David Williams - Consultant Obstetric Physician
Email: d.j.williams@ucl.ac.uk

Version 20: 29.05.2018 REC reference: 12/LO/1449

Appendix 3 – Non-pregnant consent form

University College London Hospitals NHS
NHS Foundation Trust

NON-PREGNANT CONSENT FORM
Vasoactivity of Maternal Pregnancy Serum Study
(VAMPS 1)

Name of Researchers:

Dr David Williams, Consultant Obstetric Physician
Dr Tamara Kubba, Academic Clinical Fellow

Patient Identification Number for this trial: **Please initial boxes**

1	I agree to take part in the above study	
2	I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.	
3	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
4	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from University College London, from regulatory authorities or from the NHS Trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	
5	I agree for my blood to be gifted to UCLH and stored for future studies that would be subject to future ethics committee approval	
6	I agree to my GP being informed of any clinically significant results.	
7	I would like to receive details regarding the results of this study once it has been published	
8	I agree to be contacted after the initial study has taken place to be asked if I will consider being involved in follow-up studies.	

_____	_____	_____
Name of Participant	Signature	Date
_____	_____	_____
Name of Person taking consent	Signature	Date

1 copy for patient, 1 copy for researcher, 1 copy to be kept with hospital notes Version 10
10.08.12

REC reference: 12/LO/1449

Appendix 4 – Maternal (pregnant) consent form

University College London Hospitals NHS
NHS Foundation Trust

MATERNAL CONSENT FORM
Vasoactivity of Maternal Pregnancy Serum Study
(VAMPS 1)

Name of Researchers:

Dr David Williams, Consultant Obstetric Physician
Dr Tamara Kubba, Academic Clinical Fellow

Patient Identification Number for this trial: **Please initial boxes**

1	I agree to take part in the above study	
2	I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.	
3	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
4	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from University College London, from regulatory authorities or from the NHS Trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	
5	I agree for my blood to be gifted to UCLH and stored for future studies that would be subject to future ethics committee approval	
6	I agree to my GP being informed of any clinically significant results.	
7	I would like to receive details regarding the results of this study once it has been published	
8	I agree to be contacted after the initial study has taken place to be asked if I will consider being involved in follow-up studies.	

_____	_____	_____
Name of Participant	Signature	Date
_____	_____	_____
Name of Person taking consent	Signature	Date

1 copy for patient, 1 copy for researcher, 1 copy to be kept with hospital notes Version 10
10.08.12

REC reference: 12/LO/1449

Appendix 5 – Questionnaire for non-pregnant participants

VAMPS Questionnaire – Non pregnant

Date

Time of study

Study ID

DOB

LMP

Parity

Last ate

Last Caffeine

Last Exercise

Current medication

PMH

FH

Height

Weight

BMI

Other information

Appendix 6 – Questionnaire for pregnant participants

VAMPS Questionnaire – Pregnant

Date

Time of study

Study ID

DOB

EDD

Gestation

Parity

Last ate

Last Caffeine

Last Exercise

Current medication

PMH

FH

Height

Weight

BMI

Other information

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