Relationship between endothelial cell dysfunction and insulin signalling and resistance in pre-eclampsia.

Anshuman Ghosh

Supervisors:

Dr N.S. Freestone

Dr F.I. Arrigoni

Mr N. Anim-Nyame

Faculty of Science, Kingston University London.

Acknowledgement

I will take this opportunity to thank my supervisors, Dr N Freestone, Dr FI Arrigoni, and Mr N Anim-Nyame, for giving me the opportunity to undertake this research project. All my supervisors have been very helpful in their encouragement and support throughout the project. I'm also grateful to them, for reading and making valuable changes to my thesis. They were also helpful in teaching me all the methods used in this project. Mr Anim-Nyame also allowed me to use his plethysmograph for the study.

I'm also thankful to Dr A Snabaitis, for helping me master Western blotting. I'm also thankful to my fellow researchers who helped to keep up my morale and suggested very useful tips throughout the whole project. I also want to thank the participants of the study, who fasted for me during the study. I also want to thank my wife, Dr Madhusree Ghosh, for taking time to do the illustrations in this thesis. I also thank all the authors who kindly granted me permission to use the illustrations from their work. The photos are of machines actually used in this study.

Lastly, I want to thank Kingston University Research Grant and the Insulin Dependent Diabetes Mellitus Trust for valuable funding for this study.

Abstract

Aim: The aim of the thesis was to investigate the hypothesis that (i) Changes in endothelial cell insulin signalling occur in pre-eclampsia, secondary to underlying endothelial dysfunction, resulting in insulin resistance, (ii) Impaired endothelial cell insulin signalling results in reduced tissue delivery of insulin and reduced GLUT-4 activation, and (iii) Impaired microvascular blood flow results in insulin resistance.

Methods: Filtrass strain-gauge plethysmograph was used to measure human calf blood flow in women with pre-eclampsia and normotensive controls. Biochemical markers of endothelial dysfunction, ICAM-1, VCAM-1. TNF, eSelectin, Thrombomodulin, and cellular marker, CEC, provided information regarding endothelial dysfunction. Insulin resistance was calculated using HOMA. Cells were cultured in normotensive and pre-eclamptic serum to study the insulin signalling pathway, using flow cytometry and western Blot.

Results: In this cross-sectional study, microvascular blood flow was reduced in the pre-eclamptic cohort, compared to normotensive controls. Insulin resistance was also increased in women with pre-eclampsia. Endothelial cell insulin receptor expression and Akt expression were reduced in the pre-eclamptic participants, compared to normotensive pregnant controls. However, there was no significant difference in total insulin receptor protein and Akt protein in between the two groups. There was also no difference in endothelial cell GLUT 4 expression in between the groups.

Conclusion: Insulin Resistance in pre-eclampsia, correlates with endothelial dysfunction and microvascular blood flow. Although expression of insulin receptors and Akt in endothelial cells, are reduced in pre-eclampsia, this does not correlate with insulin resistance. Furthermore, as there is no change in endothelial cell GLUT4 expression, in between the two cohorts, it is unlikely to explain the Insulin Resistance seen in pre-eclampsia.

Contents

List of figures:

Chapter 1

Chapter 2

Chapter 3

Appendix

List of tables:

Abbreviations

ATP	Adenosine triphosphate
APS	Ammonium persulfate
BMI	Body Mass Index
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEC	Circulating endothelia cell
cGMP	cyclic guanosine monophosphate
DBP	Diastolic blood pressure
dd H_2O	double distilled water
EDHF	Endothelium derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial Nitric Oxide synthase
ET-1	Endothelin-1
FFA	Free fatty acids
FMD	Flow-mediated dilatation
GLUT	Glucose transporters
HDL	High density lipoprotein
HDMEC	Human Dermal Microvascular Endothelial Cells
HLA-G	Human Leucocyte Antigen-G
HOMA	Homeostasis Model Assessment
ICAM-1	Inter-Cellular Adhesion Molecule-1
IL	Interleukin
IR	Insulin resistance
LDL	Low density lipoprotein
MAP	Mean arterial pressure

NK	Natural Killer cells
NO	Nitric Oxide
PBS	Phosphate buffered saline
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol-3-kinase
PlGF	Placenta growth factor
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
sEng	soluble Endoglin
sFlt-1	Soluble fms-like tyrosine kinase 1/ soluble Vascular Endothelial Growth Factor Receptor 1
SGLT	Sodium-dependent glucose transporters
STBM	Syncytiotrophoblast microvillous membrane particles
SVR	Systemic vascular resistance
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF-β	Transforming Growth Factor β
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumour Necrosis Factor-α
TXA_2	Thromboxane A_2
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

Chapter 1: General Introduction

1.1. Normal pregnancy

Pregnancy is defined as the time during which one or more offspring develops in the body of the females, from conception to delivery. A woman's body undergoes changes to facilitate the growing foetus, including cardiovascular, haematological, metabolic, endocrine, renal and respiratory changes. All these become more important in the event of any complications.

1.1.1. Maternal haemodynamic adaptation to pregnancy

Maternal circulation also undergoes marked changes during pregnancy. These changes are mechanisms that the body has adapted to support the increased metabolic demands of the mother and the foetus, and to ensure adequate uteroplacental circulation for foetal growth and development.

1.1.1.1. Cardiac output

Cardiac output increases by 40-50% during normal pregnancy (Ouzounian and Elkayam, 2012; Sanghavi and Rutherford, 2014). Most of the increase occurs during the first trimester (Hibbard *et al,* 2015), with a distinct rise observed even within the first few weeks of pregnancy (Ouzounian and Elkayam, 2012; Carling and Alfirevic, 2008). Cardiac output increases with gestational age, plateaus at the end of second trimester (Ouzounian and Elkayam, 2012), and then remains at this level until delivery (Ouzounian and Elkayam, 2012; Carling and Alfirevic, 2008; Sanghavi and Rutherford, 2014). The increase in cardiac output is predominantly due to an increase in stroke volume initially, with a contribution from increase in heart rate later in gestation. Stroke volume increases gradually during the first and second trimester, and then either remains constant or decreases late in pregnancy (Sanghavi and Rutherford, 2014; Liu *et al, 20*14).

1.1.1.2. Blood pressure and systemic vascular resistance

Although blood pressure falls in pregnancy, the general agreement is that the fall in systolic blood pressure (SBP) is minor (Sanghavi and Rutherford, 2014). In contrast, the fall in diastolic blood

pressure (DBP) is substantial. It starts to fall in the first trimester (6-8 weeks gestational age), reaches its nadir in the second trimester (dropping 5-10mm below baseline), and gradually returns to non-pregnant values [120/80mm Hg] near term (Mahendru *et al, 20*14). There is evidence that the changes start from the luteal phase of the preceding menstrual cycle (Fu *et al, 20*09; Liu *et al, 20*14). However, other work has challenged the concept of a decrease in blood pressure and demonstrated a progressive increase in blood pressure throughout gestation (Nama *et al, 20*11). Women with a body mass index (BMI) >25 kg/m^2 pre-pregnancy, have been shown to have significantly higher SBP, DBP and mean arterial pressure (MAP), at any point during pregnancy and postpartum, compared to women with lower BMI [<25 kg/m^2] (Grindheim *et al, 20*12). Also, there exist substantial ethnic differences in blood pressure levels observed during pregnancy, and the risk of gestational hypertension varies amongst the different ethnic groups (Bouthoorn *et al, 20*12).

The fall in blood pressure is due to reduced systemic vascular resistance (SVR) [the ratio of MAP to cardiac output], which decreases until mid-pregnancy, and then gradually rises until term. It follows the pattern of the changes observed by MAP. SVR is significantly lower than non-pregnant values as early as 5 weeks' gestation (Ouzounian and Elkayam, 2012; Carling and Alfirevic, 2008; Sanghavi and Rutherford, 2014). Although the cause of the fall in SVR remains unknown, it is attributed to the increase release of endothelium-dependent mediators (Roberts *et al, 20*02). The contribution of utero-placental circulation acting as an arterio-venous shunt and decreasing SVR is modest (Sanghavi and Rutherford, 2014; Liu *et al, 20*14).

1.1.1.3. Pulmonary circulation

Longitudinal studies have shown that there is no change in pulmonary circulation blood pressure (Liu *et al, 20*14). To accommodate the increased cardiac output in normal pregnancy, pulmonary vascular resistance falls. There is no change in pulmonary artery pressure. Pulmonary flow increases during pregnancy. Values return to pre-pregnancy level with 6 months post-delivery (Ouzounian and Elkayam, 2012; Carling and Alfirevic, 2008; Sanghavi and Rutherford, 2014).

1.1.1.4. The Microvascular System

Many of the key functions of the cardiovascular system occur at the level of the microcirculation, where nutritive exchange between blood and tissue occurs, there is a complicated relationship between maternal microvascular tissue perfusion and the imbalance of angiogenic factors during pregnancy. It has previously been shown that microvascular dysfunction occurs in pre-eclampsia (Anim-Nyame *et al, 20*03) and reduced tissue perfusion precedes the onset of the disease (Anim-Nyame *et al, 20*01).

The microvascular system is the collective name given to the smallest components of the cardiovascular system, comprising of the arterioles, capillaries and the venules. Unlike the arteries and the veins, which form distinctive anatomical entities, the microcirculation is part of the tissue they supply, both structurally and functionally. The microcirculation architecture is adapted to serve its special needs. In general, blood enters the capillary through an arteriole and leaves by way of a venule. The micro-vessels comprise of a continuous layer of endothelial cells, supported on a collagen basal lamina. These cells can perform a wide range of activities, depending on the type of micro-vessels they are in and their anatomical location (Ouzounian and Elkayam, 2012).

1.1.1.4.1. General Structure and functions

Arterioles: The arterioles (<100μm in diameter), together with small arteries (100-500μm in diameter), constitute the resistance vessels. They are important in the regulation of blood flow through the microcirculation, SVR and MAP (Simonsen and Aalkjaer, 2012; Park *et al, 20*01). They consist of one or multiple layers of vascular smooth muscle cells (VSMC) in their wall, making them capable of changing their diameter significantly. The intima consists of endothelium, basal lamina, sub-endothelium and internal elastic lamina (from inside outwards) (Latroche *et al, 20*15). There is a high density of nerve endings in the arterioles (Thomas 2011; Shoemaker *et al, 20*15), and there is pharmacological evidence that some vascular beds contain α- and β-adrenergic receptors (Jacob *et al, 20*16). Their dual control (neural and hormonal) facilitates regulation of microvascular blood flow, vascular resistance and local blood pressure, but there is marked

difference in sensitivity amongst the arteriolar branches (Tykochi *et al, 20*17; Thomas 2011; Jacob *et al, 20*16). (Figure 1.1)

Capillaries: they are the smallest of the micro vessels (5-10µm), whose wall consists of a single layer of endothelial cells, basal membrane and a few pericytes. They are marked by the disappearance of VSMC from their walls. Adventitia is present, consisting of a thin layer of connective tissue, which is continuous with the host tissue. The composition of the adventitia connective tissue may influence capillary patency, and dynamics of the fluid exchange. The structure of the capillaries varies as per the host tissue (Laganowsky *et al, 20*14; Latroche *et al, 20*15).

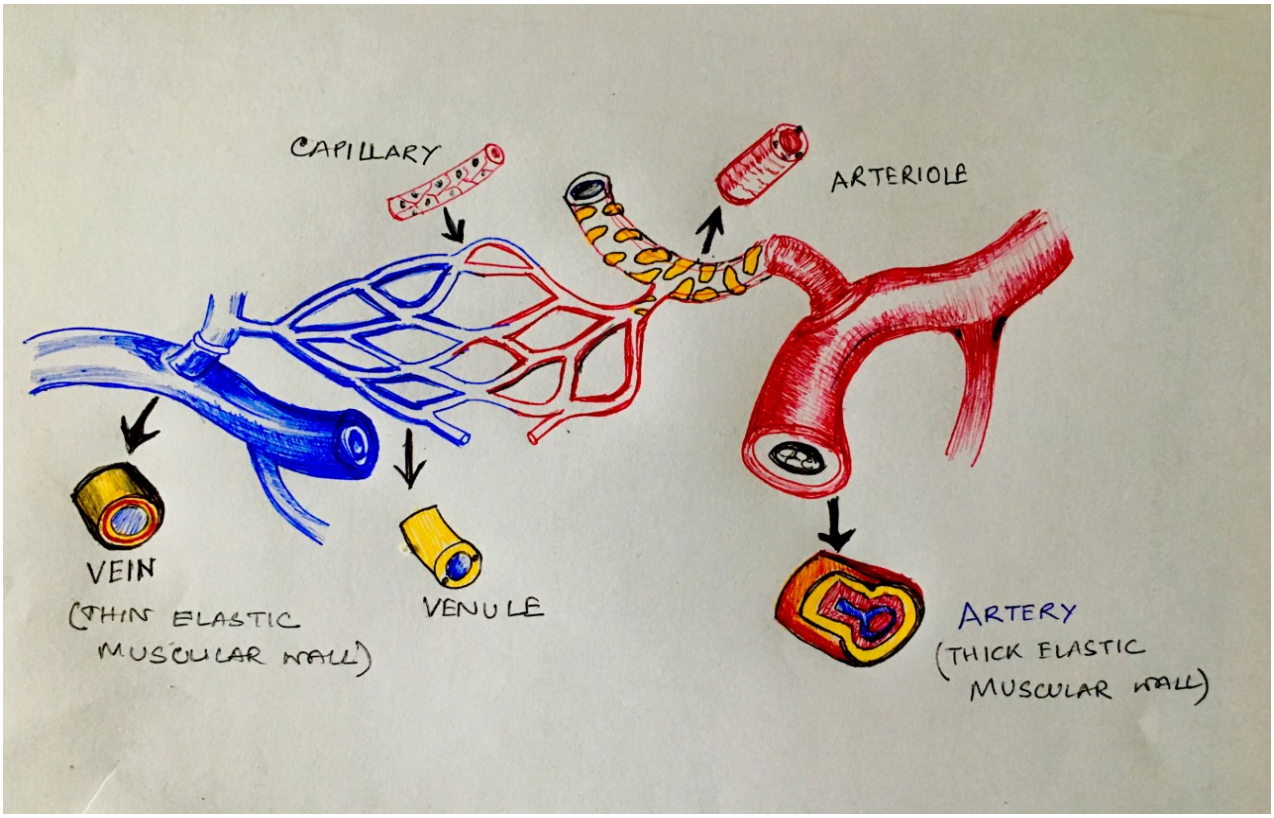


Figure 1.1: The structure of the microvascular system, showing the micro-structure of the vessels.

Venules: Blood flows from the capillaries to the venules. The transition is gradual, in terms of diameter and flow. The immediate post-capillary venules (10-50µm in diameter) are about 50-700µm in length, have pericytes embedded in the basement membrane, and continues as venules (50-200µm in diameter), which are much wider, and have one to two smooth muscle layers in their

media. The post-capillary venules are lined by a relatively thin continuous endothelium (0.2-0.3μm in diameter), which may contain lysosomes, multivesicular bodies, microfilaments, and Weibel-Palade bodies (Latroche *et al, 20*15). The intercellular junctions are loose and represent the weakest point in the entire vascular system, as maximum extravasation occurs here (Yannoustsos *et al, 20*14). The cells are particularly sensitive to prostaglandins, histamine, serotonin and bradykinin (Latroche *et al, 20*15). Here, bradykinin induce opening of the intercellular junction and it is the preferential site for extravasation and diapedesis seen in inflammation (Latroche *et al, 20*15). The venules have a thin basal lamina, which envelopes the numerous pericytes, and in some venules are extensively branched and almost appear as a continuous layer (Yannoustsos *et al, 20*14).

Venule in different muscular beds varies in ultrastructure and response to the vasoactive amines (Laganowsky *et al, 20*14; Jacob *et al, 20*16). They have a thin wall, and the lumen is lined by a continuous endothelial monolayer, which is thicker than those in post-capillary venules (Latroche *et al, 20*15). The endothelial cells of venules are particularly rich in microfilaments and organelles and have high-affinity histamine receptors. The endothelial cells rest on a thin basal lamina, which are perforated at the myo-endothelial junctions. The media consists of one to two layers of smooth muscle cells, thinner than the arterioles, and often forming an incomplete layer. The adventitial layer is thicker compared to the post-capillary venules and contains connective tissue components and specialised cells called veil cells. The veil cells are long, thin flattened fibrocytes, which are not surrounded by basal lamina (Tsioufis *et al, 20*15). Specific α- and β-adrenergic receptors are also present and are thought to promote contraction and relaxation of venular smooth muscle cells, indicating an active role in controlling capillary circulation. Unlike arterioles, venules have little sympathetic innervation (Thomas 2011; Shoemaker *et al, 20*15; Tsioufis *et al, 20*15).

1.1.1.4.2. Short-term regulation of tissue blood flow

One of the important characteristics of microcirculation is the ability of each tissue to control its own local blood flow in proportion to its needs (Shoemaker *et al, 20*15). In general, the greater the degree of metabolism, the greater is its blood flow. Similarly, it also ensures a minimal level of

blood to meet the nutritional supply, without over working the heart. In times of acute need, control is achieved by rapid changes in local constriction of precapillary smooth muscles, occurring in seconds, to maintain appropriate local tissue blood flow. Although tissue blood flow is influenced by tissue oxygen level and other metabolic requirements, the precise mechanism is unclear (Shoemaker *et al, 20*15; Tsioufis *et al, 20*15).

The largest contribution of total resistance to blood flow comes from the arterioles. Significant reduction in resistance in these arterioles must occur; to achieve the increase in blood flow required to meet the increased metabolic needs of the tissues. Sometimes, these arterioles are not in direct contact with the metabolically active tissues, indicating other mechanisms control the tissue blood flow, such as (a) conducted vasodilatation (Tsai *et al, 20*03; Tsai *et al, 20*06), (b) flow-mediated vasodilatation (Jacob *et al, 20*16; Hellsten *et al, 20*12), and (c) myogenic vasodilatation (Hudlincka, 2011).

Arterioles and venules are closely paired in most tissues, suggesting that the proximity can allow diffusible metabolites in the venous blood to have a direct effect on diameter of the arterioles (Loukas *et al, 20*09; Hudlincka, 2011). The arteriolar diameter can be influenced by several factors, such as tissue metabolites, endothelial derived factors, and changes in flow and sheer stress (Hudlincka, 2011). There appears to be an inverse relationship between venous oxygen partial pressure (PO_2) and blood flow during muscle stimulation. Although it is widely accepted that arterial endothelial cells release several vasoactive agents, such as nitric oxide (NO), prostaglandins and endothelium derived hyperpolarizing factor (EDHF) (Ozkor and Auyyumi, 2011; Félétou, 2016), there is evidence that venular endothelium also produces vasoactive agents, which influences arteriolar tone (Tsai *et al, 20*06; Hellsten *et al, 20*12), such as NO (Secomb, 2008) and prostanoids (Hammer *et al, 20*01).

The mechanism(s) by which tissue can sense decreased oxygen tension remains unexplained. There is evidence that adenosine, released from the vascular endothelium during hypoxia (Edmund *et al*, 2001), acts on the endothelial A_1 receptors to induce vasodilatation (Marshall,

2000). However, tissue oxygen partial pressure (PO_2), rarely falls to such a low level (even in severe hypoxia), for such massive release of adenosine (Conley *et al*, 2000). Moreover, NO (Edmunds *et al*, 2003) and prostaglandins (Ray *et al,* 2002), are also released in hypoxia. There is evidence that adenosine causes vasodilatation partly by releasing NO from the endothelial cells (Hellsten *et al, 20*12, Nyberg *et al, 20*10). Ray *et al* (2002) suggested that adenosine released during systemic hypoxia, acts on endothelial A_1 receptors, induces prostaglandin synthesis, which increases NO production, causing vasodilatation.

1.1.1.4.3. Long-term regulation of tissue blood flow

Long-term alteration in microvascular blood flow involves a change in tissue vascularity, by increasing or decreasing the number of micro-vessels in the tissue. It is more pronounced in new growth of tissue, than in well-established ones. Tissue hypoxia is one of the most important stimulants. The response involves angiogenesis, which is the growth of new vessels (Gutterman *et al, 20*16, Godo and Shimokawa, 2017).

1.1.1.4.4. Angiogenic factors and tissue blood flow

In normal pregnancy, there is a balance of pro-angiogenic factor, such as placenta growth factor (PlGF), and the anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) (Levine *et al, 20*04). The pro-angiogenic factors are always more predominant than the anti-angiogenic factors, throughout pregnancy, helping in the safe working of the placenta (Venkatesha *et al, 20*06). The mechanism by which sEng works is thought to be via the prevention of the binding of transforming growth factor β (TGF-β) to its receptor, reducing the production of NO and thus, NO-mediated vasodilatation, and subsequent capillary formation by endothelial cells *in vitro* (Pipp *et al, 20*03). PlGF is a vascular endothelial growth factor (VEGF) homologue, which stimulates angiogenesis. Reduced levels impair collateral artery growth in mouse limbs and neovascularization in tumors and ischemic retinas, while exogenous PlGF delivery stimulates angiogenesis and collateral growth in ischemic hearts and limbs (Pipp *et al, 20*03; Karunamichi *et al, 20*08; Mutter *et al, 20*08).

1.2. Pre-eclampsia

Pre-eclampsia is a multisystemic disorder in the second half of pregnancy, which affects about 4-5% of pregnant women (Sibai *et al, 20*03). It continues to be one of the leading causes of maternal morbidity and mortality (Knight *et al,* 2014 {CESDI. UK}). It also increases the risk of iatrogenic preterm delivery, intrauterine growth retardation (IUGR), and stillbirth (Knight *et al,* 2014 {CESDI. UK}). It is responsible for about 15% of cot occupancy in the special care baby unit (Knight *et al,* 2014 {CESDI. UK}). It is characterised by the development of hypertension, (SBP greater than 140 mm Hg or DBP greater than 90 mm Hg on at least two successive occasions 4-6 hours apart), and proteinuria (presence of more than 300 mg of protein in 24 hrs urine), after 20 weeks gestation (National Blood Pressure Education Programme 2000). Pre-eclampsia is associated with long-term consequences for both the mother and her unborn child. Despite much research, the pathophysiology of pre-eclampsia is uncertain.

Although, Hippocrates described the condition in 'On the Sacred Disease', he thought pre-eclampsia was epilepsy, which was fatal during pregnancy. The term 'eclampsia' derived from the Greek word 'eklampsia' meaning 'to flush out', was first used by Varandaeus in 1619 to describe the symptoms complained of before a fit in pregnant women. In 1843, Lever observed that eclampsia was associated with proteinuria, which disappeared after delivery, unlike those seen in renal disease (Mol *et al, 20*16). Hypertension was first described as a feature in the late 19th century, but unlike essential hypertension (called 'senile plethora'), occurred in a younger age group. Pregnancy-associated hypertension and proteinuria often preceded the convulsion and coma seen in eclampsia, therefore the term 'pre-eclampsia' was coined (Mol *et al, 20*16).

1.2.1 Abnormal placentation in pre-eclampsia

Although the primary pathology of pre-eclampsia remains unclear, there is considerable evidence that abnormal placentation might play a role in the pathogenesis of the disease. A two-stage model has been proposed to understand the pathophysiology of pre-eclampsia:

Stage 1: Reduced placental perfusion

Marked vascular remodelling occurs at the uteroplacental bed during normal pregnancy because of trophoblastic invasion of the spiral artery. This process converts these resistance vessels to dilated tortious vessels. This results in low resistance circulation at the uteroplacental bed (Myatt, 2002). The vascular pressure change is best described by using Poiseuille's equation, [$R = 8\eta / \pi r^4$, where 'R' is resistance of the vessel, 'η' is viscosity, 'r' is radius of the vessel]. Thus, resistance in a vessel is directly related to viscosity of the blood, and inversely related to the radius of the blood vessel. During pregnancy, plasma volume increases more than red cell mass, resulting in reduced blood viscosity (Ouzounian and Elkayam, 2012; Sanghavi and Rutherford, 2014). Since the blood viscosity decreases, and the radius of the blood vessel increases, the peripheral resistance decreases. In pre-eclampsia, the blood viscosity increases, secondary to extravasation of plasma in tissues. Since, the radius of the blood vessel is smaller in pre-eclampsia, it leads to increased peripheral vascular resistance (Roberts and Hubel, 2009)

Placental perfusion is decreased in pre-eclampsia. Evidence for this came originally by direct measurement with radioactive washout studies demonstrating reduced placental perfusion in pre-eclampsia (Siauve *et al, 20*15). Later, Doppler velocimetry studies have demonstrated increased resistance in the vessels that supply the intervillous spaces of women with pre-eclampsia, even in early gestation (Papageorghiou *et al, 20*02).

In pre-eclampsia, trophoblast invasion is impaired, and spiral arteries keep their endothelial lining and musculature, therefore remaining reactive to vasoactive substances (Brennan et al, 2014; Godo and Shimokawa, 2017). Trophoblastic invasion of the spiral arteries is restricted to the inner third of the myometrium. (Myatt, 2002; Brosens *et al, 20*02). Between one-third and half the length of the spiral arteries in the placental bed are not affected by the endovascular trophoblastic invasion (Brosens *et al, 20*02). Microvascular density in the placental bed among the hypertensive pregnant women was observed to be lower than for the normotensive pregnant women in the decidual and myometrial segments (Coelho *et al, 20*06).

The exact mechanism for this vascular maladaptation is unknown. It is thought that local oxygen tension and immune-mediated interactions are the primary determinants of the process, the common mechanism being through apoptosis (Hung *et al, 20*02). Apoptosis leads to the release of syncitiotrophoblast fragments into the maternal circulation, which is accelerated in pre-eclampsia (Myatt, 2002). The disturbed placentation supposedly leads to hypo-perfusion of the placenta, resulting in release of one or more yet unidentified factors (Factor 'X') from the placenta, which causes late vascular dysfunction in pre-eclampsia, because of endothelial dysfunction (VanWijk *et al, 20*00).

In pre-eclampsia, the invading cytotrophoblasts express different adhesion molecules and β_2 integrins, thereby failing to adapt their adhesion type from trophoblast cell characteristics to endothelial cell characteristics (Fisher 2015). Abnormal trophoblast invasion may also be the consequence of cytokine production by activated decidual leucocytes, such as Tumour Necrosis Factor-α (TNF-α), or altered growth factor production, like VEGF and PlGF (Shibuya 2013).

Stage 2: More than pregnancy-induced hypertension

The pathophysiology of pre-eclampsia is much more than hypertension and proteinuria, which facilitates diagnosis. Perfusion is decreased in virtually all organs, secondary to vasospasm, due to increased sensitivity to all the pressor agents (Brennan *et al, 20*14). It is further compromised due to activation of a coagulation cascade, especially platelets with attendant microthrombi, or due to decreased plasma volume, because of sequestration of fluid from the intravascular space (Roberts and Cooper, 2001; Roberts and Lain, 2002). Evidence of reduced perfusion is present in almost every organ of the body, including the uterus (Roberts and Lain, 2002). Reduced uterine blood flow further reduces placental perfusion, resulting in a feed-forward loop, consistent with the clinical course of pre-eclampsia. This is a disease, which never gets better, only worse, and when it begins to worsen, it worsens rapidly.

1.2.2. Immunology of pre-eclampsia

The risk of pre-eclampsia is reduced by prior miscarriage, longer cohabitation period before conception, and immunisation with paternal lymphocytes; while the risk is increased during first pregnancy, change of partner, donor insemination and barrier contraception (Robillard *et al, 20*11). This strongly suggests that a prior immune response against paternal antigen protects against pre-eclampsia. Hypo-responsiveness of lymphocytes seen in normal pregnancy is absent in women with pre-eclampsia (Robillard *et al, 20*11). The activity of circulating natural killer (NK) cells, neutrophils and cytokines, such as TNF-α, interleukin (IL)-6, IL-2 and IL-12, are increased (Robillard *et al, 20*11). Furthermore, human leucocyte antigen-G (HLA-G), a surrogate auto-antigen known to prevent recognition by NK cells, is not expressed as usual in the placenta in pre-eclampsia, as it is in normal pregnancy (Robillard *et al, 20*11; Fisher, 2015). Leucocyte activation in the decidua can cause release of cytokines, elastase and oxygen free radicals, all of which cause endothelial dysfunction. Whether the decreased HLA-G expression is caused by aberrant trophoblastic differentiation or results from an underlying genetic disorder is still unknown (VanWijk *et al, 20*00).

1.2.3. Genetics of pre-eclampsia

The cause of pre-eclampsia remains enigmatic, but there is a genetic component. It is a multifactorial disease in which the women's genetic background, her partner and her environment all interacts. Daughters of pre-eclamptic or eclamptic women have a 1 in 4 chance of themselves developing pre-eclampsia, two-and-a-half times higher than that of daughters-in-law (Skjærven *et al, 20*05; Williams and Pipkin, 2011). There is an increased risk of pre-eclampsia in women who became pregnant by a man who has already fathered a pre-eclamptic pregnancy in another woman; this is presumably through foetal expression of paternal gene(s) (Skjærven *et al, 20*05; Williams and Pipkin, 2011). However, the GOPEC study disputes this, and has not found any correlation between genetics and pre-eclampsia (GOPEC consortium, 2005).

1.2.5. Metabolic changes in pre-eclampsia

Profound maternal metabolic changes occur in pregnancies complicated by pre-eclampsia and the clinical picture is like that observed in other metabolic diseases, such as Syndrome "X"; a cluster of metabolic risk factors for cardiovascular disease, including hyperlipidaemia, hyperinsulinemia and hypertension (Irving *et al, 20*02). There is evidence of dyslipidaemia with elevated triglycerides, free fatty acids, LDL cholesterol, and reduced HDL cholesterol (Pignatelli *et al, 20*18). Women whose pregnancies are complicated by pre-eclampsia are therefore more likely to develop insulin resistance, Diabetes and cardiovascular disease, later in life (Wolf *et al, 20*04; Laivuori *et al,* 1996). Although the cause of this insulin resistance remains unclear there is accumulating evidence that this might be related to the underlying endothelial dysfunction of pre-eclampsia (Montagnami and Quon, 2000). There is also evidence that blood pressure increases with impaired glucose tolerance (Riemann *et al, 20*07) and that hypertension is an independent risk factor for diabetes. Elevated uric acid levels are also seen in pre-eclampsia (Robert and Lain, 2002). Uric acid has received increasing attention not only as a marker of cardiovascular disease, but also as an indicator of increased adverse foetal outcome even in the absence of proteinuria (Robert and Lain, 2002). However, the clinical utility of uric acid in pre-eclampsia is still uncertain.

In pre-eclampsia, lipid levels are increased even more than in normal pregnancy. In fact, the levels of circulating free fatty acids (FFAs) are higher in pre-eclamptic women, long before they show clinical signs of the disease (Villa *et al, 20*09). Amongst the FFAs, levels of oleic acid (18:1), linoleic acid and palmitic acid (16:0) are increased by 37%, 25% and 25% respectively (Villa *et al, 20*09). These fatty acids interfere with endothelial cell functions. Additionally, linoleic acid reduced thrombin-induced prostacyclin (PGI_2) release by 30-60%, oleic acid by 10-30%, whereas palmitic acid had no effect. The effect on PGI_2 is concentration-dependent (Villa *et al, 20*09). Endothelial levels of cGMP mainly reflect the synthesis of NO, since blocking of the endogenous production of NO with N-omega-nitro-L-arginine, resulted in about 90% reduction in cGMP-content of the endothelial cells. Incubation with linoleic acid reduced the endothelial cGMP level by 70%. Linoleic acid reduced the endothelial cells ability to inhibit platelet aggregation by 10-45% ($p=0.0019$), thus

impeding the ability of the endothelial cells to produce PGI_2 and cGMP, and to inhibit platelet aggregation (Villa *et al, 20*09).

1.2.6. Haemodynamic changes in pre-eclampsia

Pre-eclampsia is characterised by vasoconstriction, metabolic changes, endothelial dysfunction, activation of coagulation cascade, and increase in inflammatory response, to mention only a few of the changes observed in this disease. A longitudinal study has shown that women, who subsequently develop pre-eclampsia, have increased cardiac output (Ouzounian and Elkayam, 2012; Mahender *et al, 20*14). However, after development of pre-eclampsia, cardiac output variously decreases (Chaddha *et al, 20*04; Caniggia *et al, 20*00), remains the same (VanWijk *et al* 2000) or increases (Hibbard *et al, 20*15; Sanghavi and Rutherford, 2014). The haemodynamic disease model for pre-eclampsia showed that whereas the increased cardiac output was not associated with significant changes in peripheral vascular resistance, there was a cross over to a low cardiac output and high resistance circulation coinciding with the clinical onset of the disease (Hibbard *et al, 20*15; Brennan *et al, 20*14).

1.2.7. Endothelial function in pre-eclampsia

There is overwhelming evidence of generalised endothelial dysfunction in pre-eclampsia (Godo and Shimokawa, 2017). Structural changes to the endothelium occur in the uteroplacental vessels (Brosen *et al, 20*02). In addition, there is extensive evidence of functional derangements, such as increased concentration of von Willebrand's factor, endothelin, fibronectin, and an imbalance between PGI_2 and thromboxane A_2 (TXA_2) (Roberts and Lain, 2002; Roberts and Hubel, 2009). Vascular tone and thus peripheral resistance are under the continuous influence of endothelial-derived factors (Godo and Shimokawa, 2017).

Furthermore, a myriad of markers for endothelial injury or dysfunction are present in women with pre-eclampsia and, in many cases, precede clinically evident disease (Mol *et al, 20*16; Esper *et al, 20*06). Endothelial activation is only one component of a generalised activation of inflammatory responses that is characteristic of pregnancy (sometimes showing changes nearly as pronounced

as seen in sepsis) and further accentuated in pre-eclampsia (Roberts and Hubel, 2009). Thus, pre-eclampsia may represent an exaggeration of the normal inflammatory state of pregnancy. (Roberts and Lain, 2002; Germain *et al, 20*07)

The exact mechanism of widespread endothelial dysfunction encountered in pre-eclampsia is unknown. Evidence suggests the presence of several interacting factors, rather than a single agent. This might explain the heterogeneity of pre-eclampsia. One of the candidates for this is syncitiotrophoblast microvillous membrane particles (STBM), whose concentrations are increased in pre-eclampsia (Myatt, 2009). STBMs interfere with the growth of cultured endothelial cells, irrespective of whether a pre-eclamptic or normal placenta is used for their preparation (Chaddha *et al, 20*04). Oxidative stress has also been implicated as the cause of endothelial damage. Activated decidual large granulocytes produce cytokines, proteases and oxygen free radicals, induces lipid peroxidation, if not eliminated from the body (Steinberg and Baron, 2000; Villa *et al, 20*09). All these may result in endothelial damage (Mol *et al, 20*16; Esper *et al, 20*06).

In pre-eclampsia, the foetal and maternal mechanisms are not well understood. Endothelial dysfunction is considered to underlie many of the features of the disease, including hypertension and proteinuria (Robert *et al, 20*01). The strategic location of the endothelium permits it to modulate hemodynamic and humoral factors by synthesizing and releasing vasoactive substances. Thus, a critical balance exists between endothelium-derived relaxing and contracting factors that maintain vascular homeostasis. When this delicate balance is disrupted, the vasculature is predisposed to vasoconstriction, leukocyte adherence, mitogenesis, peroxidation, and vascular inflammation (Taylor and Roberts, 2007). The maternal vascular endothelium is an important target of factor(s) triggered by placental ischemia/ hypoxia in pre-eclampsia. Furthermore, markers of endothelial dysfunction may serve as predictors of pre-eclampsia, since many are elevated weeks before the clinical manifestations of the disease (Gilbert *et al, 20*08).

1.3. Introduction to Insulin

Insulin (from the Latin, 'insula' meaning 'island') is a peptide hormone produced by beta cells of the pancreatic islets. It regulates the metabolism of carbohydrates, fats and protein by promoting the uptake of glucose from the blood into fat, liver and skeletal muscle cells.

1.3.1. Historical background

Insulin was the first hormone to be discovered, synthesized and used clinically. In 1869, Paul Langerhans, a medical student in Berlin, discovered a distinct collection of cells within the pancreas. These cells would later be called the Islets of Langerhans. In 1901, Eugene Opie discovered that the Islets of Langerhans produce insulin and that the destruction of these cells resulted in diabetes. In 1916, Romanian Professor Nicolae Paulescu, developed an extract of the pancreas and showed that it lowered blood sugar in diabetic dogs, but World War I prevented the experiments from continuing and it was not until 1921 that it was published. In 1921, in Toronto, Canada, Dr Frederick Banting and medical student Charles Best performed experiments on the pancreases of dogs. Professor John Macleod provided Banting and Best with a laboratory and dogs to carry out the experiments. The pancreas of a dog was removed, resulting in the dog displaying the signs of diabetes. The pancreas was sliced and ground up into an injectable extract and injected a few times a day into the dog, which helped the dog to regain health (Rosenfeld, 2002).

1.3.2. Structure and function of Insulin

1.3.2.1. Distribution and Structure

Insulin is secreted from the β-cells of the Islets of Langerhans, present in the pancreas. It is a polypeptide hormone, containing 2 chains of amino acids, α and β, linked by disulphide bridges that connect α7 to β7 and α20 to β19. α and β chains have 21 and 30 amino acids, respectively. The gene for insulin is in the short arm of chromosome 11. Plasma glucose concentration, amino acids, free fatty acids and other hormones, like adrenal hormones, growth hormones, and placental lactogens, regulate insulin secretion. Once secreted, insulin is rapidly metabolised, mainly in the liver, kidneys and placenta (during pregnancy), and has a half-life of 3-5 minutes. It

has no carrier protein, and in fact, 50% of the circulating hormone is removed in a single pass through the liver. (Granner, 2000; Ganong, 2005)

1.3.2.2. Function

Insulin produces a wide variety of effects on endothelial cells and plays an important role in glucose and vascular homeostasis. Muscle is the main peripheral site of insulin action (Saltiel and Kahn, 2001) and this is delivered to muscle cells from the circulation by both passive diffusion and trans capillary transport mechanisms involving endothelial cell surface binding (Posner, 2017). Its action is conveniently divided into immediate (within seconds), intermediate (within minutes) and delayed (within hours) effects. Its immediate actions are increased transport of glucose, amino acids and potassium into insulin sensitive cells. Its intermediate actions are increased protein synthesis and preventing their degradation, activation of glycogen synthetase and inhibition of glycolytic enzymes, and inhibition of phosphorylase and gluconeogenic enzymes. Its delayed actions include increase in mRNAs for lipogenic and other enzymes (Granner, 2000; Ganong, 2005).

Insulin has vasodilatory effects, which indirectly regulates tissue blood flow, peripheral insulin delivery and therefore uptake of glucose by skeletal muscle. Insulin binding to its receptor activates both PI3K/AKT and the Ras-MAP kinase pathway. In endothelial cells, the PI3K/AKT pathway mediates an anti-apoptotic effect and also results in an increase in gene expression and activation of eNOS (endothelial Nitric Oxide synthase). These effects are enhanced by VEGF and fluid shear stress (Zeng *et al, 20*00; Peach *et al, 20*18). PI3K/ AKT also translocate GLUT-4 from the cytoplasm to the membranes, to enhance glucose uptake (Thong *et al, 20*05). [Figure 1.2]

1.3.3.3. Role in vascular haemostasis

Vasodilatation is achieved by relaxation of the resistance vessels, and the precapillary arterioles, thus increasing total blood flow (Grundmann *et al, 20*08, Strijbos *et al, 20*10). Insulin induces endothelial-mediated vasodilation, via PI3K/AKT/NO pathway (Roberts and Gammill, 2006) Thus, hyperinsulinemia in pre-eclampsia could be a reflex compensatory mechanism to cause the

decrease in blood flow to the tissue, that occurs in pre-eclampsia (McVeigh and Cohn, 2003). However, such an assertion has been contradicted, with an alternative explanation postulates that hyperinsulinemia is the cause of endothelial dysfunction (Wautier *et al, 20*01).

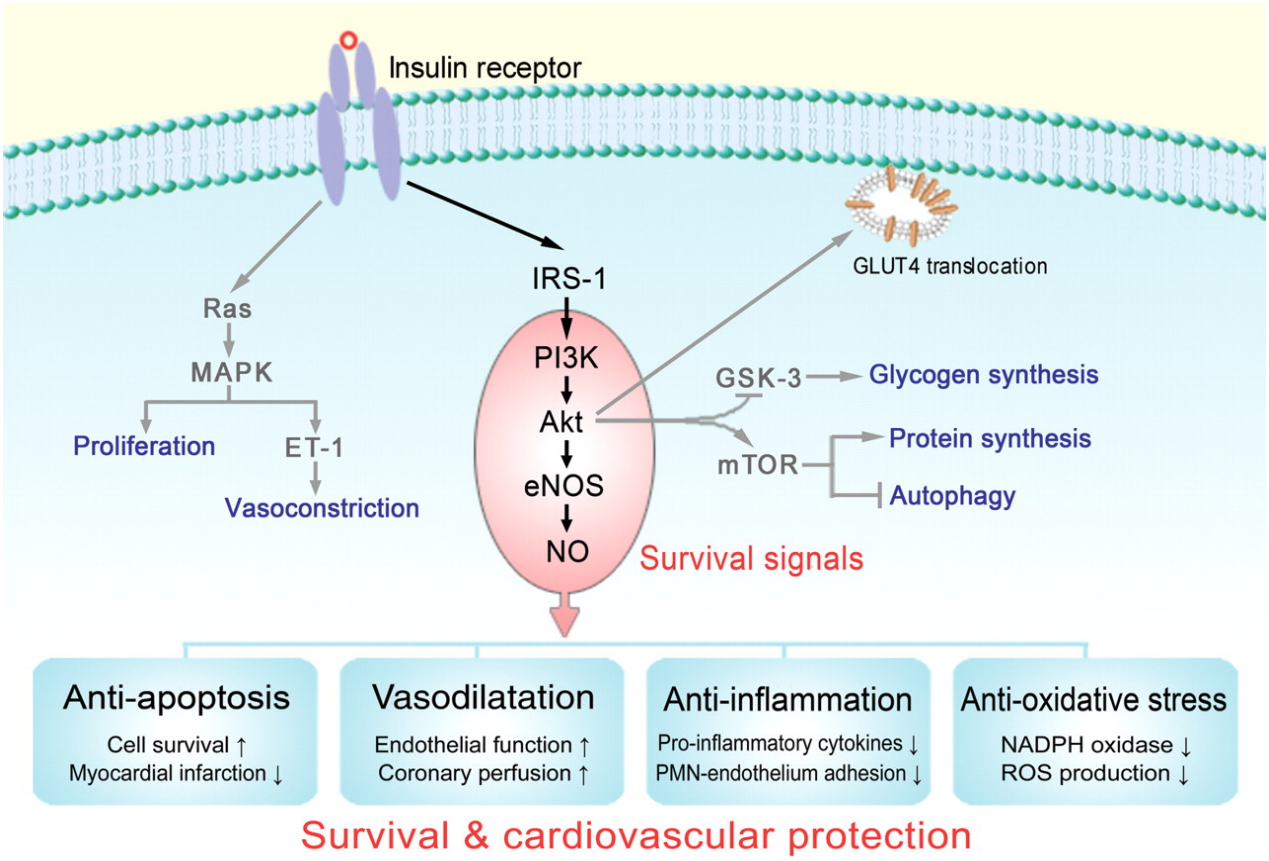


Figure 1.2: Role of the insulin-signalling pathway on endothelial function in healthy pregnancy. (Yu *et al, 20*11)

1.3.3. Insulin signalling pathway

Insulin's action begins when it binds to a specific cellular glycoprotein receptor, expressed by all tissues in the body in varying densities [Figure 1.3]. Insulin receptors have been demonstrated on endothelial cells, of both large and small blood vessels (Vincent *et al, 20*03) and participate in insulin-regulated glucose homeostasis. The insulin receptor is composed of two α- and two β-subunits, covalently linked through disulphide bonds to form a $\alpha_2\beta_2$-heterotetramer. Each subunit has a specific function; the extracellular α-subunit contains the insulin binding domain, while the transmembrane β-subunit possess an insulin-stimulated protein, tyrosine kinase, an auto-phosphorylation site, essential for signal transduction (Granner, 2000). The receptors appear to

regulate insulin action on vascular endothelium and control of glucose homeostasis by controlling trans-endothelial insulin transfer.

Insulin receptors are unique in that not all receptors are always expressed on the cellular surface. Once bound to insulin, the receptors are internalised within the cell into endosomes, and remain there until insulin is degraded (Mol *et al, 20*16; Esper *et al, 20*06). Once insulin is degraded in the endosomes, the insulin receptors may recycle back to the cell surface, or form lysosomes where the receptors are degraded. Prolonged stimulation of the receptors by insulin, caused by increased doses of insulin, appears to accelerate the degradation of insulin receptors, leading to receptor down-regulation (Roberts and Lain, 2002; Posner, 2017). Insulin binding to its receptor activates both PI3K/AKT and the Ras-MAP kinase pathway. In endothelial cells, the PI3K/AKT pathway mediates an anti-apoptotic effect and results in an increase in gene expression and activation of eNOS (Zeng *et al, 20*00; Kuboki *et al, 20*00; Hermann *et al, 20*00) [Figure 1.3]

Human cells use glucose for the generation of ATP (adenosine triphosphate), by metabolism. The lipid bilayer of the cell membrane is impermeable to carbohydrate. Glucose is transported from the blood across the cell membrane by a saturable transport system, which is of two types; 1) firstly sodium-dependent glucose transporters (SGLTs), which transport glucose against the concentration gradient, and 2) sodium independent glucose transporters (GLUTs), which transport glucose by facilitated diffusion along its concentration gradient (Jurcovicova, 2014). Currently, there are five established functional facilitative glucose transporter isoforms (GLUT1-4 and GLUTX1), with GLUT5 being a fructose transporter. The GLUT4 isoform is the major insulin-responsive transporter that is predominantly restricted to striated muscle and adipose tissue (Watson and Pessin, 2001). In the basal state, GLUT4 cycles slowly between the plasma membrane and one or more intracellular compartments, with most of the transporter residing in vesicular compartments within the cell interior (Huang and Czech, 2005; Leto and Saltiel, 2012). Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis and a smaller decrease in the rate of internalization by endocytosis (Huang and Czech, 2005; Leto and Saltiel, 2012).

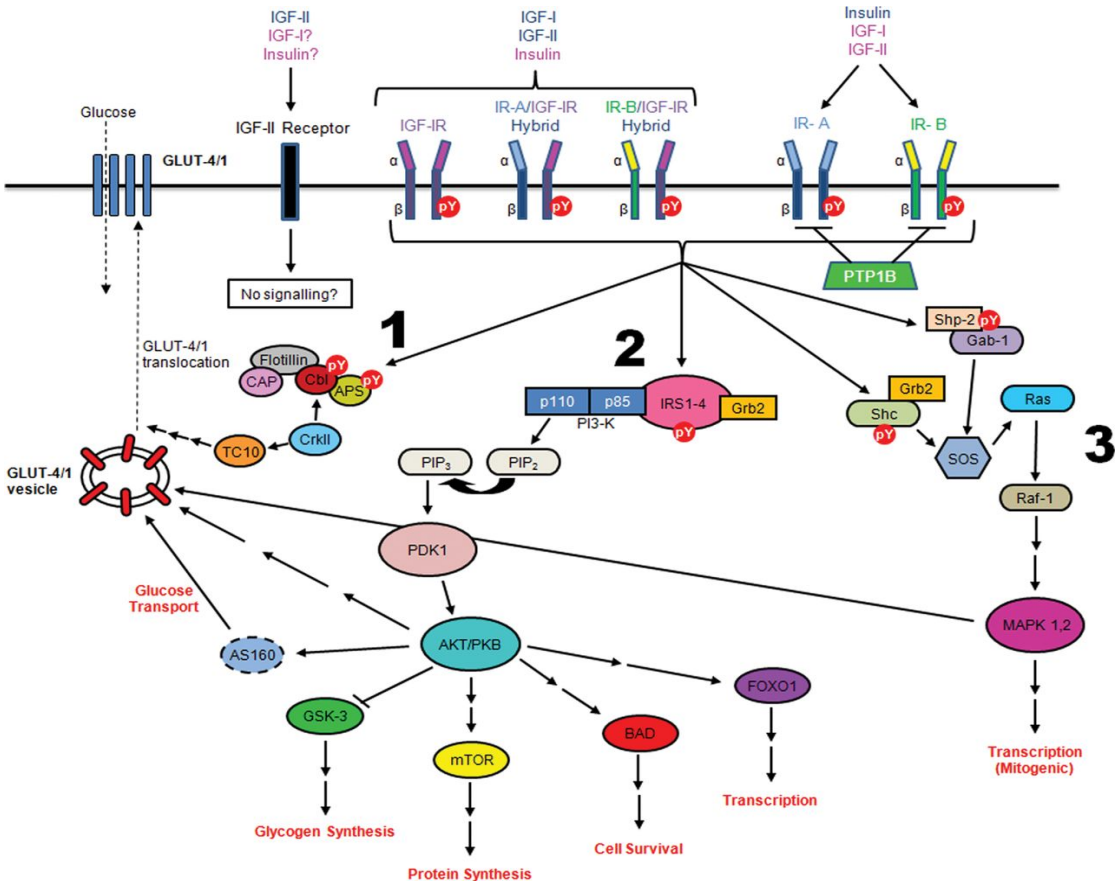


Figure 1.3: The Insulin Signalling Pathway (Hale and Coward 2013)

1.3.4. Insulin Resistance in pregnancy and pre-eclampsia

Insulin resistance is a feature of pregnancy, though it is exaggerated in pre-eclampsia (von Versen-Hoeynck and Powers, 2007; Thadhani *et al, 20*04). It is also been shown that reduced blood flow may play a role in the increased insulin resistance seen in pre-eclampsia (Anim-Nyame *et al, 20*15). Himsworth has shown previously that insulin resistance can by itself be a cause of diabetes mellitus (Himsworth, 1949). Women with pre-eclampsia and insulin resistance are at risk of developing diabetes in later life (Laivuori *et al,* 1996; Lykke *et al, 20*09; Spaan *et al, 20*10; Feig *et al, 20*13). Although the cause of this remains unclear, there is accumulating evidence, that this might be related to the underlying endothelial dysfunction (Montagnani and Quon, 2000) [Figure 1.2].

1.4. The Microcirculation in pre-eclampsia

Most of the functions of the cardiovascular system occur at the level of the microcirculation, where there is exchange of material between the plasma and the tissues.

1.4.1. Assessment of microvascular parameters

There are several methods for the clinical assessment of the tissue blood flow. Over the last few decades, several non-invasive techniques have been described, making the study of microcirculation more accurate and reproducible. In this study, Filtrass strain gauge plethysmography has been used, as described by Christ *et al* (2000a & b). A more detailed account of the technique is described in Chapter 3.

1.4.2. Clinical evidence of microvascular changes in pre-eclampsia

The microcirculation is essential for the delivery of nutrients and removal of waste products from tissues. It also plays a role in the control of the blood pressure and peripheral vascular resistance and is designed to serve each organ's needs. The clinical picture of pre-eclampsia is suggestive of reduced tissue perfusion and end organ failure, as occurs in severe forms of the disease, resembling critically ill non-pregnant patients with multi organ failure. Thus, the end organ failure in pre-eclampsia may be preceded by a deterioration of the microcirculation (Qspina- Tascón et al, 2017).

There is increasing evidence of defective tissue extraction of oxygen in pregnancies complicated by the disease, leading to tissue anaerobic metabolism. This results in a degree of base deficit, which correlates with the end-organ injury and adverse foetal outcome in pre-eclamptic patients. Severe forms of pre-eclampsia closely resemble the pathophysiology of septic shock. Generalised, widespread, endothelial dysfunction is seen in both conditions. It results in end-organ ischemic injury, secondary to vasoconstriction, hypervolemia, and impaired tissue exchange of metabolites. This may explain the pattern of maternal end-organ injury seen in pre-eclampsia (Powe *et al, 20*11; Ince *et al, 20*16).

There is evidence that endothelial dysfunction in pre-eclampsia might interfere with the regulating mechanisms for the microcirculation (Anim-Nyame *et al, 20*04). This is because the vascular endothelium acts as an organ driving vasomotor activity at the pre-capillary level, playing an

important role in sensing altered local tissue demand and adjusting flow to accommodate these needs (Hellsten *et al, 20*12; Sarelius, 2000).

1.4.3. Angiogenic factors in pre-eclampsia

Angiogenic imbalance with increased anti-angiogenic factors, such as sFlt-1 and sEng appear to play a pathogenic role in the aetiology of pre-eclampsia (Maynard *et al, 20*03; Venkatesha *et al, 20*06). A rise in sFlt-1 and sEng and a reduction in the pro-angiogenic factors, such as PlGF, have been reported in maternal serum 5-10 weeks prior to the onset of pre-eclampsia (Levine *et al, 20*04). It is proposed that these anti-angiogenic factors contribute to the maternal endothelial dysfunction seen in pre-eclampsia (Levine *et al, 20*04). Moreover, levels of sFlt-1 directly correlate with the severity of pre-eclampsia and precede the onset of the disease (Karunamichi et al, 2008; Levine *et al, 20*04), as sFlt-1 circulates freely in serum, and binds to pro-angiogenic factors, such as VEGF and PlGF.

Angiogenic imbalance is likely to affects microvascular function as the microvasculature is formed by the continuous tension between *de novo* angiogenesis and microvascular regression (rarefaction). It is possible that microvascular dysfunction in pre-eclampsia is related to the pro- and anti-angiogenic imbalance in pregnancies complicated by the disease. Furthermore, increased levels of sFlt-1 and sEng or low PIGF are associated with reduced microvascular flow whereas lower levels of the anti-angiogenic factors and higher pro-angiogenic PIGF levels correlate with a greater blood flow during normal pregnancy (Ghosh *et al, 20*17).

1.5. Aim and Objectives

1.5.1. Aims and objectives of the study

1. To compare endothelial cell insulin signalling between normal pregnancy and pre-eclampsia.
2. To investigate whether any changes in endothelial cell insulin signalling are related to insulin resistance and tissue blood flow in pre-eclampsia.
3. To evaluate the extent of endothelial cell damage by investigating circulating endothelial cell (CEC) and soluble markers of endothelial cell function.

1.5.2. Hypotheses of the study

1. Changes in endothelial cell insulin signalling occur in pre-eclampsia, secondary to underlying endothelial dysfunction, resulting in insulin resistance.
2. Impaired endothelial cell insulin signalling results in reduced tissue delivery of insulin and reduced GLUT-4 activation.
3. Impaired microvascular blood flow results in insulin resistance.

Chapter 2: Material and Methods

All the work in this study is done by the author. All clinical parts of the study, like plethysmography, blood pressure measurements and blood collection were done at Kingston Hospital, UK; by the author. The laboratory part, like blood separation, cell culture, enzyme linked immunosorbent assay (ELISA) and flow cytometry, were done at Kingston University, UK, by the author

2.1 Subjects/ Participants

This is a prospective, case controlled and collaborative study between Kingston Hospital, and Kingston University. Pregnant women were recruited during the third trimester from Kingston Hospital's maternity unit.

2.1.1. Ethical approval and consent

The London and Surrey Borders Research Ethics Committee approved the study, and informed consent was obtained from all the participants. The studies in this thesis conformed to the Helsinki Declaration.

2.1.2. Women with Pre-eclampsia

Women with pre-eclampsia were recruited in this study to compare changes in microcirculation and insulin signalling pathways with normotensive pregnant controls. Pre-eclamptic women were recruited from the antenatal ward of Kingston Hospital's maternity unit. These were women who had no history of any previous disorder that was likely to affect their microcirculation or insulin-signalling pathway, independent of pregnancy. Pre-eclampsia was defined as new-onset hypertension, (SBP greater than 140 mm Hg or DBP greater than 90 mm Hg on two successive occasions 4-6 hours apart), and new-onset proteinuria (presence of more than 300 mg of protein in 24 hrs urine), after 20 weeks gestation (National Blood Pressure Education Programme, 2000). Blood pressure was determined using the first and the fifth Korotkoff sounds (appearance and disappearance) for measuring the systolic and diastolic blood pressure. Proteinuria was assessed

by collecting 24-hour urine in plastic jars using phenyl mercuric acetate as preservative and measuring protein by calorimetric reactions in an autoanalyzer (Watanabe *et al* 1986).

2.1.3. Normal pregnant control

Normal pregnant controls were recruited in this study to compare the changes in microcirculation and insulin signalling pathways, with pre-eclamptic pregnant women. These were healthy women without any history of medical and surgical disorders that were likely to affect their microcirculation or insulin-signalling pathways, independent of pregnancy. The women were recruited from the antenatal clinic of Kingston Hospital. These women were given leaflets and information about the study. Participation in the study was voluntary, and they could withdraw from the study at any time.

2.1.4. Inclusion and Exclusion criteria

All pregnant women who were registered at Kingston Hospital, UK, were invited to participate in the study. They had to be in their third trimester of pregnancy (more than 28 weeks gestation). They were chosen to be similar in maternal age, gestational age and BMI. Women with pre-existing or gestational diabetes or any known metabolic, cardiovascular, inflammatory, immune, infectious or neoplastic conditions were excluded from the study. Smokers were also excluded from the study (Csordas and Bernhard, 2013).

2.2 Sample collection and transport

Fasting blood samples were collected from pre-eclamptic women from the antenatal ward, and from the normal pregnant controls in the antenatal clinic. Blood samples were obtained from a cubital vein of each participant aseptically, using Vacutainer™ vacuum test tubes (Becton Dickjenson, Vacutainer System, UK). Blood was collected in the tubes in the following order; Sodium Fluoride (2ml), Serum (4ml), Ethylenediaminetetraacetic Acid (EDTA) (2ml), Lithium Heparin (1ml), and Sodium Citrate (1.8ml).

After drawing blood, the women had their breakfast and were made to rest for 30 minutes. Examinations were done on a bed in the left lateral position lasting for 15 minutes. Then their blood

pressure and pulse were measured in a semi-recumbent position. Maternal tissue blood flow was estimated in the gastrocnemius muscle using a Filtrass strain plethysmograph using an established protocol previously used in other studies on pregnant women (Christ *et al, 20*00a; Christ *et al, 20*00b) (described in section 2.3).

The blood samples were transported from Kingston Hospital to Kingston University in a sealed container, over ice, in a car. The transport time was less than 30 minutes. On arrival, bloods were prepared as follows. 1 ml of EDTA was separated for determination of CEC, as described later (Section 2.5.1). 1 ml of heparinised blood and 1 ml of EDTA blood were centrifuged at 3000 rpm for 10 minutes (Thermo Scientific, UK). The supernatant was separated and stored at -80ºC in a freezer for later use. Sodium Fluoride blood was also centrifuged at 3000 rpm for 10 minutes and the supernatant stored in a -80ºC freezer for later use. Sodium citrate bloods were centrifuged at 5000 rpm for 5 minutes, the supernatant separated and centrifuged for another 5 minutes at 5000 rpm. The supernatants were stored in a -80ºC freezer for later use.

2.3 Clinical measurement of microvascular blood flow

There are different methods available for measuring microvascular blood flow. The different methods are plethysmography, of which there are different types; skin temperature and thermal clearance; clearance of radiolabelled particles, like microsomes, dyes, albumin and dextran; intravital microscopy; use of infrared spectroscopy or electrodes to study tissue oxygenation and/ or products of tissue metabolism; to name a few. The method used in the present study is plethysmography, which is described below.

2.3.1 Plethysmography

Plethysmography is a non-invasive diagnostic procedure, which measures changes in volume of certain body parts. The word ‘Plethysmograph’ is derived from the Greek words, ‘plethysmos’ (increase) and ‘graphein’ (to write). Thus, the words describe the fundamental principle of this technique, to measure any change in volume of any portion of the body.

This technique was first described by Glisson (1622) and later by Swammerdam (1737) to study muscle contraction. It was first used by François- Frank (1876) to measure blood flow in limbs, using venous occlusion plethysmography. Since then, plethysmography has undergone refinement with new methods and instruments being invented (Hyman and Windsor, 1961)

2.3.1.1 Principles of Plethysmography

Plethysmography measures changes in volume of body parts. Apart from the lungs, the change in volume of other parts of the body is related to the blood flow through the body part. Thus, plethysmography measures changes in volume of blood in the body part being examined. Venous occlusion plethysmography is a non-invasive method used to measure blood flow through the limbs or other parts of the anatomy having a circular cross-section. The venous outflow is transiently interrupted for 7 to 9 seconds, without interrupting the arterial flow. The accumulating blood causes the limb to swell at a rate, which initially is directly proportional to the arterial blood flow.

2.3.1.2 Types of Plethysmograph

Plethysmography has come a long way since Glisson first described it in 1622. There are different devices and techniques used to measure the relevant information. They fall into one of the following categories (Joyner *et al, 20*01)

- Water-filled plethysmography. It measures the amount of water displaced by a change in volume.
- Air plethysmography. This works by measuring the change in air pressure caused by air compression due to a change in the volume of body parts.
- Strain gauge plethysmography. This measures the change in circumference of a limb.
- Impedance plethysmography. This measures volume change by measuring the change in electrical resistance through tissues.
- Photo-plethysmography. This uses the reflection of light from the blood cells flowing through the vessels to determine the change in volume of the tissue.

In this experiment, the strain gauge plethysmography was used, as described below.

2.3.1.3 Principles of strain gauge plethysmography

First described by Whitney, this method uses mercury-filled strain gauge to measure changes in limb volume. Recent models use fine silastic tubes filled with mercury and sealed with molybdenum pins. These are then balanced against an adjacent temperature compensation coil on a Wheatstone bridge. The tube is wrapped around the limb, with just enough stretch to ensure good contact. As the circumference of the limb changes, the length of the gauge changes accordingly. Since the resistance of the gauge varies with its length, which changes as the gauge is stretched, a difference in the limb circumference will be reflected by variations in the voltage drop across the gauge.

A new protocol, developed by Gamble (Gamble *et al,* 1993), used the silastic strain gauge plethysmograph to measure the forces that govern microvascular exchange in the gastrocnemius muscle. It was based on Starling's principle. It follows the following equation:

$$J_v=K_f[(P_c-P_t)-\sigma(\pi_c-\pi_t)]$$

Where J_v is fluid flux per 100 ml tissue per min

P_c is capillary hydrostatic pressure,

P_t is interstitial hydrostatic pressure,

π_c is capillary oncotic pressure,

π_t is interstitial oncotic pressure

K_f and σ are co-efficients.

K_f is hydraulic conductance, and is a measure of permeability of the microvascular exchange to water, and

σ is the osmotic reflection co-efficient, an index of microvascular impermeability to protein molecules.

A σ value of 1.0 denotes total impermeability to proteins, while a value of 0.0 denotes a free permeability of proteins across the membrane. The K_f co-efficient depends on the permeability of the membrane per unit area (L_p), and the total surface area of membrane within 100g of tissue (A). Thus, changes in fluid flux (ΔJ_v) are due to either an alteration in membrane co-efficient (L_p or σ), an alteration of the total surface area available for exchange (ΔA), or variation in net transmural

forces across the membrane. However, this equation does not take lymphatic drainage (J_L) into account.

2.3.1.4 Assumptions made in this study

- The major tissue component of the gastrocnemius muscle is made up of muscle, with a high muscle to skin ratio. Thus, the measured blood flow relates more to tissue metabolism, rather than in temperature regulation.
- Since there are no visible arterio-venous malformations in the limbs, it is assumed that the arterial blood will flow through the microvascular beds. Thus, it is assumed that blood flow in the gastrocnemius muscle measures nutritive blood flow.
- The gastrocnemius muscle pressure applied is equal to the deep venous pressure, so that the venous flow is occluded (40mm Hg) (Groothuis *et al, 20*03).
- The gastrocnemius muscle pressure exerted does not occlude the arterial flow to the limbs.
- The arterial blood causes the limb to swell in proportion to the rate of arterial inflow.

2.3.1.5 Merits and Limitations

The mercury used in silastic strain gauge is very sensitive with a high frequency response, capable of reproducing the magnitude of periodic stretch without loss up to 100Hz. It is also free of any resonance effect.

The main drawback of the strain gauge plethysmography method is its sensitivity to temperature. Potentially, this could lead to a measurement error if calibration was carried out at a different temperature to that in which a recording was made. In practice, this does not happen, as the skin temperature is maintained. Another potential drawback of the technique is the mercury used in strain gauge plethysmography is toxic. The lifetimes of the gauges were variable, and there is a potential to produce an inaccurate calibration (Christ *et al, 20*00a; Christ *et al, 20*00b).

2.3.2 The Filtrass Strain Gauge Plethysmograph

First described by Christ (Christ et al, 2000a, Christ et al, 2000b), this is a novel metal-free device for venous congestion plethysmography. The Filtrass device (Filtrass, Munich, Germany) is a modification of the system produced by Gamble (Gamble *et al,* 1993). Filtrass is mercury-free, with an automated calibration device, which allows a touch-free calibration; thus, reducing artefacts by the investigator. The sensor is automatically calibrated three times during each study, by a computer driven programme. It has a pre-recorded protocol for measuring blood flow, amongst others. [Fig 2.1]

Figure 2.1: The Filtrass Strain gauge Plethysmograph (Filtrass, Munich, Germany)

The principle of the Filtrass is like the strain gauge plethysmograph, except it doesn't contain any mercury. An inelastic, flexible plastic line, with a diameter of 0.5 mm, detects the limb circumference. It spans the limb and connects the transducer with an inbuilt electric motor. It can detect changes in the limb circumference with an accuracy of ±5 µm. The plastic line glides over a silicon-coated flexible zigzag band [Fig 2.2]. This ensures low friction and good fixation of the electromechanical sensor to the limb. When the limb circumference increases, the passive transducer can be pulled out to a maximum of 4 mm, followed by the outward drive of the stepping motor, if the change in circumference exceeds 4 mm, allowing it to measure a maximum of 22 mm. The motor resets itself automatically to the initial position of passive transduction before each pressure reading, unlike the silastic tube system. This ensures that the sensor is always operating

over the same sensing range. Moreover, the calibration is touch free, thus reducing artefacts due to manipulation. Venous congestion pressure is induced with the help of an occlusion cuff, which is attached to a compressor pump built into the apparatus, and placed around the right thigh, enclosed in a tight corset, thus reducing the time by reducing the volume required to occlude the venous pressure (Christ *et al*, 2000a).

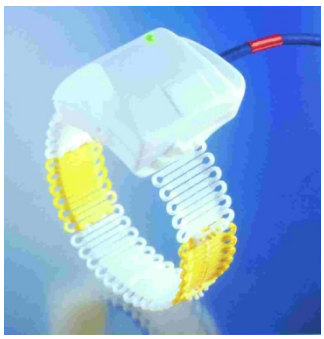


Figure 2.2: The transducer band used in Filtrass (Filtrass, Munich, Germany) [own photo]

2.3.2.1 Calibration of the Filtrass plethysmograph.

The calibration of the Filtrass is touch-free, thus reducing errors due to manipulation. Before any study, the motor applies a pre-tension pull of 1mm, followed by a calibration pull of 4 mm. The response of the inductive transducer to the pull is sampled at 10 Hz and measured in arbitrary units. Deviations from the ideally linear relationship, between motor pull and the response to the passive transducer, are included in the calibration. Three repetitive measurements are automatically performed during each calibration procedure, and the second and the third are compared for the time delay of the response of the passive transducer. The maximum time delay value that is accepted is 500 ms. The calibration and data recording procedure are fully automated, and computer driven.

Specific protocols can be written, saved and selected at the start of the study. They can also be modified during a study, thus enabling allowances to be made for changes in circumstances, e.g. for therapeutic interventions.

2.3.3 Protocol for measuring microvascular blood flow

2.3.3.1 Study environment and preparatory phase; arterial blood pressure

The studies were performed at Kingston Hospital, UK, in a quiet room at room temperature (22-24ºC). The subjects were rested for about 30 minutes in a semi-recumbent position. Pedal oedema was assessed by applying firm pre-tibial pressure for 5 seconds for evidence of pitting. After resting for 30 minutes, arterial blood pressure was measured non-invasively in the ipsilateral arm. The average values of systolic, diastolic and mean arterial blood pressures were calculated from triplicate measurements. Observations were made in the left-lateral position, to prevent aorto-caval compression, with the right mid-gastrocnemius muscle supported by pillows, at the level of the heart [Fig 2.3].

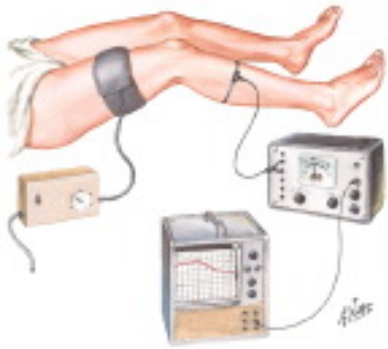


Figure 2.3 Filtrass protocol for measuring limb blood flow

2.3.3.2 Filtrass protocol for measuring limb blood flow

The strain gauge plethysmograph has been widely used for non-invasive assessment of blood flow in the limbs (Christ *et al, 20*00a &b; Gamble *et al,* 1993). Both strain gauge plethysmograph (Carberry *et al*, 1992) and Filtrass (Anim-Nyame *et al, 20*00a, b & c, 2001) have been used before in pregnancy.

In this study, the gastrocnemius muscle is used for the following reasons. Firstly, the gastrocnemius muscle is less likely to have artefacts due to involuntary movements. Secondly, in severe cases of pre-eclampsia, the arms may be used for administering intravenous therapy, and are therefore not readily available for investigation. Thirdly, the women are more rested, calm, and

co-operative when the gastrocnemius muscle is used. Finally, a large amount of control data has been gathered from the gastrocnemius muscle using strain gauge plethysmography (Anim-Nyame *et al, 20*00a, b & c, 2001; Gamble *et al,* 1998)

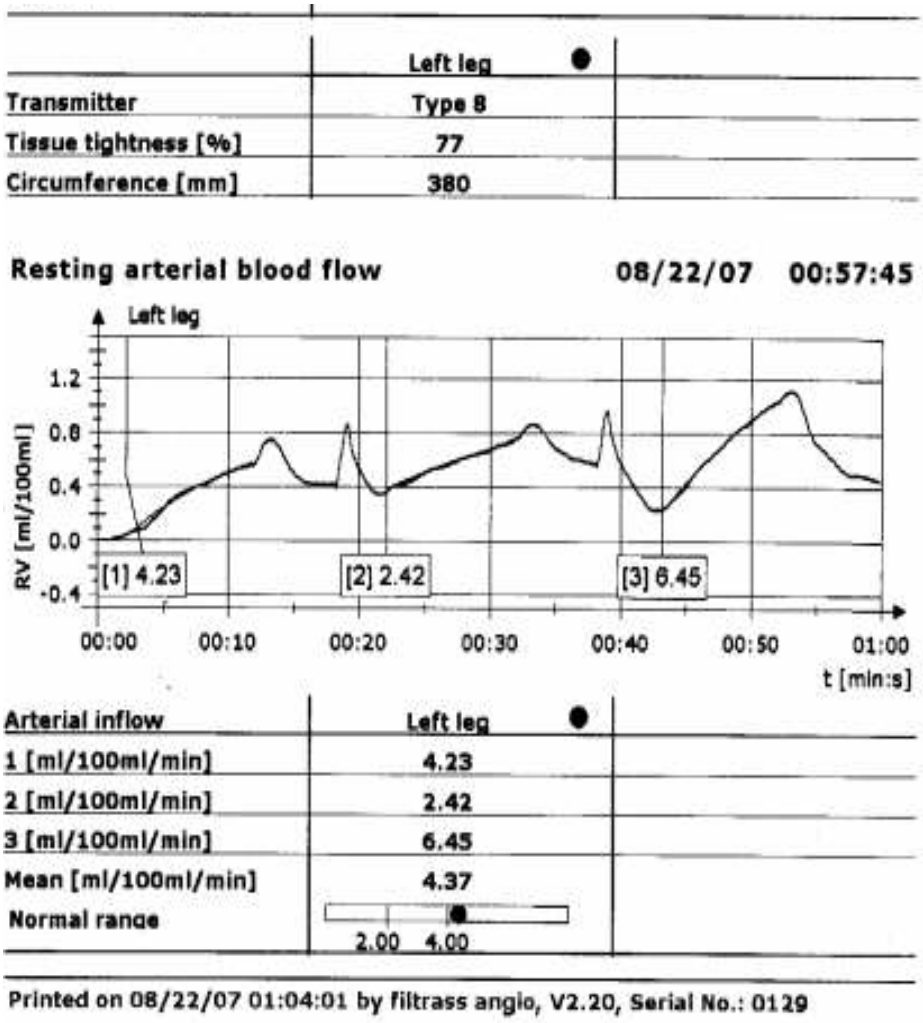


Figure 2.4. A typical plethysmograph reading. It shows the circumference of the limb, the 3 readings and the mean, calculated automatically.

The gastrocnemius muscle blood flow was measured using a protocol that has been described previously (Anim-Nyame *et al,* 2000a, b & c, 2001). As per this protocol, the venous congestion pressure was raised rapidly by 40 mmHg, and the pressure held for 10 seconds. Assuming this pressure occludes venous return without hampering the arterial flow, as described previously (2.3.1.4), the initial swelling rate will be equal to the arterial blood flow (Groothuis *et al, 20*03). To avoid discomfort to the participants and prolongation of the protocol, no attempt was made to

exclude blood flow through the foot by applying supra-systolic congestion pressure via an ankle cuff.

Blood flow was estimated from the slope of the first 3 seconds of the volume response to the pressure step. The procedure was repeated three times, with the congestion pressure kept at 0 in between each measurement. The inbuilt system analysis programme calculates the change in circumference and uses it to estimate volume change, assuming the gastrocnemius muscle to be a cylinder of uniform diameter and constant length. Units of blood flow were millilitres per 100 ml of tissue volume per minute [ml/100ml/min]. [Figure 2.4]

2.4 Biochemical assays

2.4.1 Assay of biochemical markers of endothelial dysfunction

Blood samples were collected, processed and stored as described in Section 2.2. Biochemical markers of endothelial dysfunction were measured to evaluate the relationships between insulin signalling, insulin resistance, microvascular function and endothelial dysfunction (Petrák *et al,* 2006). The biochemical markers studied were soluble Inter-Cellular Adhesion Molecule-1 (sICAM-1)/ CD54 (Cluster of Differentiation 54); soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)/ CD106; E-selectin/ CD62E; TNF-α, and Thrombomodulin/ CD141. The markers were assayed using ELISA.

Assay: All the samples were assayed as per the manufacturer's guidance. If any sample generated values higher than the highest standard, the sample was further diluted, and the assay repeated. For each assay, the mean absorbance values were calculated for each set of duplicate values. The test was done in a 96-well plate. The samples (100 µl) and the standard calibrators (100 µl) were done in duplicate. To the samples, 100 µl of sICAM-1 conjugate was added, and then covered with adhesive strips, and incubated at room temperature for 1.5 hour, with constant shaking (500±50 rpm). The wells were washed four times with a wash solution (buffered surfactant with preservatives). Then 100 µl of substrate solution (an equal volume mixture of hydrogen peroxide and chromogen) was added, covered with a new adhesive strip, and incubated at room

temperature, protected from light. After 20 minutes, 50 µl of a stop solution was added (2-N sulphuric acid), and put on a shaker for approximately 5 seconds, for proper mixing. Readings were taken within 30 minutes using a plate reader at a wavelength of 450 nm and 570 nm (Labtech International, East Sussex, UK). The reading at 570 nm was subtracted from the reading at 450 nm, to correct for optical imperfections in the plate. Concentrations obtained in each of the assays described below were determined using separate standard curves created for each assay using computer software (Graphpad Prism 7.0, CA, USA)

2.4.1.1 soluble Inter-Cellular Adhesion Molecule-1 (sICAM-1)/ CD54:

Blood was collected with EDTA as an anticoagulant. sICAM-1 was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.1.2 soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)/ CD106

Blood was collected with EDTA as an anticoagulant. sVCAM-1 was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.1.3 E-selectin/ CD62E

Blood was collected with EDTA as an anticoagulant. sEselectin was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.1.4 Tumour Necrosis factor-α (TNF-α)

Blood was collected with EDTA as an anticoagulant. TNF-α was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.1.5 Thrombomodulin/ BDCA-3

Blood was collected with EDTA as an anticoagulant. Thrombomodulin was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.2 Assessment of insulin resistance

Fasting blood glucose levels, and the amount of free insulin, was measured as described below. These were used to calculate insulin resistance by using the Homeostasis Model Assessment (HOMA) method (Matthews *et al,* 1985).

2.4.2.1 Estimation of Fasting Blood Glucose

Blood was collected in sodium fluoride tubes, centrifuged and stored as described in Section 2.2. The samples were later analysed using a GL5 Analox analyser (Alpha Laboratories, Eastleigh, UK) (Virtanen *et al,* 2002). The analyser was first calibrated with glucose standards of 5 mmol/l and 10 mmol/L. Samples (7 µl) were assayed by the analyser.

Principle of the assay: Glucose present in the sample is oxidised by oxygen, to gluconic acid and hydrogen peroxide, by the enzyme, glucose oxidase (GOD).

$$\beta\text{-}D\text{-}Glucose + O_2 \xrightarrow[(GOD)]{Glucose\ Oxidase} D\text{-}Gluconic\ Acid + H_2O_2$$

Thus, oxygen consumption is directly proportional to the glucose concentration in the sample. In this assay, oxygen consumption is used to measure glucose concentration.

2.4.2.2 Assessment of free insulin

Blood was collected with EDTA as an anticoagulant. Free insulin was measured by ELISA, as per the manufacturer's protocol (Mercodia, Sweden), as described in section 2.4.1.

2.4.2.3 Calculation of the Homeostasis Model Assessment (HOMA)

HOMA was calculated from the fasting blood glucose level (mmol/l) and fasting plasma insulin level (µU/ml), by using the following formula (Matthews *et al*, 1985)

HOMA= FI x FG/ 22.5

Where FG is fasting blood glucose level

FI is fasting plasma insulin level

2.4.2.4 Other methods of assessing insulin resistance

There are different methods used for measuring insulin resistance, as described below. In the broadest sense there are two approaches to measure insulin sensitivity and resistance: the dynamic intervention (glucose, insulin, and tolbutamide injection or infusion), and the steady-state (usually fasting) assessment (Radziuk *et al, 20*00; Wallace and Matthews, 2002).

- **The hyperinsulinemic euglycemic clamp**: Often described as the 'gold standard', it measures the amount of glucose necessary to compensate for an increased insulin level without causing hypoglycaemia. The test usually lasts 2 hours. In this test, insulin is infused in a peripheral vein at rate of 10-120 mU/m^2/min. To compensate for insulin, 20% glucose is infused, to maintain blood glucose between 5-5.5 mmol/L. The rate of glucose infusion is determined by measuring blood sugar level every 5- 10 minutes. The rate of glucose estimation in the last 30 minutes, and the glucose infusion rate (Ginf), determine insulin sensitivity.

  Though the gold standard, this method is not practicable in clinical practice, and is only used in research settings. This method is cumbersome, and lengthy, requiring frequent blood testing. It has a large operator dependent error, arising from the infusion rate (Katz *et al, 20*00).
- **Modified clamp:** This method is a modification of the above method. In this method, glucose is labelled with either a stable or radioactive isotope. This method is not suitable in pregnancy (Wallace and Matthews, 2002).
- **Quantitative insulin-sensitivity check index (QUICKI):** This is estimated from fasting blood glucose and fasting insulin levels. It has been pointed out (Skrha *et al, 20*04) that it is simply a logarithm of the HOMA equation.

$$\mathbf{QUICKI = 1/ (log[HOMA]+ log[22.5])}$$

The correlation coefficient between log (HOMA) and QUICKI is 0.98; however, the correlation is more likely between 1/QUICKI and log (HOMA) (Sarafidis *et al, 20*07). Moreover, QUICKI is a measure of insulin sensitivity, whereas HOMA is a measurement of insulin resistance (Radziuk *et al, 20*00). HOMA is used in this study, since there is insulin resistance in pre-eclampsia.

2.4.3 Angiogenic and anti-angiogenic factors

The following markers were determined from the patients' plasma by ELISA, as described below.

2.4.3.1 Human Placental Growth Factor (PlGF)

Blood was collected with EDTA as an anticoagulant. PlGF was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.3.2 Human Endoglin (sEng)/ CD105

Blood was collected with EDTA as an anticoagulant. Endoglin was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.4.3.3 Human soluble Vascular Endothelial Growth Factor Receptor 1 (sFlt-1)

Blood was collected with EDTA as an anticoagulant. sFlt-1 was measured by ELISA as per the manufacturer's protocol (R and D Systems Europe, UK), as described in section 2.4.1.

2.5 Other methods for assessment of endothelial dysfunction

Endothelial cells can slough off the wall of blood vessels in several conditions. It is a marker of endothelial damage (Erdbruegger *et al, 20*10). The numbers of sloughed endothelial cells in the circulation (circulating endothelial cells; CEC), was also used to measure the damage to the endothelium. We used a technique described previously (Woywodt *et al, 20*06).

2.5.1 Circulating Endothelial Cells (CEC)

1 ml of EDTA blood collected from patients was mixed with 20 µl of FcR blocker (Mittenyi Biotech, Germany), to prevent any non-specific binding, for 5 minutes on ice. It was then incubated with CD146 (Biocytec, France) pre-bound to Dynal® magnetic beads (Invitrogen, Paisley, UK) for 30 minutes, on a roller mixer (Appleton Woods, UK) at 4ºC. The tube was then fixed over a magnet (Invitrogen, Paisley, UK), to wash away the non-CD146 blood. The cells were then strained with the endothelial cell marker ULEX Europeus, labelled with FITC (Sigma-Aldrich, UK) for one hour, in the dark, at room temperature. The cells were washed further and then counted in a Nageotte

counting chamber (Hausser Scientific, USA), with a fluorescent microscope. CEC are defined as cells that form rosettes with at least five CD146-bound beads, bind ULEX, and are at least 15-20 μm in diameter. CEC frequency was expressed as cell count per ml of whole blood. The number of endothelial cells in a healthy individual should fall within values of 2-100 cells per ml blood, with a 10-100-fold increase reported in various disease states. (Brogan *et al, 20*06)

2.6 Endothelial cells types used for insulin signalling

Human Dermal Microvascular Endothelial Cells (HDMEC) was chosen for this study. This was the only primary adult endothelial cell line available. Human Umbilical Vein Endothelial Cells (HUVEC) was not used in this study as they were foetal in origin. HDMEC consequently were used as a model for the maternal endothelium. HDMEC has been used previously in *in vitro* studies (Bouïs *et al* 1992).

The cells were cultured as per the manufacturer's guidance (PromoCell, Germany). They were incubated in an incubator (Thermo Fisher Scientific, UK), at 37°C, at 5% Carbon dioxide (CO_2). The cells were 80% confluent, they were split into two 25-cm^2 tissue culture flasks (Becton Dickinson, UK). In this way, they were split and passaged until passage 4. The cells were plated onto 48-well plate and grown to 80% confluence.

2.7 Study of the insulin signalling pathway

For studying the insulin-signalling pathway, the cells were incubated in the serum of individual patients. Various signalling proteins were then studied in these cells. To study the effect of the serum on the viability cells, Trypan Blue assay was first undertken.

2.7.1 Trypan Blue Assay

The cells were passaged to passage 4. On passage 5, 5000 cells were incubated in each well of a 48 well plate. Once the cells were 80% confluent; the cells were incubated in either the pre-eclamptic serum, or a normotensive pregnant serum, or culture media or with 1% formaldehyde solution (to kill the cells). In each group, there were 15 wells, except for formaldehyde. While the

wells were incubated in serum for up to 60 hours, they were incubated with 1% formaldehyde for 15 minutes. The wells were incubated at varying lengths of time. A well from each group was examined at 4 hourly intervals. The cells were then strained with 1% Trypan Blue. The number of dead cells (strained positive for Trypan Blue), was counted over 10 microscopic fields (with 10x magnification factor).

Findings:- It was seen that after 44 hours the number of Trypan Blue positive cells in the sera incubated cohorts increased exponentially. That is why in this experiment, the incubation period 40 hours was chosen.

2.7.2 Preparation of samples for insulin signalling proteins

Once the cells were 80% confluent in 48-well plates, the endothelial cells were washed thoroughly with phosphate buffered saline (PBS) (Gibco, UK) and incubated in 100% sera, from different participants. These included sera from pre-eclamptic women, normotensive pregnant women, and culture media. The serum was not changed. The cells were cultured for 40 hours and then expression of proteins relevant to insulin signalling pathways was analysed by flow cytometer, and western blotting as described below.

2.7.3 Flow-cytometry

Flow-cytometry was used to analyse the insulin receptor expression and other proteins of the signalling pathway. After growing the cells as described above, the cells were detached with the help of 0.25% Trypsin (Sigma-Aldrich, UK). The cells were mixed with 20 µl of FcR blocker (Mittenyi Biotech, Germany), to prevent any non-specific binding, for 5 minutes on ice. A portion of the sample was taken for a cell count, and 10,000 cells were used for further straining. They were then strained either for surface insulin receptor expression, or for intracellular Akt or GLUT-4 expression. Flow-cytometry was done using the FACS Calibur machine (BD Biosciences, US).

2.7.3.1 Insulin receptor expression

The cells were strained with rabbit anti-human insulin receptor antibody (Santa Cruz, US) [ratio 1:25] for 30 minutes at room temperature. The cells were washed two times with buffer [PBS, bovine serum albumin (BSA), 0.1% sodium azide], and then incubated with anti-rabbit antibody-FITC (SantaCruz, US) [ratio 1:25] and 1% providone iodide (Sigma-Aldrich, UK) [ratio 1:50]. The cells were also incubated with VE-Cadherin-PerCP (SantaCruz, US) [ratio 1:15], at the same time. VE-Cadherin is a cell surface marker for endothelial cells, while PerCP is the immunofluorescent marker. The cells were incubated in the dark for 1 hour. Afterwards, the cells were washed two times with buffer (PBS, BSA, 0.1% sodium azide), and then fixed with 200 µl of 1% formaldehyde (Becton Dickinson). Acquisition was done with the CellQuest software (Becton Dickinson, US) within 1 hour.

2.7.3.2 Expression of intracellular Akt and GLUT-4

The cells were washed with buffer (PBS, BSA, 0.1% azide) and centrifuged at 2500 rpm for 5 minutes. Then ice-cold permeabilization buffer [1% Saponin in buffer (PBS, BSA, 0.1% azide)] was added drop by drop, over a vortex. The cells were incubated in the permeabilization buffer for 10 minutes at 4ºC, and then washed with buffer two times to remove the excess permeabilization buffer. The pellet was suspended in 100µl of permeabilization buffer containing primary antibody, either rabbit anti-human Akt antibody (Santa Cruz, US) [ratio 1:25], or rabbit anti-human GLUT-4 antibody (Santa Cruz, US) [ratio 1:25]. The cells were incubated in the primary solution for 30 minutes at room temperature, and then washed two times with buffer [PBS, BSA, sodium azide], and incubated with anti-rabbit antibody-FITC (SantaCruz, US) [ratio 1:25] and Vimentin-PE (SantaCruz, US) [ratio 1:15], at the same time. Vimentin is an intracellular marker, while PE is the immunofluorescent marker. The cells were incubated in the dark for 1 hour. Afterwards, the cells were washed two times with buffer (PBS, BSA, Sodium Azide), and then fixed with 200 µl of 1% formaldehyde. Acquisition was done with the CellQuest software (Becton Dickinson, US) within 1 hour.

2.7.4 Estimation of signalling proteins by western blot.

2.7.4.1 Preparation of samples.

After the cells were incubated in the patient's serum for 40 hours, they were washed with PBS three times to remove any trace of serum. To each 48-well plate 55 µl of sample buffer (Tris, SDS, ß-marcaptoethanol, glycerol, dd H_2O, and Bromophenol blue) was added. Each sample was then heated to 100ºC for 2 minutes. The samples were kept at -80ºC until analysed.

2.7.4.2. Estimation of insulin receptor protein

Western blotting was done using a 9% SDS-PAGE gel (dd H_2O, Tris, Bisacrylamide, SDS, APS, TEMED), using a molecular weight marker (Bio Rad, UK) in the first lane. 25 µl of sample were used in each well. Cells grown in culture media were used as control in each gel. The transfer was done using a Mini-Protean Tetra electrophoresis system (Bio Rad, UK). The proteins were transferred onto hydrophobic polyvinylidene diflouride (PVDF) membranes (Amersham GE Healthcare UK), using a semi dry blotting system (Amersham GE Healthcare UK). After transfer, the membrane was blocked with 3% BSA (Sigma UK) in Tween buffer solution [1% Tween 20 in Tris, sodium chloride solution], for 2 hours. Following blocking, the membrane was cut into two at the level of the 70kD molecular weight marker. The top part of the membrane was incubated with rabbit anti-human IgG insulin receptor antibody [ratio 1:500] (Santa Cruz, Germany), while the bottom part of the membrane was incubated with goat anti-human IgG actin antibody [ratio 1:1000] (Santa Cruz, Germany).

The membranes were incubated overnight at 4ºC on a rotatory shaker. The next morning, the membranes were washed with 1% Tween buffer solution every 10 minutes for 1 hour. After washing, they were incubated in their respective secondary antibodies; the top part with goat anti-rabbit IgG- Horse radish peroxidase (HRP) [ratio 1:1000] (Santa Cruz, Germany), while the bottom part of the membrane was incubated with donkey anti-goat IgG- HRP [ratio 1:1000] (Santa Cruz, Germany), for 1 hour at room temperature, with gentle shaking. The membranes were again washed with Tween buffer solution every 10 minutes for 1 hour. The membranes were analysed within 30 minutes using a chemiluminescent buffer (Amersham GE Healthcare UK), on a

GeneGnome system (Syngene, UK), with software Gene Snap image acquisition software (Syngene, UK). The results were analysed using the Gene Tool image analysis software (Syngene, UK).

Stripping the membrane

Following image capture, the bottom part of the membrane was washed two times with 1% Tween buffer. It was then incubated in stripping buffer (Thermo Scientific, UK), on a rotatory mixture for 15 minutes. The membranes were then checked on a Gene Gnome to make sure that the stripping was complete. The membranes were stripped for estimation of Akt on the same blot as Actin.

2.7.4.3. Estimation of Akt.

Following stripping, the membranes were again blocked with 3% BSA (Sigma UK) in Tween buffer solution [1% Tween 20 in Tris, sodium chloride solution], for 2 hours. After blocking, the membrane was incubated with rabbit anti-human IgG Akt antibody [ratio 1:500] (Santa Cruz, Germany). The membranes were incubated overnight at 4ºC on a rotatory shaker. The next morning, the membranes were washed with Tween buffer solution every 10 minutes for 1 hour. After washing, they were incubated in secondary antibodies; the top part with goat anti-rabbit IgG- HRP [ratio 1:1000] (Santa Cruz, Germany), for 1 hour at room temperature, with gentle shaking. The membranes were again washed with Tween buffer solution every 10 minutes for 1 hour. The membranes were analysed within 30 minutes using a chemiluminescent buffer (Amersham GE Healthcare UK), on the GeneGnome system (Syngene, UK), with Gene Snap (Syngene, UK). The results were analysed using the Gene Tool software programme (Syngene, UK).

2.8 Statistical Analysis

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula, since the data was non-parametric. P-values of <0.05 were

considered statistically significant. Statistical analysis was performed using Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, ILL, USA) and Graphpad Prism Version 7.0 (Graphpad Prism 7.0, CA, USA).

Chapter 3: Results and Discussion

Chapter 3.1

Microvascular Tissue Blood Flow in Pre-eclampsia

3.1.1 Introduction

Pre-eclampsia is a multi-system disorder of the second half of pregnancy characterised by hypertension and proteinuria and is a leading cause of maternal and perinatal morbidity and mortality (Sibai *et al, 20*03). It is characterised by increased peripheral vascular resistance (Anim-Nyame *et al, 20*15), and the clinical presentations are suggestive of impaired blood flow to the affected vascular beds (Bosens *et al, 20*02). Although the exact cause(s) are unknown, abnormal implantation of the foetus has been implicated. It results in impaired placental perfusion (Papageorghiou *et al, 20*02; VanWijk *et al, 20*00). The mechanism by which the impaired placental perfusion translates into deranged maternal physiology and metabolism is still unclear. It was suggested that unidentified factor(s) released by the ischemic placenta into the maternal circulation causes generalised endothelial cell dysfunction, causing widespread circulatory changes, leading to the changes commonly seen in pre-eclampsia (Roberts and Lakin, 2002). Though there has been ongoing extensive research in this field, the factor(s) are still elusive.

Peripheral blood flow increases in both the resting condition (Liang *et al, 20*18; Oyama-Kato *et al, 20*06) and under stress challenges (Jacob *et al, 20*16; Hellsten *et al, 20*12) in normal pregnancy. The mechanism of regulation of microvascular blood flow has been described earlier in this work (Chapter 1.1.2.4). This mechanism of control of peripheral blood flow under stress, mediated by the endothelium, is reduced in pre-eclampsia (Anim-Nyame *et al,* 2003). Thus, the control of the microcirculation is very much dependent on the presence of an intact endothelium. Pre-eclampsia is associated with endothelial dysfunction (Levine *et al, 20*04; Levine *et al, 20*06). Thus, the mechanism of fine-tuning the microcirculation control is impaired in pre-eclampsia. The end organ failure associated with severe pre-eclampsia may be a result of severe impaired blood flow to these organs; although this has yet to be demonstrated.

In the present study, strain gauge plethysmography was used to compare peripheral blood flow in pre-eclampsia and normal pregnancies. This technique was first proposed by Whitney (1953) and has been described in detail by both Gamble (Gamble *et al* 1998) and Christ (Christ *et al,* 2000a). It is a non-invasive technique to assess limb blood flow used previously in normal pregnant women and women with pre-eclampsia (Anim-Nyame *et al,* 2003). It has also been used previously to study endothelial-dependent vascular response to pharmacological agents (Christ *et al,* 2000b). The gastrocnemius muscle was chosen over skin to study the peripheral microvascular blood flow, because the gastrocnemius muscle has a high muscle to skin ratio, which means that the blood flow through it relates more to the support of metabolism. Moreover, since the skeletal muscle vasculature lacks visible arterio-venous channels, most of the blood flow will traverse microvascular beds, representing microvascular blood flow. Skin, on the other hand, provides an accessible and convenient organ for investigating peripheral haemodynamics. However, its usefulness as a measure of metabolic flow, is limited by the dual function of skin, which is both nutritional and thermoregulatory. Moreover, the surrounding temperature regulates the cutaneous perfusion. In the present study, the resting blood flow was measured in pre-eclamptic and normotensive pregnancies, using strain gauge plethysmography as described in Chapter 2.3.

3.1.2 Method

Participants

In this study, microvascular blood flow was compared between pre-eclamptic women (n=16), and women with normotensive pregnancies (n=18), who were recruited from the maternity department at Kingston Hospital, UK, as described previously (Section 2.1)

Measurement of Microvascular blood flow

Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany) was used as described previously (Chapter 2.3). Briefly, the patients were rested for about 30 minutes in a semi-recumbent position. Observations were made in the left-lateral position, to prevent aorto-caval compression, by the gravid uterus. The right mid-gastrocnemius muscle was supported by pillows, at the level of the heart. (Figure 2.3)

Power calculations were based on a previous cross-sectional study showing that resting blood flow was significantly reduced in pre-eclampsia compared to normal pregnant controls (1.95 ± 0.9 ml/min/100ml versus 3.9 ±1.4 ml/min/100ml, p = 0.004, for pre-eclampsia and normal pregnancy respectively) (Anim-Nyame *et al, 20*00). This study showed that a sample size of 10 in each group was sufficient to achieve statistical significance with α of 0.05 and β of 0.02.

Statistical Analysis:

The demographic data were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the data was not normally distributed (P-P plots). Cor-relation was done using the Spearman's formula. The demographic data between the groups was compared using t-tests. P-values of <0.05 were considered statistically significant.

3.1.3 Results

Participants were only recruited in this study, if they were healthy pre-pregnancy. Smokers were also excluded from the study, because smoking alters endothelial cell function (Ozaki *et al, 20*10; Zeiher *et al, 20*05; Csordas and Bernherd 2013). Some of the participants suffered from asthma, but their disease was well controlled. None of them had any exacerbation within the last 3 years. None of them had used their inhalers within a year prior of becoming pregnant.

The clinical and demographic characteristics of the participants are shown in Table 3.1. There was no significant difference in age, booking BMI, gestational age or haematocrit between the two groups. Babies born to the pre-eclamptic women were smaller in weight than the normal pregnant group, which was statistically significant (p = 0.023). As expected from the recruitment criteria, women with pre-eclampsia, had higher systolic, diastolic, and mean arterial pressure, compared with the normotensive pregnant women (p<0.001).

Tissue blood flow was significantly reduced in the pre-eclamptic group when compared with the normal pregnant controls [1.13 (0.94– 1.54) and 4.03 (3.05 -5.35) $ml \cdot min^{-1} \cdot 100\ ml^{-1}$, p <0.0001; for

pre-eclampsia and normal pregnancy respectively] (Figure 3.1). None of the participants in the normotensive pregnancy control group tested positive for protein in a urinary dipstick test. The 24-hour urinary protein concentration was increased in all the pre-eclamptic women [0.64(0.37- 1.66)] {p-value was not calculated as 24-hour urinary protein was not done in the normal pregnant cohort}; although it did not correlate with the microvascular blood flow [r_s=-0.05 (p=0.854)]. In the pre-eclamptic group, microvascular blood flow showed statistically significant correlations with gestational age [r_s=0.558 (p=0.025)], systolic blood pressure [r_s= -0.96 (p<0.001)], mean arterial pressure [r_s= -0.73 (p=0.001)], and platelet count [r_s=0.674 (p=0.004)]. There was no statistically significant correlation with any other parameters in the pre-eclamptic group. In the normotensive pregnancy group, there was no statistically significant correlation with any of the parameters.

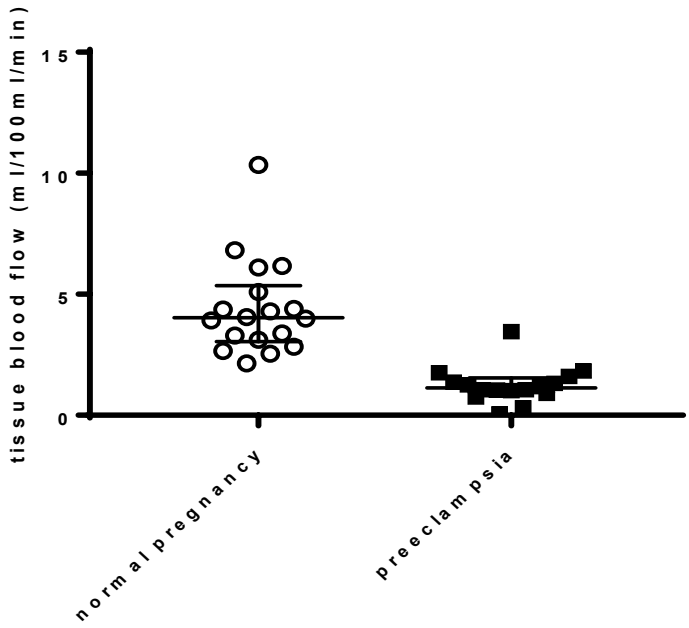


Figure 3.1: Comparison of resting maternal gastrocnemius muscle blood flow in pre-eclamptic pregnancies and normal pregnant controls.

There was no difference in liver and renal functions in between the two groups. The serum albumin level was lower in the pre-eclamptic group [22 (19.25-23) g/L;)], than normal pregnant control [24 (23-26.5) g/L]. There was no statistically significant correlation of albumin with microvascular blood flow (r_s= 0.01; p=0.97), in the pre-eclamptic group. In the pre-eclamptic group, serum urate level was higher than the normal pregnant women [0.34 (0.285- 0.425) mol/L]. There is a significant

direct correlation with microvascular blood flow and serum urate level (r_s= 0.512; p=0.043). [Appendix 3]

Variable	Normal Pregnancy (n=18)	Pre-eclampsia (n=16)	P value *
Age (years)	32.94± 1.24	32.31± 1.16	0.7145
Gestational age (weeks)	34.42± 0.48	34.11± 0.52	0.6705
BMI (kg/m^2)	25.97± 0.91	23.87± 0.91	0.1125
Systolic BP (mmHg)	113.6± 2.83	140.6± 2.06	<0.0001*
Diastolic BP (mmHg)	75.83± 1.81	94.56± 1.16	<0.0001*
Mean Arterial Pressure (mmHg)	88.43± 1.88	109.9± 1.24	<0.0001*
Haematocrit	0.341± 0.01	0.3259± 0.01	0.2425
Platelet (x10^6/ml)	270.9± 11.98	147.1± 9.2	<0.0001*
Birth weight (g)	3337.78± 158.3	2742+ 197.99	0.0238*

*= P- value less than 0.05 are considered significant.

Table 3.1: Clinical and demographic characteristics of the subjects in the cross-sectional study.

3.1.4 Discussion

In this present study, the hypothesis was tested that nutritive microvascular blood flow is reduced in pregnancies complicated by pre-eclampsia. The result shows that there was a significant reduction in the tissue blood flow in pre-eclamptic patients, supporting the hypothesis. There was a strong inverse correlation with both SBP and MAP. Since SBP is an accepted index of disease severity, a decrease in microvascular blood flow is also an indicator of the disease severity.

In normal pregnancy, tissue blood flow is increased by vasodilatation resulting from relaxation of the resistance vessels, and relaxation of precapillary arterioles (Thadbani *et al, 20*04, Barret *et al, 20*09, Vincent *et al, 20*04). In pre-eclampsia, there was a marked reduction in the peripheral blood flow. As expected, the results showed an inverse relationship with SBP and MAP. There was also

a direct relationship between tissue blood flow and platelet function and serum urate level. Reduction in the microvascular circulation in pre-eclampsia, mirrors the reduction in perfusion of other vital organs in the body (Christ *et al* 1998). This may also explain the impaired blood flow in other organs such as liver and kidneys and result in intrauterine growth reduction in pregnancies complicated by pre-eclampsia. Therefore, this data strengthens the case for inclusion of measurement of resting blood flow in the estimation of severity of pre-eclampsia. This might help clinicians in planning the management of pre-eclampsia.

In this study, gastrocnemius muscle was chosen for measuring microvascular blood flow. It is assumed that the blood flowing through the gastrocnemius muscle is mainly microvascular and used for nutritive purposes. Previous plethysmography studies on gastrocnemius muscle microvascular function demonstrated that blood flow changes in the gastrocnemius muscle tissue provided a realistic index of parallel changes in vital organs of patients in critical care (Christ *et al* 1998). Gamble *et al* (1993) showed similar results using measurements in the forearm and gastrocnemius muscle; however Altenkirch *et al* (1989) demonstrated that the results obtained from the gastrocnemius muscle were more reproducible than from the forearm.

Pregnancy is associated with considerable changes in the circulation of the mother. This is to facilitate the growing foetus. Maternal blood flow increases gradually during normal pregnancy, because of the vaso-relaxing effect of oestrogen and other substances (Nevo *et al, 20*10). The peripheral vascular resistance is reduced in normal pregnancy. Women who have chronic hypertension or pre-eclampsia, have impaired autoregulation (van Veer *et al, 20*15). Because of reduced blood flow, tissues suffer from chronic hypoxia, resulting in intrauterine growth retardation of the foetus (Karanam *et al, 20*14). It may also affect other organs, and in severe disease can lead to HELLP (Haemolysis, Elevated Liver enzymes, and Low Platelet) syndrome (Aloizos *et al, 20*13). Delivery is the only effective treatment of pre-eclampsia.

All the changes seen in pre-eclampsia, revert to the pre-pregnancy state post-delivery. Tissue blood flow also returns to normal. All the organ functions revert back, except the maternal

endothelium. Markers of endothelial dysfunction will always be elevated in women with a history of pre-eclampsia (Tuzcu *et al, 20*15). Women with previous history of pre-eclampsia are at increased risk of hyperinsulinaemea and diabetes (Laivuori *et al* 1996), or cardiovascular disease (Wolf et al, 20*04*).

Chapter 3.2

Relationship of endothelial dysfunction and microcirculation in pre-eclampsia

3.2.1. Introduction

In pre-eclampsia, structural changes of the endothelium have been seen in the uteroplacental vessels (Brosens *et al, 20*02). There is extensive evidence of biochemical changes, such as increased concentration of von Willebrand's factor, endothelin, fibronectin, and an imbalance between PGI_2 and TXA_2 in pre-eclampsia (Roberts 1998). Vascular tone and thus peripheral resistance are known to be under the continuous influence of endothelial-derived factors. Furthermore, many markers for endothelial injury or dysfunction, present in women with pre-eclampsia, precede clinically evident disease (Esper *et al, 20*06, Mol *et al, 20*16). Endothelial activation is only one component of a generalised activation of inflammatory responses that is characteristic of pregnancy (sometimes showing changes nearly as pronounced as seen in sepsis) and further accentuated in pre-eclampsia (Robert and Hubel 2009). Thus, pre-eclampsia may represent an exaggeration of the normal inflammatory state of pregnancy (Germain *et al, 20*07).

There are ever-evolving bodies of evidence for using different markers in determining the endothelial dysfunction. One of these is the decrease/ derangement in microcirculation function in pregnancies complicated by pre-eclampsia (Anim-Nyame *et al, 20*00a, 2001), as described previously (Chapter 3.1). The other evidence is an increased soluble marker of endothelial activation and injury, which will be assessed in this chapter. The aim of this chapter is to investigate whether a relationship exists between soluble markers of endothelial dysfunction and changes in microvascular function in pregnancies complicated by pre-eclampsia.

3.2.2. Method

Participants, Blood Sampling and Assays

In this study, participants were recruited from the maternity department at Kingston Hospital, UK, to compare microvascular blood flow and correlate it with markers of endothelial dysfunction as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each

participant aseptically, as described in Chapter 2.2. Markers of endothelial dysfunction, s-ICAM-1, s-VCAM-1, e-Selectin, Thrombomodulin and TNF-α were measured by ELISA (R and D Systems Europe, UK), as described previously in Chapter 2.4.

Measurement of blood flow

In this study, Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany) was used as described previously (Chapter 2.3).

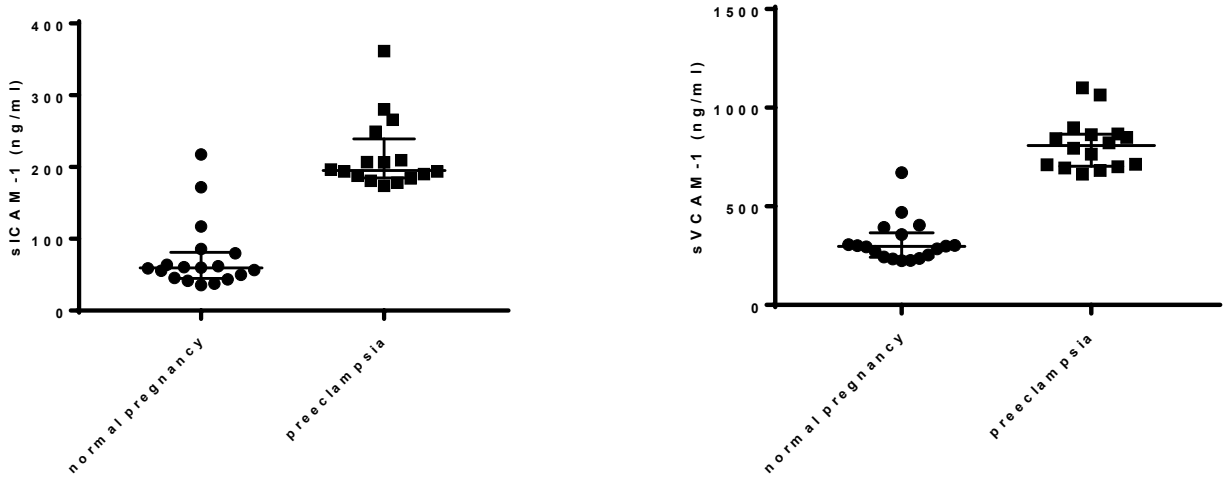
Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

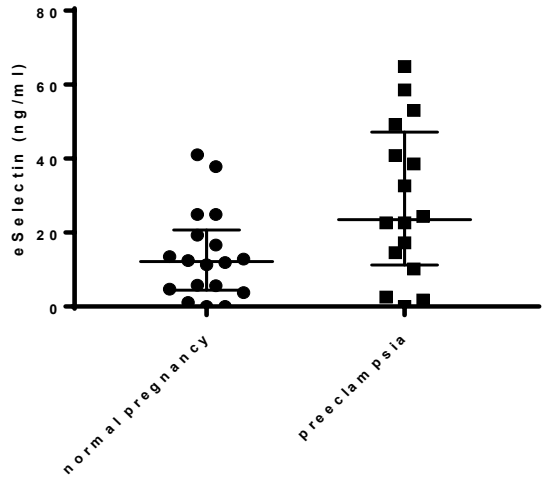
3.2.3. Results

The clinical and demographic characteristics of the participants are shown in Table 3.1 (Chapter 3.1). There was no significant difference in age, BMI, gestational age or haematocrit between the two groups. Babies born to the pre-eclamptic women were smaller in weight than the normal pregnant group, which was statistically significant. ($p = 0.023$). As expected from the recruitment criteria, women with pre-eclampsia, had higher systolic and diastolic blood pressures, and mean arterial pressure compared with the normal pregnant controls ($p<0.001$).

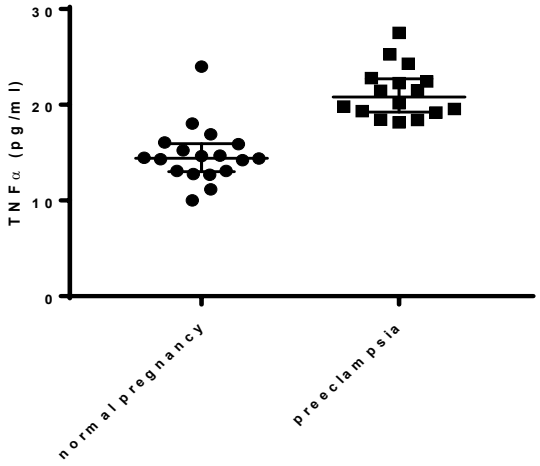
Tissue blood flow and markers of endothelial dysfunction are shown in Table 3.2. As already described and discussed in the previous chapter (Chapter 3.1), tissue microvascular blood flow was significantly reduced in the pre-eclamptic group in comparison to the normal pregnant cohort. As expected, all the biochemical markers of endothelial dysfunction were significantly raised in the pre-eclamptic group (Table 3.2; Figure 3.2.1). In the normal pregnant cohort, the biochemical endothelial markers did not show any significant statistical correlations with maternal age,



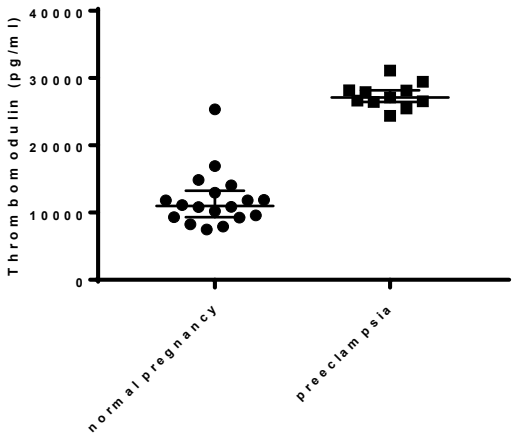
(b)



(c)



(d)



(e)

Figure 3.2.1: Comparison of (a) ICAM-1, (b) VCAM-1, (c) e-Selectin, (d) TNF-α, and (e) Thrombomodulin in pre-eclamptic pregnancies and normal pregnant controls.

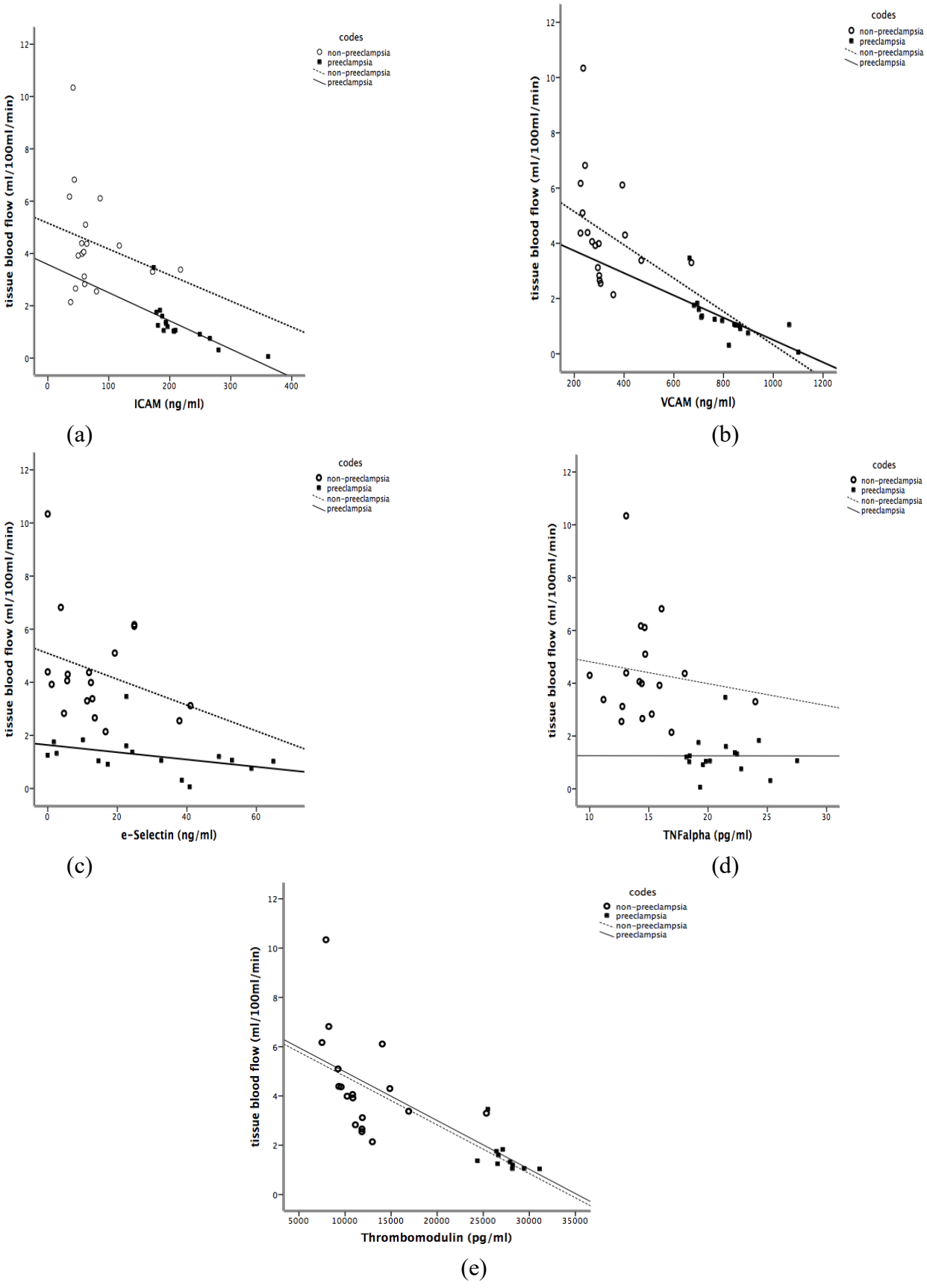


Figure 3.2.2: Correlation of microvascular blood flow with (a) ICAM-1, (b) VCAM-1, (c) e-Selectin, (d) TNF-α, and (e) Thrombomodulin in the two cohorts.

gestational age, BMI, MAP, haematocrit, and the birth weight. However, in the pre-eclamptic group, ICAM-1 showed a statistically significant inverse correlation with gestational age [r_s= -0.676; p=0.004], and platelet count [r_s= -0.694; p=0.003], but a direct correlation with systolic blood pressure [r_s= 0.883; p<0.0001], and MAP [r_s= 0.588; p=0.017]. Furthermore, VCAM-1 had a statistically significant inverse correlation with platelet count [r_s= -0.559; p=0.024], but a direct correlation with systolic blood pressure [r_s= 0.854; p<0.0001], and MAP [r_s= 0.673; p=0.004]. Thrombomodulin showed a statistically significant inverse correlation with platelet count [r_s= -0.764; p=0.006], and a direct correlation with systolic blood pressure [r_s= 0.91; p<0.0001], in the pre-eclamptic cohort.

Variable	**Normal pregnant (n=18)**	**Pre-eclampsia (n=16)**	**P value ***
Tissue Blood Flow ($ml \cdot min^{-1} \cdot 100\ ml^{-1}$)	4.03 (3.05 -5.35)	1.13 (0.94– 1.54)	<0.0001*
sICAM-1 (ng/ml)	59.35 (44.94 – 81.28)	195.2 (184.9 - 239.3)	<0.0001*
sVCAM-1 (ng/ml)	296.55 (241.33- 365.85)	808.3 (703.37- 866.11)	<0.0001*
sEselectin (ng/ml)	12.63 (5.64 – 23.48)	24.35 (14.6 – 49.25)	0.042*
TNF- α (pg/ml)	14.43 (13 – 15.94)	20.82 (19.23 – 22.71)	<0.0001*
Thrombomodulin (pg/ml)	10984 (9301- 13234)	27115 (26448- 28192)	<0.001*

*= P- value less than 0.05 are considered significant.

Table 3.2: Showing the Tissue blood flow and markers of endothelial dysfunction in subjects in the cross-sectional study.

Microvascular tissue blood flow showed a statistically significant correlation with the endothelial markers. In the pre-eclamptic cohort, microvascular blood flow had an inverse correlation with ICAM-1 [r_s= -0.915; p<0.0001], VCAM-1 [r_s= -0.903; p<0.0001], eSelectin [r_s= -0.546; p=0.028], and Thrombomodulin [r_s= -0.773; p=0.005]. However, TNF-α did not show statistically significant correlation with microvascular tissue blood flow [r_s= 0.062; p=0.82]. In the normal pregnant control group, microvascular tissue blood flow has a statistically significant inverse correlation with VCAM-1 [r_s= -0.591; p=0.01], and Thrombomodulin [r_s= -0.637; p=0.004], but there is no statistically

significant correlation with ICAM-1 [r_s= -0.193; p=0.443], eSelectin [r_s= -0.162; p=0.549], and TNF-α [r_s= -0.034; p=0.893] (Figure 2.2.2).

3.2.4. Discussion

In this present study, the hypothesis that endothelial dysfunction present in pregnancies complicated by pre-eclampsia correlates with impaired microvascular blood flow, was tested. The results demonstrated that there was significant increase in the soluble markers of endothelial dysfunction in pre-eclampsia, supporting this hypothesis. Furthermore, this correlates with MAP, platelet count and systolic blood pressure. As described previously (Chapter 3.1), microvascular blood flow was also reduced in pre-eclampsia.

Normal pregnancy is associated with marked anatomical and functional changes of the cardiovascular system to accommodate the increased demands of pregnancy. Generalised vasodilatation begins developing from the luteal phase after conception, and peripheral vascular resistance starts falling substantially after 5 weeks gestation (Liu and Arany, 2014; Fu and Levine, 2009). As a result, peripheral blood flow increases substantially, particularly in the cutaneous, renal and uteroplacental circulation (Hibbard *et al, 20*15; Caniggia *et al, 20*00). Alteration in synthesis or response to vasoactive substances, like NO, prostaglandins, endothelins and angiotensins, may be involved in the fall in peripheral resistance in normal pregnancy (Chaddha *et al, 20*04). During early pregnancy, trophoblast cells invade the placental bed, leading to remodelling of the spiral arteries into maximally dilated low resistance vascular channels, which are unable to respond to vasoactive mediators, thereby guaranteeing a high flow volume to the uteroplacental bed (Brosens *et al, 20*02). In pre-eclampsia, this vascular remodelling seen in normal pregnancy is impaired. This results in reduction of uteroplacental circulation, with the placenta becoming increasingly ischemic as pregnancy progresses (Frusca *et al, 20*03). This is evidenced by observations that placentae from women with pre-eclampsia have infarcts on histology, and there is rapid recovery from this condition following delivery (Mol *et al, 20*16; Fisher 2015).

The vascular endothelium has many important functions, including control of smooth muscle tone through release of vasoconstrictor and vasodilatory substances, regulation of anticoagulation, antiplatelet, and fibrinolysis functions via release of different soluble factors (Roberts and Lain 2002). Release of factors, from the placentae, results in endothelial dysfunction of maternal circulation in pre-eclampsia (Roberts and Hubel 2009). Since evidence of endothelial dysfunction precedes the clinical onset of the disease; it has been suggested to be the cause, and not the result, of pre-eclampsia. Additionally, in women with pre-eclampsia, preexisting maternal factors such as chronic hypertension, diabetes and hyperlipidemia, predisposes the maternal endothelium to further damage (Roberts and Hubel 2009).

Pre-eclampsia is thought to be an exaggerated inflammatory response of pregnancy, to yet unknown factor(s). Inflammatory endothelium may, in response to unknown mediators, express new cell surface molecules that are adhesive for leukocytes, helping its adhesion and extravasation. Several such molecules are known, like ICAM-1, VCAM-1, eSelectin, which have specific ligands on the leukocytes. These molecules are expressed after stimulation of endothelial cells with cytokines, such as TNF-α and inlerleukin-1β (Chaiworapongsa *et al, 20*02, Chavarria *et al, 20*08). In pre-eclampsia, there is an increase in proteins of the coagulation cascade. Circulating levels of fibronectin are significantly increased in women who develop pre-eclampsia, as early as 20 weeks gestation (Chaiworapongsa *et al, 20*02). Thrombomodulin, an anticoagulant factor, also increases in pre-eclampsia, and is detected as early as 24 weeks gestation. Von-Willebrand factor is also elevated in pre-eclampsia. Platelets play an important role in the etiology of pre-eclampsia. Increased platelet activation occurs in the disease (Roberts and Lain, 2002). The increased expression of endothelial inflammatory factors and cell adhesion molecules will increase post-capillary pressure resulting in reduced microvascular blood flow (Anim-Nyame *et al, 20*04).

Biomarkers of endothelial dysfunction, like ICAM-1, VCAM-1, are elevated several weeks before the onset of the disease. Their levels are higher in early onset pre-eclampsia, than in late onset disease (Dogan *et al, 20*14). If the levels are elevated at 20 weeks gestation, it can predict the development of severe pre-eclampsia later (Chavarria *et al, 20*08). Since our pre-eclamptic cohort

had mild late-onset pre-eclampsia, that's why the levels reported here are lower than those reported elsewhere (Szarka *et al, 20*10). They are also elevated a few weeks before in patients with preterm delivery (Chen and School 2014). All these are markers of endothelial dysfunction in pre-eclampsia (Szarka *et al, 20*10). These markers are present in the sera from pre-eclamptic patients, but there is no detectable increase in the supernatant of cultured endothelial cells, suggesting that sera from the pre-eclamptic participants stimulate the endothelial cells (Heyl *et al,* 1999). Therefore in this study, the cells were incubated in 100% sera from the participants.

Patients with history of pre-eclampsia are at increased risk of cardiovascular disease in later life (Wolf *et al, 20*04). However, Gaugler-Senden *et al* (2012) showed that there was no difference in the endothelial markers between pre-eclamptic and uncomplicated pregnancy, 10 years later. Therefore, these angiogenic factors will not contribute to the early detection of women at risk for future cardiovascular disease. This finding has been disputed by others. Another study showed that the markers were markedly increased in pre-eclamptic pregnancy, than uncomplicated pregnancy, even after 20 years (Freeman *et al, 20*04, Tuzcu *et al, 20*15)

In summary, results from this chapter shows that reduced microvascular blood flow in pre-eclampsia, might be related to the endothelial dysfunction seen in pregnancies complicated by the disease. There is a positive correlation between microvascular blood flow and markers of endothelial dysfunction.

Chapter 3.3

Relationship between microvascular blood flow and angiogenic factors in pre-eclampsia.

3.3.1 Introduction

Pre-eclampsia is a multi-systemic disorder of the second half of pregnancy. Angiogenic imbalance with increased anti-angiogenic factors, such as sFlt-1 and sEng, appear to play a pathogenic role in the aetiology of pre-eclampsia (Maynard *et al, 20*03; Venkatesha *et al, 20*06). A rise in sFlt-1 and sEng and a reduction in the pro-angiogenic factors, like PlGF, have been reported in maternal serum 5-10 weeks prior to the onset of pre-eclampsia (Levine *et al, 20*04). It is proposed that these anti-angiogenic factors contribute to the maternal endothelial dysfunction seen in pre-eclampsia (Levine *et al, 20*04). Moreover, levels of sFlt-1 directly correlate with the severity of pre-eclampsia and precede the onset of the disease (Levine *et al, 20*04; Karunamichi *et al, 20*08), as sFlt-1 circulates freely in serum, and binds to pro-angiogenic factors, such as VEGF and PlGF.

The mechanism by which sEng works is thought to be via the prevention of the binding of TGF-β to its receptor, reducing the production of NO and its mediated vasodilatation, and subsequent capillary formation by endothelial cells *in vitro* (Pipp *et al, 20*03). Conversely, a reduction in PlGF has been reported in pre- eclampsia (Levine *et al, 20*04). PlGF is a VEGF homologue and stimulates angiogenesis. Reduced levels impair collateral artery growth in mouse limbs and neovascularization in tumors and ischemic retinas, while exogenous PlGF delivery stimulates angiogenesis and collateral growth in ischemic hearts and limbs (Karunamichi *et al, 20*08; Mutter *et al, 20*08).

There is evidence that an alteration in the balance of pro- and anti- angiogenic factors contributes to the generalized endothelial dysfunction and increased vascular resistance in pre-eclampsia (Venkatesha *et al, 20*06; Levine *et al, 20*04). However, Noori *et al* (2011) did not observe any correlation between sFlit-1, sEng and PIGF levels and maternal endothelial function, as measured by brachial artery flow-mediated dilatation (FMD). It was proposed that the lack of correlation was

possibly due to the differential effects of these circulating angiogenic factors on large and resistance vessels.

Angiogenic imbalance is likely to affect microvascular function as the microvasculature is formed by the continuous tension between *de novo* angiogenesis and microvascular regression (rarefaction). Several anti-angiogenic cancer therapies have been implicated in the development of hypertension by inducing microvascular rarefaction (Mourad *et al, 20*08; Steeghs *et al, 20*08). The multisystem manifestations of pre-eclampsia with end-organ dysfunction suggest underlying microvascular dysfunction. Microvascular dysfunction occurs in pre-eclampsia (Anim-Nyame *et al,* 2003) and reduced tissue perfusion precedes the onset of the disease (Anim-Nyame *et al,* 2001). Although many of the key functions of the cardiovascular system occur at the level of the microcirculation, where nutritive exchange between blood and tissue occurs, there are yet no reported studies on the relationship between maternal microvascular perfusion and angiogenic balance.

In this study, it was hypothesized that there is an imbalance in the levels of the pro- and anti-angiogenic factors in pre-eclampsia, which affects microvascular function, and these circulating factors correlate with reduced tissue blood flow. The aim of the following study was to determine the relationship of microvascular tissue blood flow to circulating angiogenic factors in pre-eclampsia. Although the role of sFlt-1, PlGF, and its ratio in pre-eclamptic patients has been reported before (Verlohren *et al, 20*12, Chaiworapongsa *et al, 20*13), this is the first time the correlation of the disease with microvascular blood flow, has been investigated.

3.3.2. Method

Participants, Blood Sampling and Assays

In this study, participants were recruited from the maternity department at Kingston Hospital, UK, to compare microvascular blood flow and correlate this with markers of endothelial dysfunction as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant aseptically, as described in Chapter 2.2. Human PlGF, s-Eng, sFlt-1, and markers of

endothelial dysfunction, s-ICAM-1, s-VCAM-1, e-Selectin, Thrombomodulin and TNF-α were measured by an ELISA (R and D Systems Europe, UK), as described previously in Chapter 2.4.

Measurement of blood flow

In this study, Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany) was used as described previously (Chapter 2.3).

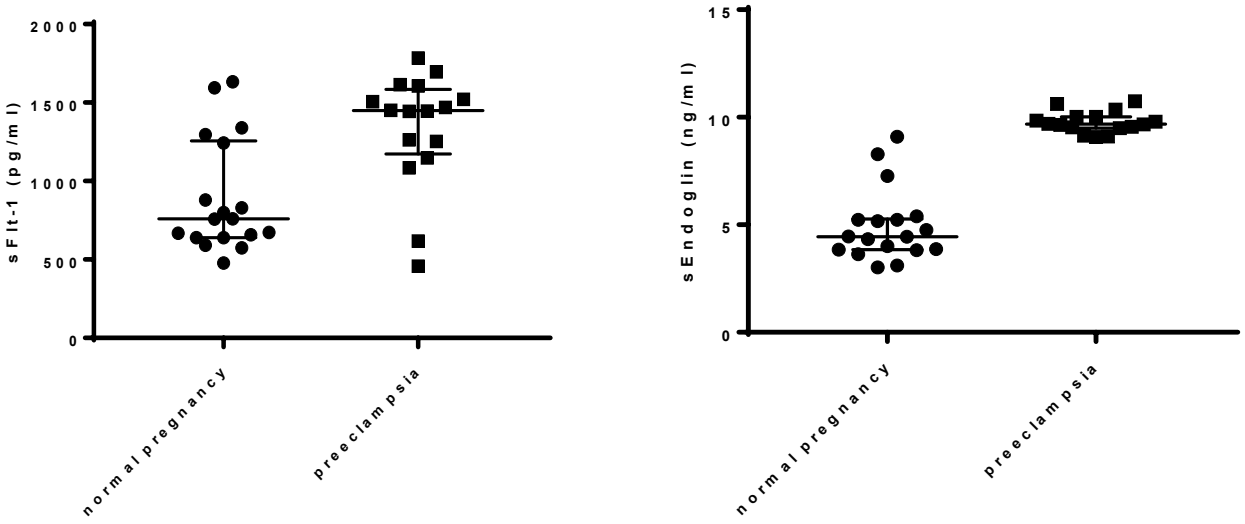
Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

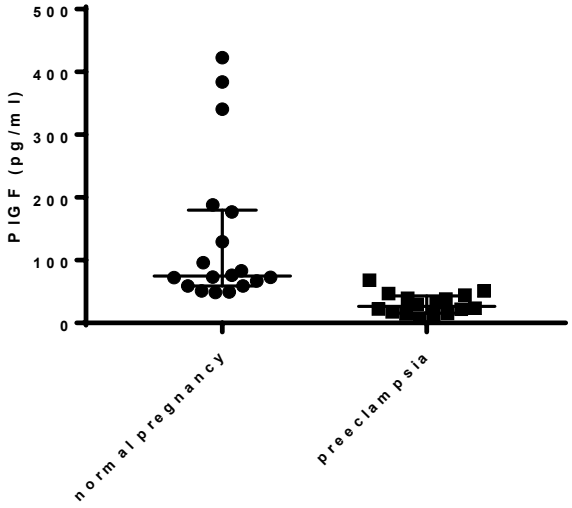
3.3.3. Results

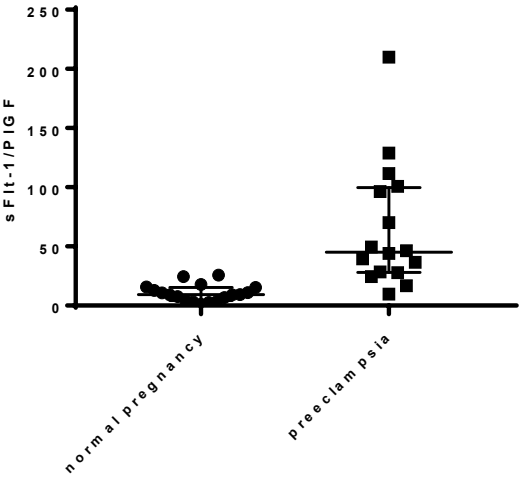
The clinical and demographic characteristics of the participants are shown in Table 3.1 (Chapter 3.1). There was no significant difference in age, BMI, gestational age or haematocrit between the two groups. Babies born to the pre-eclamptic women were smaller in weight than the normal pregnant group, which is statistically significant. ($p = 0.023$). As expected from the recruitment criteria, women with pre-eclampsia, had higher systolic and diastolic blood pressures, and mean arterial compared with the normal pregnant controls ($p<0.001$).

Tissue blood flow, angiogenic factors and markers of endothelial dysfunction are shown in Table 3.3. Microvascular tissue blood flow and pro-angiogenic factor, PlGF, were significantly reduced in the pre-eclamptic group compared to the normal pregnant controls. The anti-angiogenic factors, sFlt-1 and sEng, were raised in the pre-eclamptic group compared to the normal pregnant controls (Figure 3.3.1). The ratio of anti- and pro- angiogenic factors, sFlt-1: PlGF and (sFlt-1+ sEng): PlGF, was elevated in the pre-eclamptic group compared to the normal pregnant controls. As expected,

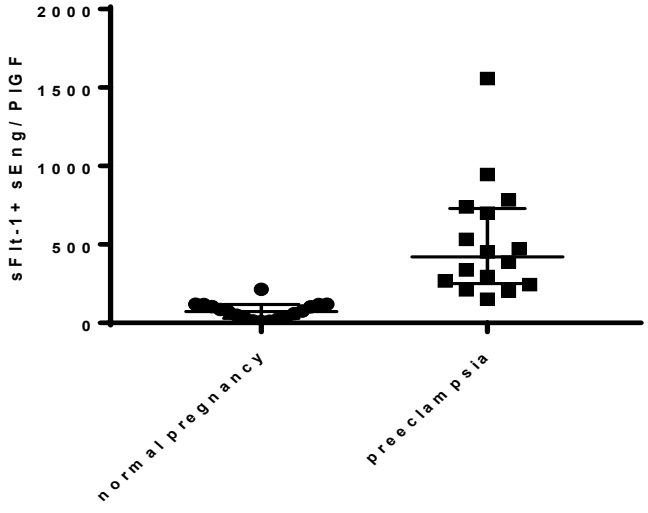


(b)



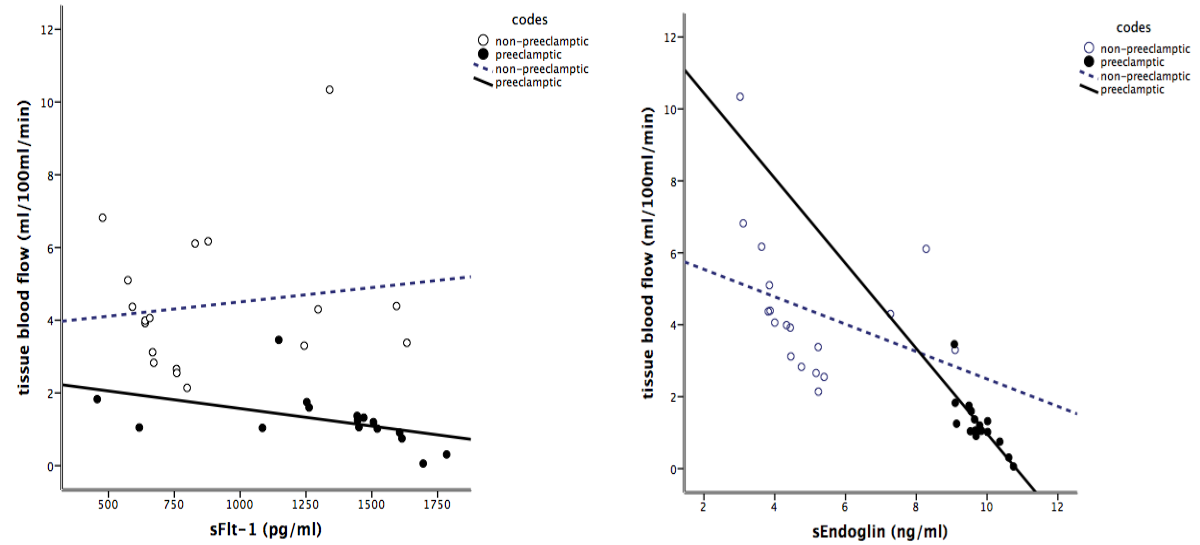


(c) (d)

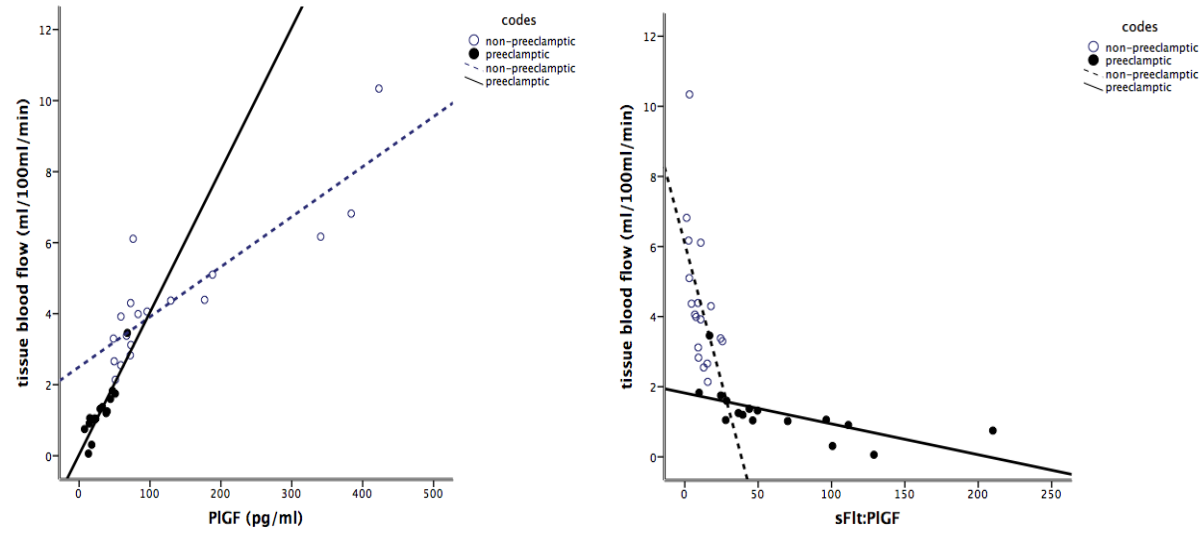


(e)

Figure 3.3.1: Comparison of (a) sFlt-1 (b) sEndoglin (c) PlGF (d) sFlt-1/PlGF and (e) sFlt-1+ sEng/PlGF in normal pregnant controls and pre-eclamptic pregnancies.

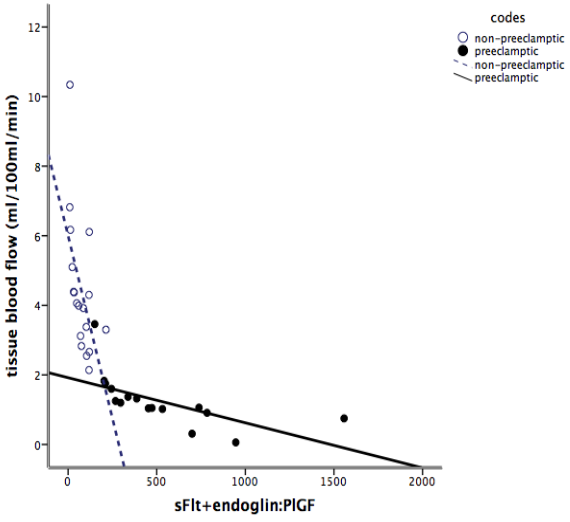


(b)



(c)

(d)



(e)

Figure 3.3.2: Graph showing the correlation of microvascular blood flow, in the study groups, with (a) sFlt-1, (b) sEndoglin, (c) PlGF, (d) sFlt-1:PlGF, and (e) sFlt-1+ sEndoglin: PlGF.

all the markers of endothelial dysfunction, such as sICAM-1, sVCAM-1, sEselectin and TNF-α, were elevated in the pre-eclamptic group.

Variable	Normal Pregnancy (n=18)	Pre-eclampsia (n=16)	P value *
Tissue Blood Flow ($ml \cdot min^{-1} \cdot 100\ ml^{-1}$)	4.03 (3.05 -5.35)	1.13 (0.94– 1.54)	<0.0001*
sFlt-1 (pg/ml)	758.8 (639.2 – 1256.5)	1449 (1173 – 1585)	0.0056*
sEndoglin (ng/ml)	4.448 (3.841 – 5.274)	9.678 (9.498 – 10.02)	<0.0001*
PlGF (pg/ml)	74.61 (58.98 – 179.8)	26.54 (15.72 – 43.07)	<0.0001*
sFlt-1: PlGF	9.227 (4.219 – 15.39)	45.16 (28.06 – 99.59)	<0.0001*
(sFlt-1+ sEng): PlGF	72.68 (29.03 – 117.9)	420.3 (250.2 – 728.7)	<0.0001*
sICAM-1 (ng/ml)	59.35 (44.94 – 81.28)	195.2 (184.9 - 239.3)	<0.0001*
sVCAM-1 (ng/ml)	296.55 (241.33- 365.85)	808.3 (703.4 – 866.1)	<0.0001*
sEselectin (ng/ml)	12.63 (5.637 – 23.48)	24.35 (14.6 – 49.25)	0.0404*
TNF- α (pg/ml)	14.43 (13 – 15.94)	20.82 (19.23 – 22.71)	<0.0001*

*= P- value less than 0.05 are considered significant.

Table 3.3: Showing the tissue blood flow, angiogenic factors and markers of endothelial dysfunction in subjects in the cross-sectional study.

There was a strong inverse correlation between microvascular tissue blood flow and sFlt-1 in the pre-eclamptic group [r_s= -0.738 (p=0.001)], but there was no such significant correlation in the normal pregnant controls [r_s= -0.02 (p= 0.938)] (Figure 3.3.2a). A strong inverse correlation was also observed between blood flow and sEng in the two groups of women [r_s= -0.806 (p<0.001) and r_s= -0.641 (p= 0.004); for pre-eclamptic and normal pregnant controls, respectively] (Figures 3.3.2b). There was a positive correlation between microvascular blood flow and PlGF in normal pregnancy [r_s= 0.882 (p< 0.001)] and pre-eclampsia [r_s= 0.921 (p<0.001)] (Figures 3.3.2c). Blood flow also showed a strong inverse correlation with the sFlt-1: PlGF ratio in normal pregnancy [r_s= -0.692 (p= 0.001)] and pre-eclampsia [r_s= -0.868 (p<0.001)] (Figures 3.3.2d). Microvascular tissue

blood flow also showed a strong inverse correlation with the sFlt-1+ Eng: PIGF ratio in normal pregnancy [r_s= -0.697 (p= 0.001)] and pre-eclampsia [r_s= -0.924 (p<0.001)] (Figures 3.3.2e).

In the pre-eclamptic group, microvascular blood flow showed a significant inverse correlation with SBP [r_s= -0.96 (p < 0.001)] and MAP [r_s= -0.73 (p= 0.001)], and a direct correlation with platelet count [r_s= 0.674 (p=0.004)]. Levels of sFlt-1 showed significant positive correlations between SBP [r_s= 0.685 (p= 0.003)], and an inverse correlation with gestational age [r_s= -0.586 (p=0.017)]. Similarly, serum levels of Endoglin showed significant direct correlations with SBP [r_s= 0.753, (p= 0.001)], and MAP [r_s= 0.541 (p= 0.031)], but an inverse correlation with platelet count [r_s= -0.679, (p= 0.004)]. Serum PlGF showed significant inverse correlations with SBP [r_s= -0.874 (p <0.001)], and MAP [r_s= -0.546, (p= 0.029)], but a direct correlation with platelet count [r_s= 0.624, (p= 0.01)] and gestational age [r_s= 0.666 (p=0.005)]. The sFlt-1: PlGF ratio showed a significant direct correlation with SBP [r_s= 0.805 (p <0.001)], but inverse correlation with platelet count [r_s= -0.562, (p= 0.024)] and gestational age [r_s= -0.742 (p=0.001)]. The sFlt-1+ Eng: PlGF ratio showed a direct correlation with SBP [r_s= 0.869 (p <0.001)], and MAP [r_s= 0.538, (p= 0.032)], but inverse correlation with platelet count [r_s= -0.609, (p= 0.012)] and gestational age [r_s= -0.682 (p=0.004)]. In the normal pregnant controls, there was no correlation between SBP and DBP, MAP, platelet count and gestational age with either the microvascular tissue blood flow or any of the angiogenic factors. [Appendix 3]

3.3.4. Discussion

This study provides the first report on the relationship between maternal microvascular tissue perfusion and the imbalance of angiogenic factors during pregnancy. Data shows that angiogenic factors correlate inversely with microvascular blood flow during normal pregnancy and pre-eclampsia. Increased levels of sFlt-1 and sEng or low PIGF are associated with reduced microvascular flow whereas lower levels of the anti-angiogenic factors and higher pro-angiogenic PIGF levels correlate with a greater blood flow during normal pregnancy.

Increased tissue blood flow is a feature of normal pregnancy as an adaptive response to meet increased metabolic requirements. This implies that a pro-angiogenic profile during normal pregnancy would enhance angiogenesis, resulting in the vascular changes seen in normal pregnancy such as increased microvascular perfusion and a fall in peripheral vascular resistance and blood pressure. Elevations in sFlt-1+sEng: PlGF ratio, as observed in pre-eclampsia would impair angiogenesis, resulting in reduced capillary density and tissue blood flow. This would lead to increased peripheral vascular resistance and hypertension (Humar *et al* 1989), reduced microvascular perfusion (Verlohren *et al, 20*12), impaired end-organ function and possibly contribute to the multi-system manifestations of pre-eclampsia. Indeed, strong correlations between elevated mean arterial pressures and elevated sFLT-1 and sEng and decreased PlGF have been demonstrated in pre-eclamptic patients (Noori et al, 2010). Since impaired tissue perfusion precedes organ dysfunction and correlates with severity of pre-eclampsia (Anim-Nyame *et al, 20*01), measurement of these angiogenic factors could provide a simple clinical test for assessing the severity of end-organ dysfunction, which is a feature of pre-eclampsia.

Augmented levels of anti-angiogenic factors and decreased levels of pro-angiogenic factors in pre-eclampsia correlated with a decreased microvascular blood flow in this study. This decrease in blood flow in pre-eclampsia was also associated with elevations in circulating markers of endothelial activation and inflammation. Increases in circulating inflammatory markers like TNF-α are associated with endothelial cell activation in pre-eclamptic patients. Indeed, serum from pregnant women with pre-eclampsia can induce endothelial dysfunction and injury *in-vitro*, suggesting that circulating factors underlie the pathology of the disease (Myers *et al, 20*05).

Increases in sFlt-1 are associated with decreases in circulating PlGF and VEGF, attributable in part to sFlt-1 binding (Levine *et al, 20*04). Thus standardising this relationship by expressing a ratio of anti-angiogenic: pro-angiogenic factors in maternal plasma is a better prognostic marker for pre-eclampsia than either measure alone (Levine *et al, 20*04, Verlohren *et al, 20*12a & b) and is better at identifying pre-eclamptic patients in the third trimester (Chaiworapongsa *et al, 20*13). Increases in the anti-angiogenic: pro-angiogenic factor ratio in the maternal circulation have been associated

with endothelial dysfunction, related to the pathogenesis of pre-eclampsia (Noori *et al, 20*10) although no direct correlations have been observed between circulating factors and brachial artery endothelial function (Myers *et al, 20*05). The proportion of sFlt-1+sEng: PlGF not only increased with pre-eclampsia but also correlated inversely with microvascular perfusion in both cohorts, with PlGF significantly predicting microvascular blood flow.

Angiogenic factors and their receptors are important regulators of placental vascular development, and neovascularisation (Savvidou *et al, 20*08). Among the angiogenic factors expressed by the placenta, VEGF and PlGF appear to play a central role in vascular development. Increased circulating levels of angiogenic receptor inhibitor (sFlt-1) are believed to compromise angiogenesis by inhibiting mutagenic and homeostatic actions on endothelial cells (Kendall *et al,* 1993). Angiogenic imbalance contributes to abnormal placenta vascular development and also endothelial cell function in pre-eclampsia (Levine *et al, 20*04), although others have failed to show a direct correlation between angiogenic factors and large vessel endothelial dysfunction assessed by brachial artery FMD (Savvidou *et al, 20*08).

Currently no validated test exists that reliably predicts progression of pre-eclamptic pathology and therefore pre-eclamptic women who do not require immediate delivery are monitored as inpatients until timely delivery (Visintin *et al, 20*10). The ratio of sFlt-1:PlGF has been used previously to assess the severity (Verlohren *et al, 20*12a) and prognosis (Verlohren *et al, 20*12b) of pre-eclampsia, as well as to assess the increased risk of stillbirth (Chaiworapongsa *et al, 20*13). The data presented here demonstrates that microvascular blood flow (which precedes end-organ dysfunction) inversely correlates with both sFlt-1:PlGF and sFlt-1+ sEng:PlGF. Thus, the data supports the clinical use of an anti-angiogenic: pro-angiogenic ratio to identify pre-eclamptic women who may be at risk of underlying organ dysfunction and more likely to deteriorate, requiring urgent intervention and early delivery (Verlohren et al, 2012a &b, Chaiworapongsa *et al, 20*13).

This study did not investigate the mechanism underlying the inverse correlation between the anti-angiogenic factors sFlt-1 and sEng and microvascular blood flow. However, it is possible that the

elevated sFlt-1 and sEng could reduce capillary formation in pre-eclampsia leading to a decreased capillary density. Increased levels of sFlt-1 and sEng or reduced levels of PlGF in pre-eclampsia could impair angiogenesis, resulting in reduced capillary density and tissue blood flow. Reduced capillary density (microvascular rarefaction) is a feature of arterial hypertension and increases or aggravates peripheral resistance (Humar *et al, 20*09). Microvascular rarefaction also occurs in pre-eclampsia and precedes the onset of the disease (Myers *et al, 20*05). In pregnant rats, administration of sFlt-1 and sEng causes a pre-eclampsia-like syndrome and vasoconstriction of renal micro vessels (Levine *et al, 20*05). Moreover, anti-angiogenic cancer therapy inhibits angiogenesis and results in rarefaction leading to increased peripheral vascular resistance and hypertension (Mourad *et al, 20*08; Steeghs *et al, 20*08). Thus, increased sFlt-1 and sEng and reduced PlGF levels in pre-eclampsia could increase peripheral resistance, reduce microvascular perfusion, impair end-organ function and possibly contribute to the multi-system manifestations of pre-eclampsia.

There is evidence that adipose tissue expresses sFlt-1 (Herse *et al, 20*11), and that levels of anti-angiogenic factors vary with gestational age (Noori *et al, 20*11) and impaired angiogenesis increases with age (Wagatsuma, 2006). However, it is unlikely that the differences in sFlt-1, sEng and PlGF between the two groups are due to any of these variables as there is no difference in maternal age, BMI and gestational age between the groups. In spite of the significant correlation between the angiogenic factors and microvascular blood flow reported in this study, there are potential limitations to these findings. Firstly, the pre-eclampsia group in this study had mild hypertension and ideally a 24-hour ambulatory blood pressure monitoring would have allowed a full evaluation between blood pressure and the angiogenic factors. Secondly, gastrocnemius muscle blood flow was presumed to represent nutritive flow blood flow, and although the blood flow values are similar to those in other reports (Anim-Nyame *et al, 20*01), an ankle occlusion cuff was not used to exclude arterio-venous shunts of the feet. This was done to prevent discomfort to the participants that might have had hemodynamic consequences and interfered with other aspects of the protocol. Furthermore, although the gastrocnemius muscle circumference between the two

groups were similar, differences in adipose tissue composition could, by adding heterogeneity, influence the applicability of the findings more generally.

In summary, this study has provided convincing evidence of an inverse correlation between anti-angiogenic factors and microvascular blood flow in pre-eclampsia. Lower levels of anti-angiogenic and higher levels of pro-angiogenic factors are associated with a greater blood flow during normal pregnancy. Measurement of circulating angiogenic factors could be used to assess the severity of multisystem dysfunction in pre-eclampsia as reduced tissue perfusion precedes end organ dysfunction.

[Data from this chapter is published. Ghosh A, Freestone N et al . Microvascular function in pre-eclampsia is influenced by insulin resistance and an imbalance of angiogenic mediators. *Physiol Rep.* 2017 Apr;5(8). pii: e13185. doi: 10.14814/phy2.13185. Epub 2017 Apr 28.*]*

Chapter 3.4

Relationship between insulin resistance, microvascular blood flow and endothelial dysfunction in pre-eclampsia

3.4.1. Introduction

Pre-eclampsia, a multi-systemic disorder of the second half of pregnancy is a leading cause of maternal and perinatal morbidity and mortality (Sibai *et al, 20*03). Generalised endothelial dysfunction is also a feature of the disease (Levine *et al, 20*04; Levine *et al, 20*06). In previous chapters, reduced blood flow in pre-eclampsia (Chapter 3.1), and its relationship with endothelial dysfunction (Chapter 3.2), has been demonstrated. Insulin resistance is a feature of the disease (Laivuori *et al* 1996; Anim-Nyame *et al, 20*15), and persists after delivery (Laivuori *et al,* 1996). It has also been attributed to endothelial dysfunction (Montagnani and Quon, 2000; Ranganath and Quon, 2007).

Muscle is the main peripheral site of insulin action (Saltiel and Kahn 1988), where insulin is delivered by both passive diffusion and trans-capillary transport after binding to the receptors on the endothelial surface (Posner 2017). It also helps in glucose uptake by the muscles. The insulin delivery process is the rate-limiting factor for insulin's action (Ranganath and Quon, 2007; Barrett *et al, 20*11). Although insulin uptake is not related to the blood flow, glucose uptake does correlate to the microvascular blood flow (Wallis *et al, 20*02). Thus, changes in the microvascular environment, including endothelial dysfunction and tissue blood flow, may affect insulin delivery and insulin resistance (St-Pierre *et al, 20*10).

In this chapter, it will be investigated whether a relationship exist between insulin resistance, microvascular blood flow and endothelial dysfunction in pregnancies complicated by pre-eclampsia.

3.4.2. Method

Participants, Blood Sampling and Assays

In this study, participants were recruited from the maternity department at Kingston Hospital, UK, to compare microvascular blood flow and correlate with markers of endothelial dysfunction as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant aseptically, as described in Chapter 2.2. Human PlGF, s-Eng, sFlt-1, and markers of endothelial dysfunction, (s-ICAM-1, s-VCAM-1, e-Selectin, Thrombomodulin and TNF-α) were measured by an ELISA (R and D Systems Europe, UK), as described previously in Chapter 2.4. Fasting insulin and blood glucose were measured to estimate insulin resistance by HOMA as described previously in Chapter 2.4.1.

Measurement of blood flow

In this study, Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany) was used as described previously (Chapter 2.3).

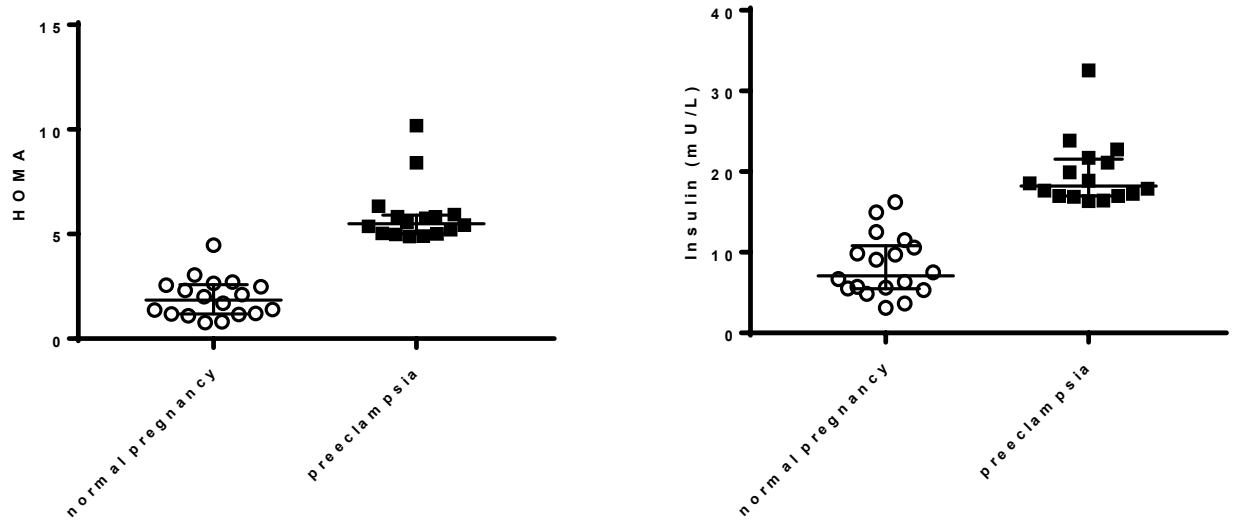
Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

3.4.3. Results

As already discussed in Chapter 3.1, there was no significant difference in age, BMI, gestational age or haematocrit between the two groups. As already discussed previously, microvascular tissue blood flow was reduced in the pre-eclamptic cohort, than in the normal pregnant cohort (Chapter 3.1). Also, levels of biochemical markers of endothelial dysfunction were elevated in the pre-eclamptic cohort, than in the normal pregnant cohort (Chapter 3.2). The insulin resistance (IR), as calculated from HOMA, was higher in the pre-eclamptic cohort [5.496 (5.015 – 5.906)], than in the normal pregnant cohort [1.849 (1.18 – 2.58); $p < 0.001$] (Figure 3.4.1a). The fasting free serum insulin level was higher in the pre-eclamptic group [18.22 (16.985 – 21.559)], in comparison to the

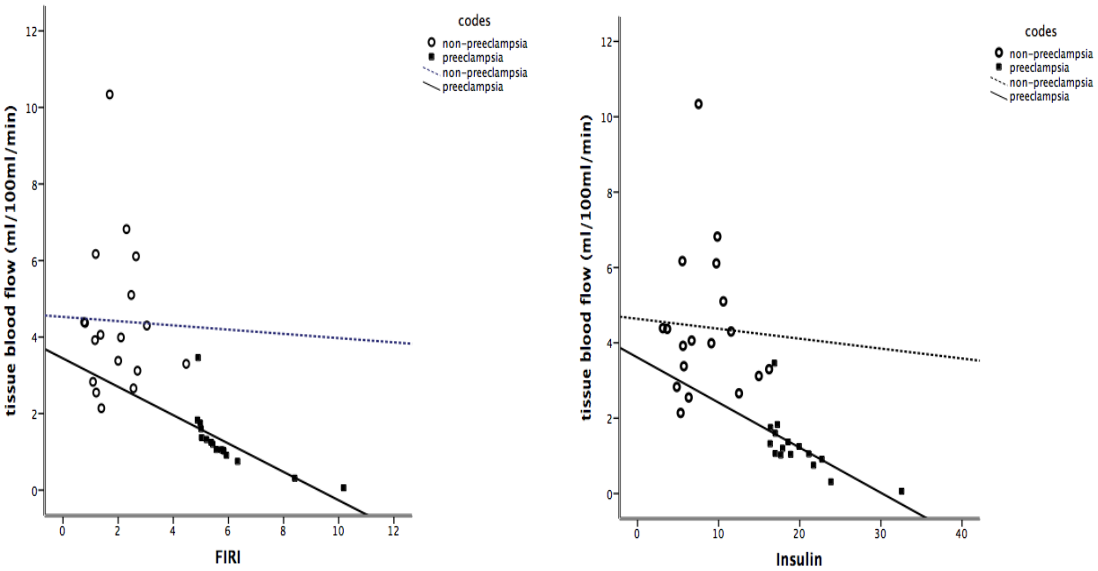
normal pregnant controls [7.09 (5.471 – 10.816); p <0.001] (Figure 3.4.1b). Fasting serum glucose was also elevated in the pre-eclamptic cohort [6.733 (6.192 – 7.134)], compared to the normal pregnant cohort [5.134 (4.65 – 5.908); p <0.001).



(a) (b)

Figure 3.4.1: Comparison of (a) HOMA (Homeostasis Model Assessment) and (b) Serum fasting insulin in normal pregnant controls and pre-eclamptic pregnancies.

There is a statistically significant inverse correlation between microvascular tissue blood flow and both fasting insulin concentration (r_s= -0.8, p<0.0001), and HOMA (r_s= -0.997, p<0.0001), in the pre-eclamptic group. No significant relationship was observed in the normal pregnant controls, between the microvascular blood flow and either the serum fasting insulin concentration (r_s= -0.013, p=0.958), or with HOMA (r_s= -0.03, p=0.906) [Figure 3.4.2]. Serum fasting glucose did not show any statistically significant correlation with microvascular blood flow in nether the pre-eclamptic group (r_s= -0.334, p=0.206), nor in the normal pregnant group (r_s= -0.206, p=0.413). HOMA showed a statistically significant correlation with gestational age (r_s= -0.57, p=0.021), platelet count (r_s= -0.65, p=0.006), and MAP (r_s= 0.719, p=0.002); but there was no correlation in the normal pregnant group with these measures. Serum fasting insulin showed a positive correlation with MAP (r_s= 0.719, p=0.002), in the pre-eclamptic group, but this relationship was not observed in the normal pregnant group. Fasting plasma glucose did not show any significant correlation with any of the groups.



(a) (b)

Figure 3.4.2: Correlation of microvascular blood flow with (a) HOMA, (b) insulin, in the two cohorts.

Insulin resistance showed statistically significant correlations with the markers of endothelial dysfunction. In the pre-eclamptic group, HOMA elicited statistically significant positive correlations with ICAM-1 (r_s= 0.906, p<0.0001), VCAM-1 (r_s= 0.897, p<0.0001), eSelectin (r_s= 0.561, p=0.03), and Thrombomodulin (r_s= 0.736, p=0.01). In the normal pregnant group, HOMA exhibits correlation with only VCAM-1 (r_s= 0.507 p=0.032), and Thrombomodulin (r_s= 0.511, p=0.03). Similarly, in the pre-eclamptic group, serum fasting free insulin displayed a statistically significant positive correlation only with ICAM-1 (r_s= 0.641, p=0.007), and VCAM-1 (r_s= 0.759, p=0.001), however there was no statistically significant correlation in the normal pregnant group. Serum glucose failed to elicit any correlation in either of the groups. [Appendix 3]

3.4.4.Discussion

This study found a significant correlation between insulin resistance, microvascular blood flow and markers of endothelial dysfunction in the pre-eclamptic group, which was absent in the normal pregnant cohort. The results showed an inverse correlation between microvascular blood flow with both insulin resistance (HOMA) and hyperinsulinemia in pre-eclamptic pregnancies, but not in the normal pregnant cohort. Insulin resistance, a feature of pre-eclampsia, has been reported before (Laivuori *et al,* 1996; Anim-Nyame *et al, 20*15), and other studies (Anim-Nyame *et al, 20*04) have

reported a relationship with microvascular blood flow. The finding in this study supports these findings. There is a strong relationship between insulin resistance and blood flow, in pre-eclampsia (Anim-Nyame *et al, 20*15). Since muscle is the main site of peripheral insulin action, blood flow is important for the peripheral utilisation of insulin and glucose. A healthy endothelium is required for effective transport of insulin to the muscles across the capillary cell wall (Saltiel and Kahn 1988). Reduced blood flow, endothelial dysfunction and insulin resistance are seen in pre-eclampsia. Thus, the endothelial dysfunction seen in pre-eclampsia, may contribute to insulin resistance, by altering the trans-capillary transport of insulin across the endothelial cells.

Vasodilatation is achieved by relaxation of the resistance vessels, following relaxation of precapillary arterioles, thus increasing total blood flow (Barret *et al, 20*09, Vincent *et al, 20*04). Insulin induces endothelial-mediated vasodilation, via a NO pathway (Kuboki *et al, 20*00). Thus, hyperinsulinemia in pre-eclampsia can be a reflex compensatory mechanism to increase the blood flow to the tissue (Vincent *et al, 20*03, Barrett *et al, 20*11) (Figure 3.4.3). However, such an assertion has been contradicted, with an alternative explanation postulated as the cause of endothelial dysfunction (Arcaro *et al, 20*02). Thus Arcaro *et al* (2002) has shown that modest hyperinsulinemia causes endothelial dysfunction, as seen in insulin resistant states, predisposing to atherosclerosis. Irving *et al* (2002) showed that there was no significant correlation between tissue blood flow and insulin resistance in healthy control, which is the same as the data obtained in this study. The changes in microcirculation observed by Irving *et al* (2002) were measured in skin capillaries, whereas, the findings in this study reflected changes in skeletal muscle blood flow, which are the main peripheral site of insulin action. Gastrocnemius muscle blood flow, used here, mainly reflects flow through the muscles. Skin circulation serves the dual purpose of nutrition and temperature regulation, functions which are facilitated by the presence of arteriovenous shunts. In contrast, flow through the calves lacks these arteriovenous shunts, and are therefore, predominantly nutritional (Anim-Nyame *et al, 20*15).

The skeletal muscle is a major target organ of insulin action and plays an essential role in insulin-induced glucose uptake, and therefore insulin resistance. In obesity and type 2 diabetes (T2DM),

insulin delivery to the skeletal muscles, as well as insulin-dependent glucose uptake by skeletal muscles is delayed and impaired (Kubota *et al, 20*11). Insulin is delivered by the blood to the skeletal muscles, by the blood vessels. In obesity and T2DM, insulin delivery through the endothelial cells is the rate-limiting step in insulin-mediated glucose uptake by cells, leading to insulin resistance. This is thought to be due to reduced insulin-receptor substrate 2 (IRS2) expression and reduced insulin-induced eNOS production, resulting in reduced insulin-induced capillary recruitment and insulin delivery [Fig 3.4.3] (Kubota *et al, 20*11, Yu *et al, 20*11). This in turn, reduces glucose uptake by skeletal muscles, increasing insulin resistance. Studies have shown, that restoration of insulin-induced phosphorylation in the endothelial cells, completely reverses the process, restoring normal glucose delivery to the muscles (Kubota *et al, 20*13).

As expected, insulin resistance also showed a statistically significant correlation with gestational age (Anim-Nyame *et al, 20*15). There is some degree of insulin resistance in normal pregnancy, which is exacerbated in pre-eclampsia and it becomes more severe as the gestational age progresses. Factors known to affect insulin resistance (as described in Chapter 1.3 and 1.4), such as BMI and age, did not appear to show any statistically significant correlation in this stydy. This can be explained by the strict inclusion criteria, intended to exclude the confounding effects of these variables on gastrocnemius muscle blood flow. There is evidence that insulin resistance is increased in obesity and older age. Furthermore, several medical disorders and smoking predisposes to either endothelial dysfunction or insulin resistance. By choosing our criteria for patient selection, these confounding variables were excluded.

Although this study has shown a significant relationship between insulin resistance, microvascular blood flow and endothelial dysfunction, it is possible that the actual mechanism of insulin resistance is multifactorial (Hseuh *et al, 20*03). Other factors, such as the presence of hypertension and other atherosclerotic risk factors, like increased vascular angiotensin II generation and activity (Dzau 2001), abnormal lipid profile (Laight *et al, 20*00), increased leptin levels and oxidative stress (Nick-Anim *et al, 20*00a), may all play significant roles in the development of insulin resistance in pre-eclampsia. The role of reduced blood flow and endothelial dysfunction, in the development of

insulin resistance in pre-eclampsia, may represent just one facet of a multifactorial disorder. Further studies are required to investigate the interaction between any such factor(s), and the relationship between insulin resistance and the evolution of pre-eclampsia.

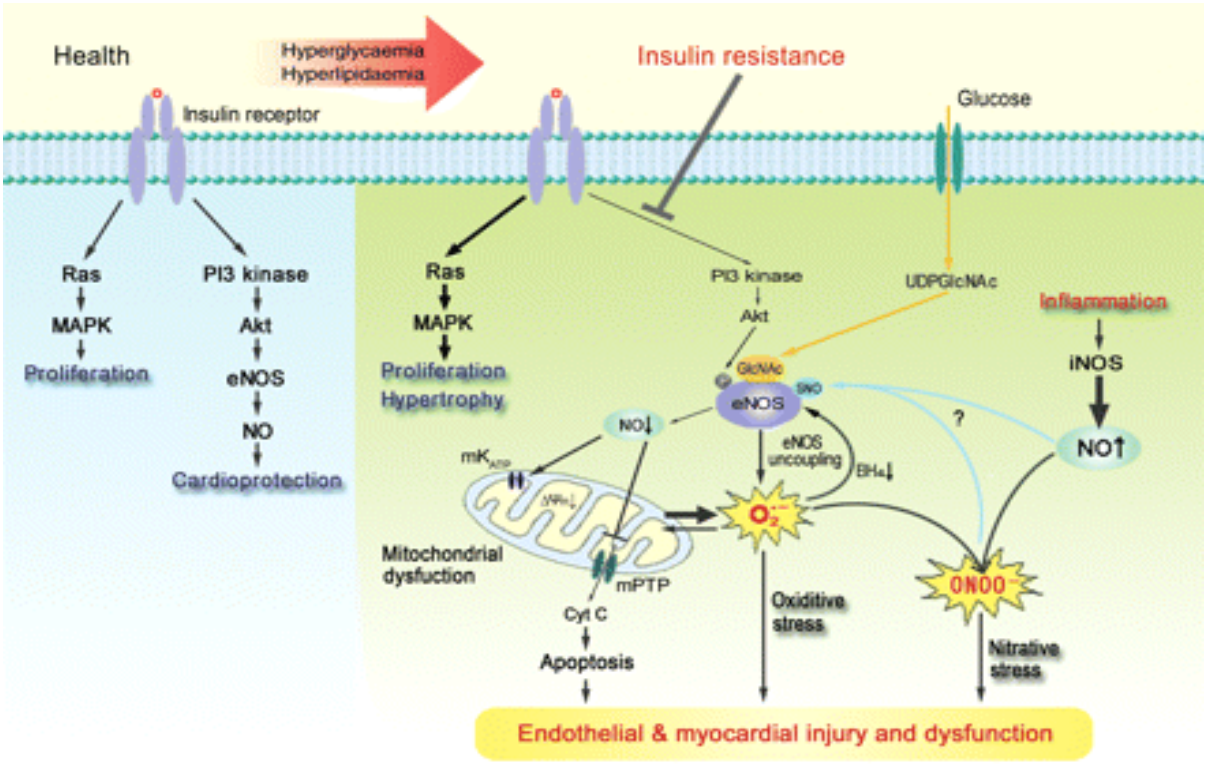


Figure 3.4.3: Function of the insulin-signalling pathway in normal conditions and in insulin resistance. (Yu *et al, 20*11)

In summary, insulin resistance in pre-eclampsia is related to microvascular blood flow and endothelial dysfunction. Insulin resistance in pre-eclampsia is more pronounced than that in normal pregnancy. Also, there is reduced microvascular blood flow in pre-eclampsia. All these are thought to be secondary to endothelial dysfunction. The cause of endothelial dysfunction is unknown in pre-eclampsia. In obesity and T2DM, it is thought to be secondary to the insulin-signalling pathway. Whether that is true for pre-eclampsia is unknown. Once the endothelial dysfunction can be rectified, insulin resistance and microvascular blood flow returns nearly back to normal (Kubato *et al, 20*11, 2013). The same happens in pre-eclampsia, so that after delivery, insulin resistance and microvascular blood flow nearly return to the pre-pregnancy state.

Chapter 3.5

Relationship between insulin resistance and circulating endothelial cells in pre-eclampsia

3.5.1. Introduction

In pre-eclampsia, generalized endothelial cell dysfunction appears to underlie all the pathological manifestations of the disease (Roberts and Hubel 2009). Insulin resistance is a feature of the disease (Laivuori *et al,* 1996; Anim-Nyame *et al, 20*15). Furthermore, profound metabolic changes occur in pre-eclampsia, like those observed in metabolic syndrome (Scioscia *et al, 20*09). Insulin resistance (IR) is more pronounced in pre-eclamptic women than normotensive women (Stefanovic´ *et al, 20*09) and there is evidence mid-trimester insulin resistance may predict subsequent development of pre-eclampsia (Hauth *et al, 20*11). Although the mechanism of increased insulin resistance in pre-eclampsia remains unexplained, this has been attributed to the underlying generalised endothelial dysfunction (Montagnani and Quon, 2000; Ranganath and Quon, 2007).

CEC are sloughed endothelial cells with a low count in healthy individuals (Woywodt *et al, 20*06). The number in circulation is significantly increased with age (Fabbri-Arrigoni *et al, 20*12) and in conditions associated with endothelial dysfunction, such as systemic lupus erythomatosus (Clancy *et al, 20*01), myocardial infarction (Mutin *et al,*1999), vasculitis (Clarke et al, 2010) and familial hypercholesterolemia (Fabbri-Arrigoni *et al, 20*12). The CEC count appears to correlate with the degree of endothelial dysfunction (Gignat-George *et al, 20*00), and inversely with endothelial repair (Fabbri-Arrigoni *et al, 20*12). Recent evidence suggests the CEC count may be used as a diagnostic biomarker of myocardial infarction (Bethel *et al, 20*14). CEC count is increased in Type II Diabetes independent of HbA_{1c} level (McClung *et al, 20*05) and this is attributed to the underling endothelial dysfunction.

There is also increasing evidence that circulating endothelial cell levels are higher in pre-eclampsia compared to normal pregnancy (Canbakan *et al, 20*07) and may be used as a surrogate marker to assess the degree of endothelial damage (Karthikeyan *et al, 20*11). However, it is unclear whether

the elevated circulating CEC numbers correlate with insulin resistance in pre-eclampsia as both are related to endothelial dysfunction. If this was the case, then the CEC count in pre-eclampsia could be a simple test for assessing the degree of insulin resistance in pre-eclampsia and may be used to predict the risk of type II diabetes in pregnancies complicated by the disease. In this chapter, the hypothesis that circulating endothelial cells correlates with insulin resistance in pre-eclampsia, will be investigated.

3.5.2. Method

Participants and insulin resistance

In this study, 10 women with pre-eclampsia and 10 normal pregnant controls were recruited from the maternity department at Kingston Hospital, UK, as described in Chapter 2.1. (This was a subset of the total participants). Fasting blood samples were obtained from women to measure CEC count and insulin resistance from insulin and glucose measurements. Blood was obtained from the ante-cubital vein of each participant using aseptic techniques, as described in Chapter 2.2. Insulin resistance was calculated in each patient as described previously in Chapter 2.4.2, using serum free Insulin and fasting blood glucose.

.

Measurement of CEC

CEC was measured using an established technique, as described previously in Chapter 2.5.

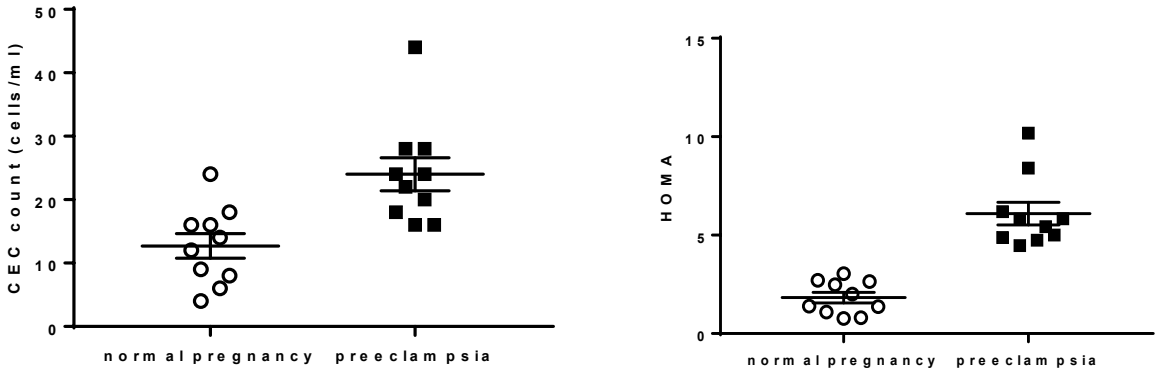
Statistical Analysis:

Circulating endothelial cell numbers and insulin resistance were summarised as mean and standard error of mean and the differences between the groups were calculated using t-test. Correlation was performed using Pearson's correlation coefficient. P-values of <0.05 were considered statistically significant. Statistical analysis was performed using Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, ILL, USA) and Graphpad Prism Version 6.0 (Graph Pad Software, Inc. CA, USA).

3.5.3. Results

The clinical and demographic characteristics of the two groups are shown in Table 3.5. There were no significant differences in maternal age, BMI, ethnicity and gestational age between the two groups. Each group had eight Caucasian women, one Asian and one black African woman. As expected from the recruitment criteria, women with pre-eclampsia had higher systolic and diastolic blood pressure, and lower platelet counts compared to the normal pregnant controls. There was no significant difference in haematocrit between the two groups.

CEC numbers were significantly increased in pre-eclampsia compared to normal pregnancy (21.54± 3.4 versus 14.2± 2.35; p=0.035 for pre-eclampsia and normal pregnancy respectively). Insulin resistance was also significantly greater in pre-eclampsia compared to the normal pregnant controls (5.06± 0.13 versus 2.2± 0.4; p<0.0001, for pre-eclampsia and normal pregnancy respectively) [Fig 3.5.1].



(a) (b)

Figure 3.5.1: Comparison of (a) CEC count and (b) HOMA in normal pregnant controls and pre-eclamptic pregnancies.

However, there was no significant correlation between CEC count and insulin resistance, in pregnancies complicated by pre-eclampsia, (r=0.56; p=0.96). There was also no correlation between the CEC and insulin resistance in the normal pregnant controls (r=-0.21, p=0.56) [Fig 3.5.2].

Variable	Normal Pregnancy (n=10)	Pre-eclampsia (n=10)	P value *
Gestational age (weeks)	32.61± 0.29	32.44± 0.25	0.677
Age (years)	32.75± 0.7	32.57± 0.83	0.85
BMI (kg/m^2)	24.34± 0.58	24.62± 0.57	0.73
Systolic BP (mmHg)	109.2± 3.25	146± 2.18	0.0002*
Diastolic BP (mmHg)	72.3± 2.8	95.2± 1.85	0.0002*
Haematocrit	0.339± 0.009	0.351± 0.016	0.54
Platelet (x10^6/ml)	252.7± 9.91	143.9± 7.66	0.0002*
Birth weight (g)	3735± 187.65	2358+ 204.1	<0.0001*

*= P- value less than 0.05 are considered significant.

Table 3.5: Clinical and demographic characteristics of the subjects in the cross-sectional study between IR and CEC.

3.5.4. Discussion

This study investigated the relationship between insulin resistance and CEC count during the third trimester of pregnancies complicated by pre-eclampsia. Although CEC count (Canbakan et al, 2007) and insulin resistance (Laivuori *et al,* 1996; Anim-Nyame *et al, 20*15) in pre-eclampsia have been reported, to our knowledge this is the first study to investigate the relationship between them in pregnancies complicated by the disease. Previous studies have shown that CEC count correlates with endothelial dysfunction and severity of pre-eclampsia **(**Karthikeyan *et al, 20*11**).** Data from this study shows that there is no significant correlation between insulin resistance and the CEC count in pre-eclampsia.

In a similar fashion to previous studies (Grundmann *et al, 20*08), CEC numbers reported here increased significantly in pre-eclamptic women when compared to normotensive pregnancies, with the baseline CEC number in normotensive pregnancies also comparable. Normal CEC levels are around the 20 cells/ml mark in healthy individuals (Woywodt *et al, 20*06) and under pathological

conditions can increase up to 204 cells/ml, as seen in cases of vasculitis (Clarke *et al, 20*10). In pre-eclampsia, a range of CEC numbers has been reported from between 53- 88 cells/ml (Grundmann *et al, 20*08; Strijbos *et al* 2010). Increased CEC numbers correlate with markers of increasing endothelial dysfunction (Clarke *et al, 20*10) as they represent the sloughed endothelial lining; although that is not always the case at lower levels (Fabbri-Arrigoni *et al, 20*12). Pre-eclamptic women in this study had CEC numbers that although elevated from control groups would not be considered overtly pathophysiological, as seen in other inflammatory conditions (Fabbri-Arrigoni *et al, 20*12). This may be because the pre-eclamptic women in this study only had moderate disease and consequently less severe endothelial injury. In fact, Strijbos *et al* (2010) did not observe any significant difference in CEC counts between pre-eclampsia and the control groups. He observed more correlation of the disease frequency with the biochemical markers of endothelial dysfunction, than with CEC count.

Intuitively, it is expected that CEC count would correlate with insulin resistance because the two are related to endothelial dysfunction in pre-eclampsia. Data from this study has shown this is not the case. This may reflect the lower CEC levels in our pre-eclamptic group compared to previous studies (Grundmann *et al, 20*08), and therefore the disease severity. Strijbos *et al* (2010) did not observe any correlation between CEC and markers of endothelial dysfunction in a cross-sectional study.

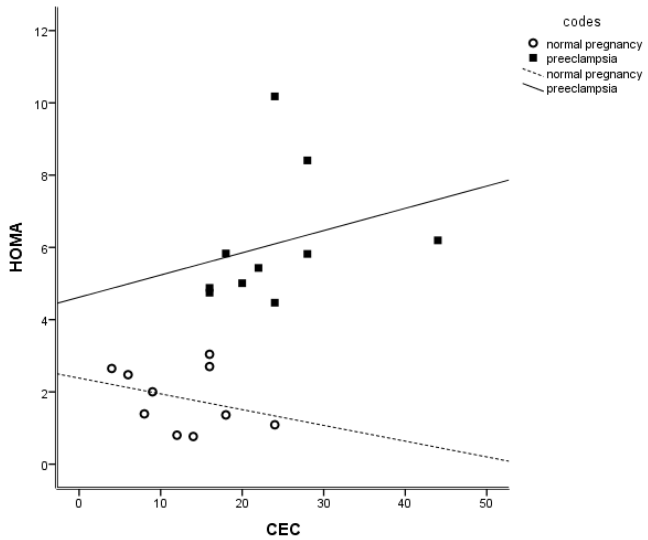


Fig 3.5.2: Correlation of CEC count with HOMA between the two groups.

The results from the present study show that the pathological levels of IR can exist independently of marked endothelial dysfunction as there was limited endothelial sloughing in the pre-eclamptic women. In general, endothelial dysfunction can be found in both pre-eclampsia and insulin resistance states (Cousins, 1991), like obesity and dyslipidemia in the absence of diabetes mellitus (Roberts and Gammill, 2006). Moreover, endothelial dysfunction and IR have been found to correlate independently with the severity of pre-eclampsia (Roberts and Gammill, 2006), although this has not always been observed (McVeigh *et al,* 2003). Hyperglycemia causes endothelial dysfunction in IR states via oxidative stress and the formation of advanced glycation end-products (AGEs) (Wautier *et al,* 2001). However, hyperglycemia is unlikely to explain the endothelial dysfunction and increased CEC in pre-eclampsia since hyperglycemia is not a common feature of pre-eclampsia, in the absence of diabetes. As in type II diabetes, it is possible the elevated CEC in pre-eclampsia reflect ongoing vascular injury, independent of glucose control (McClung *et al,* 2005).

This study suggests that in the cohorts, high levels of IR can exist alongside an initiation of vascular damage, but that they might be independent of each other at low levels of damage. In a similar fashion, while the CEC count from the normal pregnant group in this study was similar to that reported for non-pregnant women by other studies (Gignat-George *et al,* 2000), in spite of relative IR (Powe *et al,* 2011), there was no correlation between IR and CEC number. In normal pregnancy, the mechanism of IR may be different and unlikely to be related to endothelial dysfunction (Powe *et al,* 2011).

It is more than likely that the levels of IR in the normotensive and pre-eclamptic pregnancies did not correlate with vascular dysfunction, as in these subjects, the levels of vascular damage, although detectable was less severe. Further studies are required to investigate this relationship in early onset pre-eclampsia, which represents severe disease. It is unlikely that the lack of correlation between CEC count and IR is due to the small number of women in the study as others have reported significant differences between CEC counts (Canbakan *et al,* 2007) and IR (Stefanovic´ *et al,* 2009) using similar sample sizes and the same methodology. It is also unlikely

that the relationship between CEC and IR would have been different if the blood samples had been obtained at later gestational ages since levels of endothelial dysfunction in term pre-eclampsia are lower than those found in preterm disease (Powers *et al,* 2012).

In summary, it has been shown that although IR and CEC counts are increased in pre-eclampsia, there is no correlation between the two. However, the CEC count on its own in pre-eclampsia is unlikely to be useful as a surrogate measure of IR and not appropriate for detecting persistent IR or predicting the risk of type II diabetes in women whose pregnancies are complicated by pre-eclampsia. (Anim-Nyame *et al, 20*15). Post-delivery, CEC numbers are similar to the age-matched non-pregnant women with a history of normal pregnancy (Tuzcu *et al,* 2015).

[Data from this study is published in Anim-Nyame N, Ghosh A, Freestone N, Arrigoni FI. Relationship between insulin resistance and circulating endothelial cells in pre-eclampsia. *Gynecol Endocrinol.* 2015; 31(10): 788-91*]*

Chapter 3.6

Comparison of endothelial cell insulin receptors expression in normal pregnancy and pre-eclampsia

3.6.1. Introduction

Although the pathophysiology of pre-eclampsia remains an enigma, generalized endothelial cell dysfunction appears to underlie all the pathological manifestations of the disease (Levine *et al,* 2004; Levine *et al,* 2006). This is evident from increased endothelial cell sloughing (Grundmann *et al,* 2008) and increased inflammatory and angiogenic markers in pre-eclampsia (Grundmann *et al,* 2008; Erdbruegger *et al,* 2010; Brennan *et al,* 2014; Masoura *et al, 20*14). Profound metabolic changes occur in pre-eclampsia, like those observed in metabolic syndrome (Scioscia *et al,* 2009). Women whose pregnancies are complicated by pre-eclampsia are therefore more likely to develop diabetes and cardiovascular disease, later in life (Laivuori *et al,* 1996; Wolf *et al,* 2004; Lykke *et al,* 2009). Although the cause remains unclear, there is accumulating evidence that it might be related to the underlying endothelial dysfunction (Montagnani and Quon, 2000), which persists after the pre-eclamptic delivery (Sandvik *et al, 20*13).

Insulin resistance is a feature of the disease and may persist after delivery. Insulin plays an important role in glucose and vascular homeostasis (Saltiel and Kahn, 2001). Insulin receptors have been demonstrated on endothelial cells of both large and small blood vessels (Vincent *et al,* 2003), which participate in insulin-regulated glucose homeostasis. Although muscle is the main peripheral site of insulin action (Saltiel and Kahn, 2001), insulin is delivered to muscle cells from the circulation via both passive diffusion and trans-capillary transport mechanisms involving endothelial cell surface binding (Vincent *et al,* 2003; Posner 2017).

This chapter investigates the hypothesis that there is a change in endothelial cell insulin receptor expression in women with pre-eclampsia, because of underlying endothelial dysfunction. These changes may predispose these women to long-term risk of type II diabetes and cardiovascular disease, in pregnancies complicated by pre-eclampsia.

3.6.2. Method

Participants, Blood Sampling and Assays

In this study, participants were recruited from the maternity department at Kingston Hospital, UK, to compare microvascular blood flow and correlate with markers of endothelial dysfunction as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant aseptically, as described in Chapter 2.2. Human PlGF, s-Eng, sFlt-1, and markers of endothelial dysfunction, (s-ICAM-1, s-VCAM-1, e-Selectin, Thrombomodulin and TNF-α) were measured by an ELISA (R and D Systems Europe, UK), as described previously in Chapter 2.4.

Cell culture

HDMEC were grown as per the supplier's guidance (Promo Cell, Germany), as described in detail in Chapter 2.6. The cells were then incubated separately in sera from the different participants.

Study of insulin signalling pathway

Endothelial cell insulin receptor expression was studied using flow-cytometry (Chapter 2.7.3) and western blotting (Chapter 2.7.4). Flow cytometry helped to estimate the amount of insulin receptor expression on the surface of HDMEC cells, while western blot estimated the total amount of receptor protein in the endothelial cells.

Statistical Analysis:

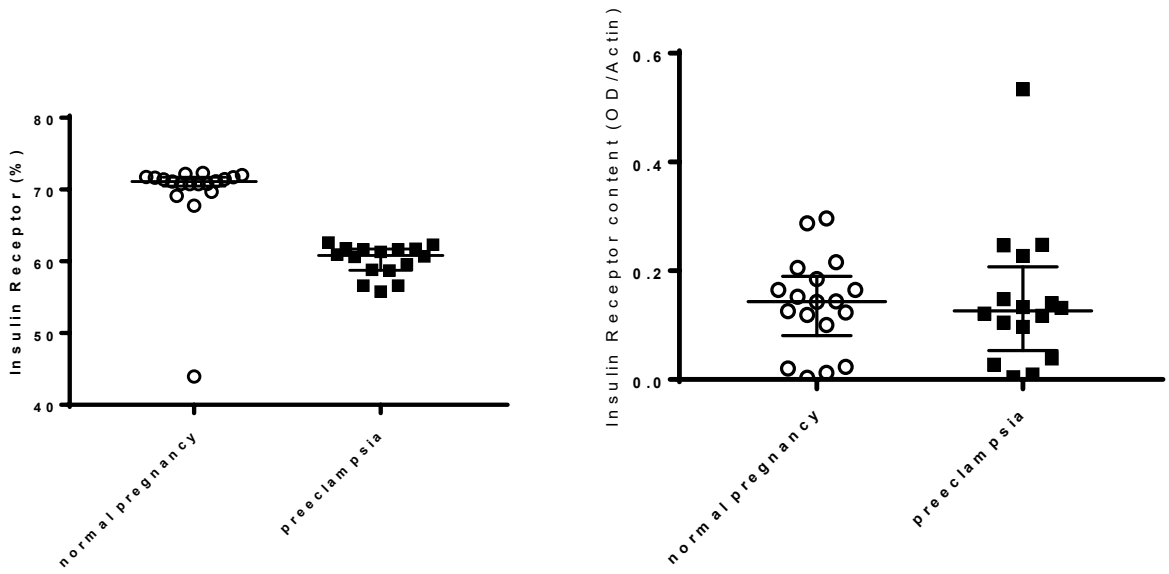
The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

3.6.3. Results

The clinical and demographic characteristics of the participants are shown in Table 3.1 (Chapter 3.1). There were no significant differences in age, BMI, gestational age or haematocrit between the

two groups, all the biochemical markers of endothelial dysfunction were significantly raised in the pre-eclamptic group (Table 3.2; Figure 3.2.1; Chapter 3.2). Briefly, levels of endothelial markers, like sICAM-1, sVCAM-1, eSelectin, TNF- α and Thrombomoduin levels, were significantly higher in the pre-eclamptic cohort compared to the normal pregnant controls.

Using flow-cytometry, the endothelial cell surface insulin receptor expression in the pre-eclamptic group [60.82 (58.75- 61.73)] was found to be statistically significantly lower than the normal pregnant cohort [71.13 (70.47 – 71.73); p<0.0001]. Using western blotting, there was no statistically significant difference in the total amount of insulin receptor protein between the two groups [0.1264 (0.0533 - 0.2073) versus 0.1434 (0.0809 - 0.1899); p= 0.646 for pre-eclampsia and normal pregnancy respectively].



(a) (b)

Figure 3.6.1: Comparison of (a) Flow-cytometry and (b) western blot data on insulin receptors in normal pregnant controls and pre-eclamptic pregnancies.

Endothelial surface insulin receptors showed an inverse correlation with haematocrit in the pre-eclamptic group (r_s= -0.732, p= 0.001) only. Amongst the normal pregnant women, there was a statistically significant correlation between the surface insulin receptors and foetal birth weight (r_s=0.553; p=0.017). This was not present in the pre-eclamptic group. Endothelial surface insulin receptors do not show any other statistically significant correlation with any other parameters, in

either group. The total cellular protein of the cells also did not show any statistically significant correlation with any parameters, in either group. Neither the endothelial surface insulin receptors, nor the total receptor proteins, showed any statistically significant correlation with any biochemical markers of endothelial dysfunction, in either group.

[Appendix 1A for FACs graph, and Appendix 2 for western blot pictures]

3.6.4. Discussion

This study was designed to investigate the hypothesis that a change in insulin receptor expression by endothelial cells occurs during pre-eclampsia because of endothelial dysfunction. Since endothelial dysfunction in pre-eclampsia appears to result from a yet unknown circulatory factor(s), the effects of sera from pre-eclamptic and normotensive pregnancies, on insulin receptor expression by vascular endothelial cells were studied. The study showed that surface insulin receptor expression on the vascular endothelial cells was down regulated in pre-eclamptic pregnancies compared to those in normotensive pregnancies; however there was no change in the total amount of receptor protein between the two groups. This is the first study to report changes in insulin receptor expression in pregnancies complicated by pre-eclampsia.

Insulin receptors have been demonstrated on endothelial cells of both large and small blood vessels (Vincent *et al,* 2003) and participate in insulin-regulated glucose and vascular homeostasis. Although muscle is the main peripheral site of insulin action (Kubota *et al, 20*11), insulin is delivered to muscle cells from the circulation via both passive diffusion and trans-capillary transport mechanisms (Kubota *et al,* 2013). Insulin receptor binding is the initial step for trans-endothelial insulin transport (Kubota *et al,* 2013). Insulin receptors are unique in that not all receptors are expressed on the cellular surface at all times. Once bound to insulin, the receptors are internalised within the cell into endosomes, and remain there until insulin is degraded (Posner 2017). Once insulin is degraded in the endosomes, the insulin receptors may recycle back to the cell surface, or form lysosomes where the receptors themselves are degraded. Prolonged stimulation of the receptors by insulin, caused by increased doses of insulin, appears to accelerate

the degradation of insulin receptors, leading to receptor down-regulation (Roberts and Gammill, 2002; Posner 2017).

Although it was hypothesized changes in endothelial cell insulin receptor expression resulted from endothelial dysfunction, there was no correlation between the insulin receptors expression and any of the biochemical markers of endothelial dysfunction. It possible that altered endothelial function alone is not enough to result in changes in insulin receptor expression regardless of the severity of endothelial dysfunction/ injury. In normal pregnant participants, the surface insulin receptors have a direct correlation with the birth weight of the baby. However, this relation was not seen in the pre-eclamptic group, showing that there are other factors that influence the birth weight. Reduced placental blood flow and microcirculation (Brandão *et al,* 2012), seen in pre-eclampsia, may result in intrauterine growth retardation (IUGR) (Karanam *et al,* 2014).

In addition to the effects of endothelial cell function on insulin receptor expression, it is also possible that the impaired microvascular blood flow seen in pre-eclampsia may have contributory effects on insulin receptor expression (Anim-Nyame *et al,* 2015; Posner, 2017). In this study, flow-cytometry was used to demonstrate endothelial surface insulin receptors, and western blot for the total cellular receptor protein.

Insulin plays a major role in placental modulation in pregnancy (Cvitic *et al,* 2014). In normal pregnancy, maternal insulin levels increase during the third trimester, which stimulates placental vascular angiogenesis, to meet the increasing demand of the rapidly growing foetus (Cvitic *et al,* 2014; Posner 2017). In diabetic pregnancies, elevated foetal insulin levels stimulate placental hyper-vascularisation via the phosphatidylinositol 3-kinase/ Akt/ eNOS pathway (Hiden *et al,* 2009; Lassance *et al,* 2013). Scioscia *et al* (2006) has demonstrated decreased levels of inositol phosphoglycan P type (P-IPG) in the pre-eclamptic placenta, thus demonstrating a down-regulation of the phosphatidylinositol 3-kinase/ Akt/ eNOS pathway. But Ferreira *et al* (2011) have demonstrated that there is no difference in insulin response to Akt/ PKB phosphorylation in between the placentae of the two groups. Thus, in pre-eclampsia, there is widespread

vasoconstriction, despite increased serum insulin levels. This may be due to altered cellular insulin receptor expression, which in turn fails to stimulate the phosphatidylinositol 3-kinase/ Akt/ eNOS pathways, resulting in vasoconstriction, so widespread in the disease (Kubota *et al*, 2013) (Fig 3.6.2).

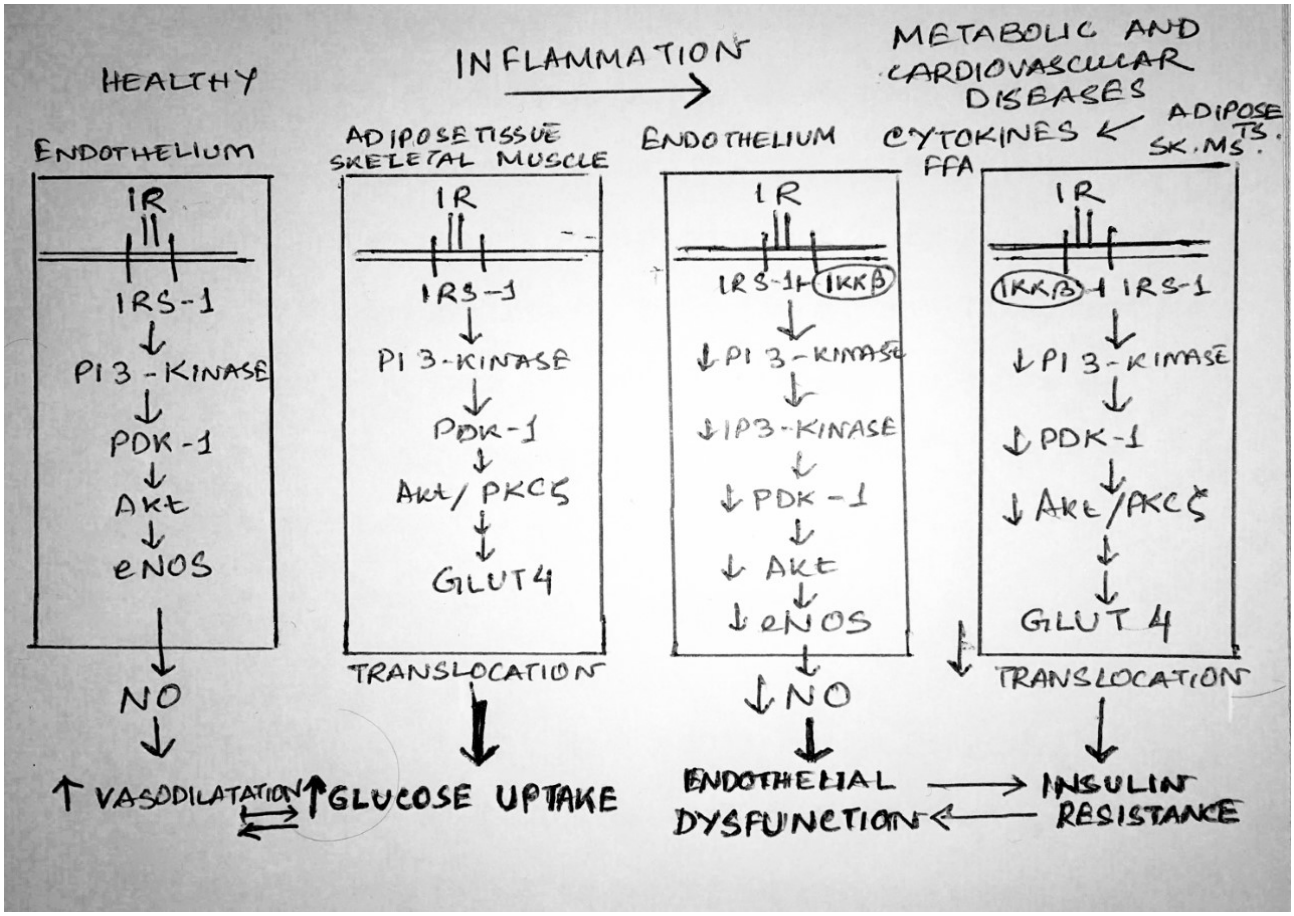


Figure 3.6.2: Diagram showing the importance of Insulin receptors and the signalling pathway in healthy and in inflammatory conditions.

Endothelial dysfunction is one of the reasons for insulin resistance seen in obesity and T2DM (Bakker *et al,* 2009). Endothelial dysfunction also plays a key role in the development of hypertension (Konukoglu and Uzun, 2017), and is a major risk factor for cardiovascular disease (Ormazabai *et al*, 2018). Our study failed to demonstrate any correlation between the markers of endothelial dysfunction and insulin receptor expression. Insulin receptors and the insulin signalling pathway are downregulated in hypoxia (Regazzetti et al, 2009). In our study, microvascular blood

flow is reduced in pre-eclamptic cohort, but it has no correlation with the blood flow. This can be due to the fact, that the patients in the pre-eclamptic cohorts had only mild disease.

It is known that women whose pregnancies are complicated by pre-eclampsia are more likely to develop diabetes and cardiovascular disease, later in life (Laivuori *et al,* 1996; Wolf *et al,* 2004; Lykke *et al,* 2009). Though the cause is unknown, it is thought to be the sequence of events encountered during the development of pre-eclampsia, which makes them more prone to diabetes and cardiovascular disease. Further studies are required to investigate how down-regulation of endothelial cell insulin expression in pre-eclampsia might affect insulin-mediated vascular and metabolic homeostasis. Further studies are also required to investigate whether down-regulation of insulin receptor expression affects downstream down insulin signalling pathways.

In summary, it has been shown that there is reduced expression of surface insulin receptors in pre-eclampsia compared to normal pregnancy, although the total amount of insulin receptor protein in the cells in the two groups was equal. This was hypothesized to be due to the widespread endothelial cell dysfunction seen in pre-eclampsia, but it shows that there are other factor(s) affecting the insulin receptors in this condition. It is possible the effect on insulin receptor expression explains the long-term risk of cardiovascular disease in women whose pregnancies are complicated by pre-eclampsia.

Chapter 3.7

Relationship between endothelial insulin receptor expression and insulin resistance in normal pregnancy and pre-eclampsia

3.7.1. Introduction

Insulin Resistance is a feature of pregnancy, although it is exaggerated in pre-eclampsia (von Versen-Hoeynck *et al, 20*07; Thadhani *et al, 20*04). It is also been shown that reduced blood flow may play a role in the in increased insulin resistance seen in pre-eclampsia (Anim-Nyame *et al, 20*15). Himsworth (1949) have shown previously that insulin resistance can by itself be a cause of diabetes mellitus. There is now evidence that women with pre-eclampsia and insulin resistance are at risk of developing diabetes in later life (Laivuori *et al,* 1996; Wolf *et al, 20*04; Lykke *et al, 20*09). Although the cause of this remains unclear, there is accumulating evidence that this might be related to the underlying endothelial dysfunction (Montagnani and Quon, 2000).

Muscle is the main peripheral site of insulin action (Feig *et al, 20*13) and insulin is delivered to the muscle cells from the circulation via both passive diffusion and trans capillary transport mechanisms involving endothelial cell surface binding (Kubota *et al, 20*11; Kubota *et al, 20*13). Insulin stimulates glucose uptake by its action to increase cellular permeability to glucose largely by recruiting and activating glucose transport proteins to the cell membrane (Reaven, 2011). In additional to increasing its own ability to promote glucose uptake (Barrett *et al, 20*11), it also increases skeletal muscle blood flow (Montagnani and Quon, 2000; Ranganath and Quon, 2007). Insulin exhibits a gradient from the feeding capillary to the interstitium (Barrett *et al, 20*11); therefore, a large intercapillary distance will lead to a greater insulin action gradient. Insulin-mediated increases in skeletal muscle blood flow are accompanied by capillary recruitment, as seen in obese individuals (Barrett *et al, 20*11; Saltiel and Kahn, 2001). This would increase functional capillary density and reduce the insulin action gradient, resulting in enhanced access of insulin to muscle for metabolism and an amplification of insulin action.

In this chapter, it is hypothesized that in pre-eclampsia, down regulation of endothelial cell insulin receptor expression, reported in the previous chapter (Chapter 3.6), will result in insulin resistance.

3.7.2. Method

Participants and Measurement of Insulin Resistance

In this study, participants were recruited from the maternity department at Kingston Hospital, UK, to compare microvascular blood flow in pre-eclamptic and normotensive pregnancies, and correlate them with markers of endothelial dysfunction as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant aseptically, as described in Chapter 2.2. Fasting insulin and blood glucose were measured to estimate insulin resistance by HOMA as described previously in Chapter 2.4.1.

Cell Culture

HDMEC were grown as per the supplier's guidance (Promo Cell, Germany), as described in detail in Chapter 2.6. The cells were then incubated separately in sera from the participants.

Study of Insulin Signalling Pathway

Endothelial cell insulin receptor expression was studied using flow-cytometry (Chapter 2.7.3) and western blotting (Chapter 2.7.4). Flow cytometry helped to estimate the amount of insulin receptor expression on the surface of HDMEC cells, while western blot estimated the total amount of receptor protein in the endothelial cells.

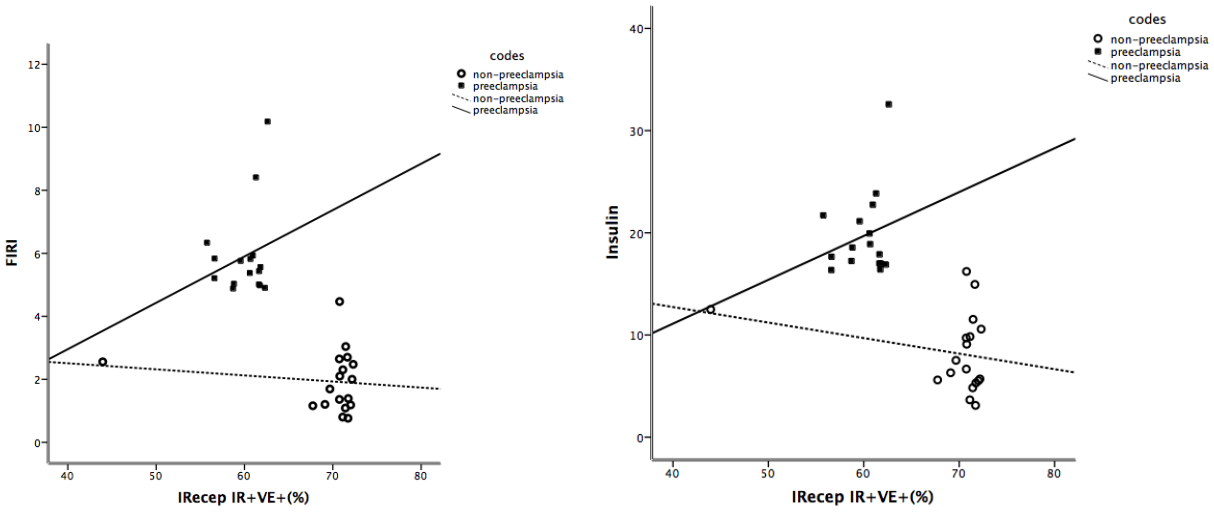
Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

3.7.3. Results

As discussed, there was no significant difference in age, BMI, gestational age or haematocrit between the two groups. Insulin resistance, measured by HOMA, was raised in the pre-eclamptic group, than in the normal pregnant cohort (Chapter 3.4), and endothelial cell surface insulin receptor expression was reduced in the pre-eclamptic group, then in the normal pregnant cohort. However, there was no significant difference in total expressions of receptor proteins in the endothelial cells between pre-eclampsia and normal pregnancy (Chapter 3.6).

In the normal pregnant cohort, there was no statistically significant correlation between endothelial cell surface expression of insulin receptors (as detected by flow-cytometry), with insulin resistance (r_s= -0.034, p= 0.893), or serum fasting insulin level (r_s= -0.201, p= 0.423), or fasting serum glucose level (r_s= 0.402, p= 0.098). Similarly, in the pre-eclamptic cohort, there was also no statistically significant correlation between endothelial cell surface expression of insulin receptors with either insulin resistance (r_s= -0.029, p= 0.914), or serum fasting insulin level (r_s= -0.035, p= 0.897), nor fasting serum glucose level (r_s= 0.163, p= 0.546) [Fig 3.7.1].

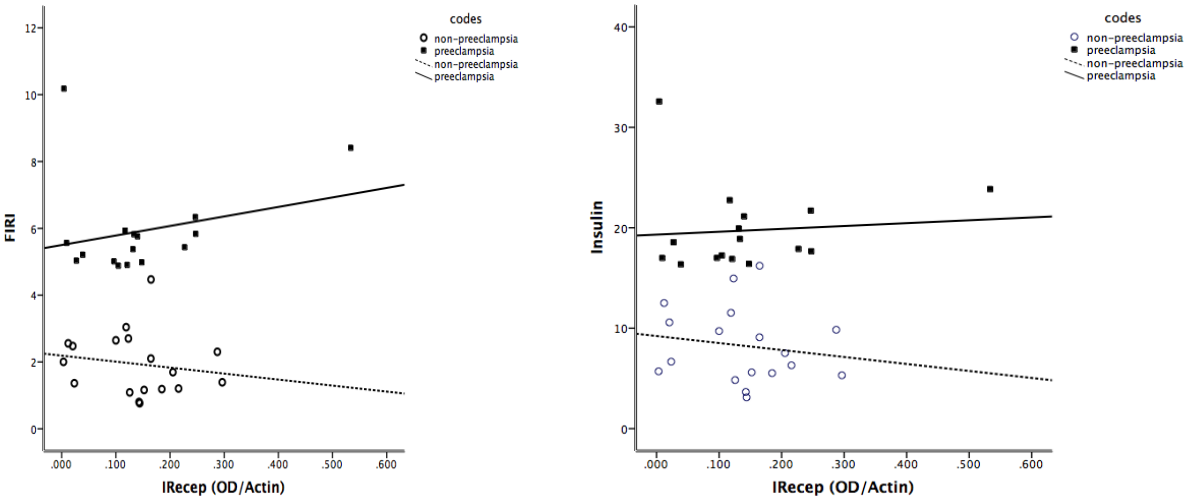


(a) (b)

Figure 3.7.1: Correlation of cell surface Insulin receptors determined by flow-cytometry with (a) HOMA and (b) fasting Insulin level.

In terms of total cellular insulin receptor protein (as measured by western blot), in the pre-eclamptic group, there is no statistically significant correlation with any of the variables, like insulin resistance

(r_s= 0.262, p= 0.327), serum fasting insulin level (r_s= 0.2, p= 0.458), or serum fasting glucose level (r_s= 0.172, p= 0.524). In the normal pregnant controls, there was no statistically significant correlation with any of the variables, like insulin resistance (r_s= -0.247, p= 0.324), serum fasting insulin level (r_s= -0.234, p= 0.349), or fasting serum glucose level (r_s= -0.054, p= 0.832) [Fig 3.7.2]. The analysis was repeated, by removing the outlier, but the correlation was unchanged. [Appendix 3]



(a) (b)

Figure 3.7.2: Correlation of total Insulin receptors protein determined by western blotting with (a) HOMA and (b) fasting Insulin level.

[Appendix 1A for FACs graph, and Appendix 2 for western blot pictures]

3.7.4. Discussion

This study tests the relationship between cellular insulin receptor, and insulin resistance in pre-eclamptic pregnancies, compared to normal pregnancies. It tested the hypothesis that altered endothelial insulin receptor expression can explain the potential mechanism for insulin resistance in pre-eclampsia. As explained previously, tissue perfusion is reduced (Chapter 3.1), while insulin resistance is increased (Chapter 3.4) in pre-eclampsia. Surface insulin receptor expression is down regulated in pre-eclampsia, while the amount of total insulin receptor protein in the endothelial cells, remains unchanged (Chapter 3.6). There was a statistically inverse correlation between tissue blood flow and insulin resistance in pre-eclampsia (Chapter 3.4). There was no statistically significant correlation between insulin receptor expression and biochemical markers of

endothelial dysfunction (Chapter 3.6). This study also shows no statistically significant correlation in between insulin receptor expression (either cell surface expression or the total protein) with insulin resistance.

Maternal tissue perfusion decreases during pre-eclampsia (Anim-Nyame *et al, 20*01; Anim-Nyame *et al, 20*15). Insulin resistance is seen in normal pregnancy; however this is far greater in pre-eclampsia (Hodson *et al, 20*13). Although, Salamalekis *et al* (2005) have reported that there is no association between insulin resistance and pre-eclampsia, others have not reported such an association (von Versen-Hoeynck *et al, 20*07; Thadhani *et al, 20*04). Insulin resistance is seen to some extent in the first trimester in all pregnancies, its rise is more pronounced by the third trimester in pre-eclampsia (Abhari *et al, 20*14); and precedes the onset of pre-eclampsia (Hauth *et al, 20*11). It is suggested that pre-eclampsia per se is not a risk factor for development of insulin resistance (Sinha *et al, 20*14), thus the cause and effect relationship between insulin resistance and pre-eclampsia is unclear.

The association between insulin resistance and hypertension is well established but the mechanism remains unexplained (Reaven, 2011; Barrett *et al, 20*11). One theory postulates that insulin resistance causes blood pressure elevation, and there is compensatory hyperinsulinemia to overcome generalized vasoconstriction (Reaven, 2011). An alternative theory states that microvascular function, as a common antecedent, determines both blood pressure and insulin sensitivity (Roberts and Hubel, 2009; Brandão *et al, 20*12). In several tissues, capillary density has been found to correlate inversely with blood pressure and peripheral resistance in hypertensive and normotensive subjects (Nama et al, 2012), and a decrease in capillary density may contribute to an increase in vascular resistance (Humar *et al, 20*09; Pozrikidis 2009). Although previous investigations in skeletal muscle preparations from insulin-resistant subjects have convincingly shown the existence of insulin receptor and post receptor defects (DeFronzo, 2010), there is also evidence that both reduced capillary surface area and impaired microvascular endothelial function may contribute to insulin resistance (Kim *et al, 20*06; Thadhani *et al, 20*04). Muscle capillary density is positively correlated with insulin sensitivity, and diffusion distance of insulin and glucose

from capillary to muscle cells (which increases with decreased capillary density) may play a role in determining insulin sensitivity (Snijders *et al, 20*17; Fisher *et al, 20*17; Barrett *et al, 20*11). This decrease in capillary density may be a consequence of reduced endothelium-dependent vasodilatation at the pre-capillary level. Small pre-capillary vessels are considered the main regulators of capillary recruitment and in addition contribute to total peripheral resistance. Indeed, reduced endothelium-dependent vasodilatation of resistance vessels is associated with insulin resistance (McVeigh and Cohn, 2003; Barrett *et al, 20*11) and hypertension (Tsioufis et al, 2015). In pre-eclampsia, it has been shown that there is structural rarefication of the capillary density, before the actual onset of the disease (Nama *et al, 20*12). This may explain the reported inverse correlation between insulin resistance and microvascular perfusion (Anim-Nyame *et al, 20*15).

This study clearly shows that there are altered insulin receptors on the endothelial cell surface in pre-eclampsia. There is growing evidence that hyperinsulinemia is the link between diabetes and hypertension (Montagnani and Quon, 2000; Ranganath and Quon, 2007; Lykke *et al, 20*09). This link is also implicated in dyslipidaemia, obesity and cardiovascular disease (Palanjappan *et al, 20*04). Hyperinsulinemia is observed secondary to decreased insulin receptor expression (Obisi *et al, 20*02). However, cellular inflammatory changes have also been implicated as the cause of insulin resistance (Wolf *et al, 20*04), including those seen in pre-eclampsia (Anim-Nyame *et al, 20*03). It might also be the cause of endothelial dysfunction (Ridker *et al, 20*03). Kubota *et al* (2011, 2013), have suggested that defects in cellular insulin receptors and post receptor defects are the main reason for insulin resistance in Type 2 diabetes mellitus.

In normal pregnancy, during the third trimester, increased levels of maternal insulin greatly stimulate the placental vascular angiogenesis, to meet the increased demand of the rapidly growing foetus (Hiden *et al, 20*09). There is evidence that insulin plays a major role in this placental modulation. The placental arterial endothelial cells have a high expression of insulin receptors (Hiden *et al, 20*09). In diabetic pregnancies, elevated foetal insulin level may stimulate placental hyper vascularisation via the phosphatidylinositol 3-kinase/ Akt/ eNOS pathway (Lassance *et al, 20*13). In pre-eclampsia, there is widespread vasoconstriction, and there is

increased insulin resistance and subsequently increased serum insulin levels. This may be due to altered cellular insulin receptor expression, and post-receptor defects. This in turn fails to stimulate the phosphatidylinositol 3-kinase/ Akt/ eNOS pathways, which causes vasodilatation (Lassance *et al, 20*13).

In summary, this study provides some evidence that there is decreased endothelial cell insulin receptor expression in pre-eclampsia. Insulin delivery to the skeletal muscle occurs through the endothelial cells, which is the rate-limiting step (Kubota *et al*, 2011). Therefore, this study tested the hypothesis of a correlation between the insulin receptors and insulin resistance, but there is no correlation between insulin receptor expression and insulin resistance, in pre-eclampsia. Further research is required to explain the downstream effect of this on insulin signalling pathway and its clinical implications.

Chapter 3.8

Differential expression of Akt by endothelial cell in normal pregnancy and pre-eclampsia, and its relationship with microcirculation and insulin receptor expression.

3.8.1. Introduction

In pre-eclampsia, there is impaired blood flow to the affected vascular beds (Brosens *et al,* 2002), and generalised endothelial dysfunction (Levine *et al,* 2004; Levine *et al*, 2006). It is a leading cause of maternal and perinatal morbidity and mortality (Sibai *et al*, 2003) and its association with long-term risk of Type 2 Diabetes.

Insulin-mediated glucose uptake occurs principally in skeletal muscles (Kubota *et al, 20*11, 2013), and there is convincing evidence that Insulin increases skeletal muscle perfusion (Barrett *et al,* 2011). Insulin receptors have been demonstrated on endothelial cells of both large and small blood vessels (Vincent *et al,* 2003). They are also found in vascular smooth muscle cells; and modulate vascular tone and tissue blood flow (Barrett *et al,* 2011). Insulin binding to its receptor activates both PI3K/AKT and the Ras-MAP kinase pathways. In endothelial cells, the PI3K/AKT pathway mediates an anti-apoptotic effect and results in an increase in gene expression and activation of eNOS (Kuboki *et al,* 2000; Zeng *et al,* 2000; Hermann *et al,* 2000). (Figure 1.3)

In chapter 3.6, it was demonstrated that down-regulation of endothelial surface insulin receptors occurs, without any change in total insulin receptor proteins in pre-eclampsia. In this chapter, it is hypothesized, that there will be changes in the Akt protein, which might affect the tissue perfusion in pre-eclampsia.

3.8.2. Method

Participants

To investigate the hypothesis, endothelial cell Akt expression was measured in 16 women with pre-eclampsia and 18 normal pregnant controls and correlated with endothelial cell insulin receptor expression and microvascular blood flow as described in chapter 3.6 and 3.1 respectively. All the

women were recruited from the maternity department at Kingston Hospital, UK, as already described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant using aseptic techniques, as described in Chapter 2.2.

Measurement of blood flow

In this study, I used Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany) as described previously (Chapter 2.3).

Culture

HDMEC were grown as per the supplier's guidance (Promo Cell, Germany), as described in detail in Chapter 2.6.

Study of Insulin Signalling Pathway: Insulin

Endothelial cell insulin receptor expression was studied using flow-cytometry (Chapter 2.7.3) and western blotting (Chapter 2.7.4). Flow cytometry helped to estimate the amount of insulin receptor expression on the surface of HDMEC cells, while western blot estimated the total amount of receptor protein in the endothelial cells.

Study of Insulin Signalling Pathway: Akt

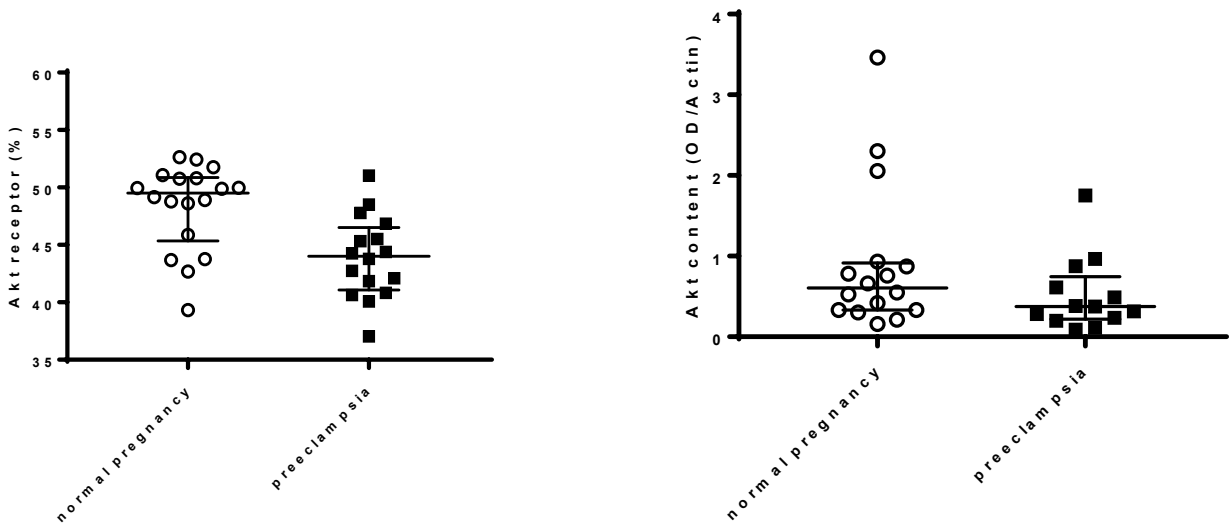
Endothelial cell Akt receptor expression was studied using flow-cytometry (Chapter 2.7.3) and western blotting (Chapter 2.7.4). Flow cytometry estimated the active receptor protein, while western blot estimated the total amount of receptor protein in the endothelial cells.

Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

3.8.3. Results

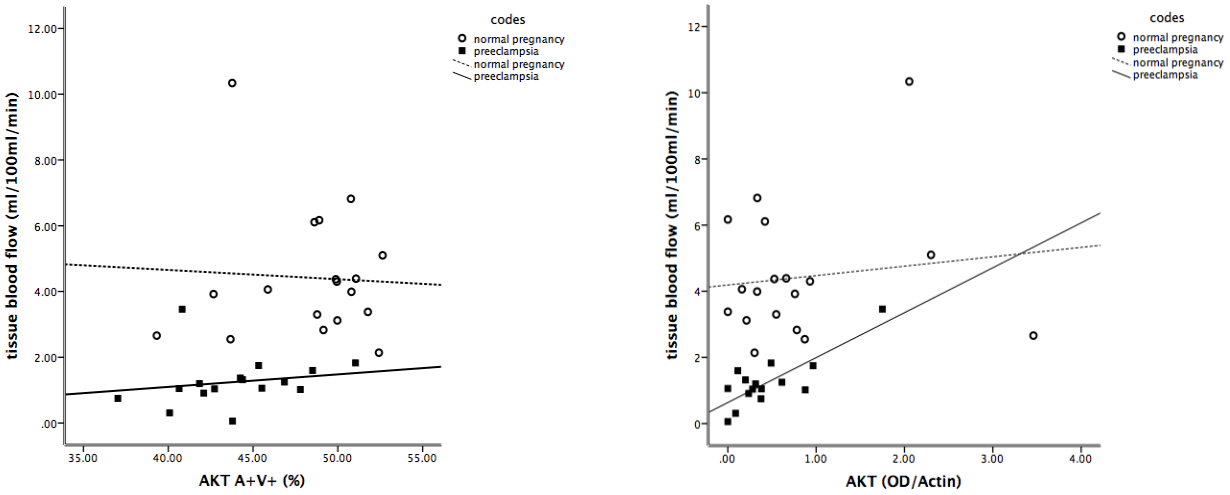
The clinical and demographic characteristics of the participants are shown in Table 3.1. As already discussed, there were no significant difference in age, BMI, gestational age or haematocrit between the two groups. Microvascular tissue blood flow was reduced in pre-eclamptic group, compared to normal pregnant control (Chapter 3.1). Cell surface insulin receptor expressions were reduced in the pre-eclamptic group compared to normal pregnancy, though there was no change in the total amount of cellular receptor protein (Chapter 3.6). Intracellular Akt was measured by both Flow-cytometry and western blot. Flow cytometry estimated the active receptor protein, while western blot estimated the total amount of receptor protein in the endothelial cells. Using Flow-cytometry, the endothelial cell intracellular Akt receptor expression in the pre-eclamptic group [44.02 (41.08 – 46.52)] was significantly lower, when compared to the normal pregnant group [49.52 (45.35 – 50.87; p=0.002)] {Fig 3.8.1a}. However, using western blotting, there is no statistically significant difference in total amount of Akt protein in between the two groups, i.e. pre-eclampsia [0.3757 (0.2178 - 0.744)], and normal pregnant [0.6042 (0.332 - 0.9156); p= 0.144] {Fig 3.8.1b}



(a) (b)

Figure 3.8.1: Comparison of (a) Flow-cytometry and (b) western blot data on Akt protein in normal pregnant controls and pre-eclamptic pregnancies.

In the normal pregnant group, Akt shows statistically significant correlation with foetal birth weight (r_s= 0.545, p= 0.019), using Flow-cytometry. In normal pregnant cohort, there was no significant correlation of Akt with both methods with haematocrit, BMI, MAP, gestational age, and maternal age. In the pre-eclamptic cohort, there was no statistically significant correlation with any parameters using either Flow-cytometry or western blotting.

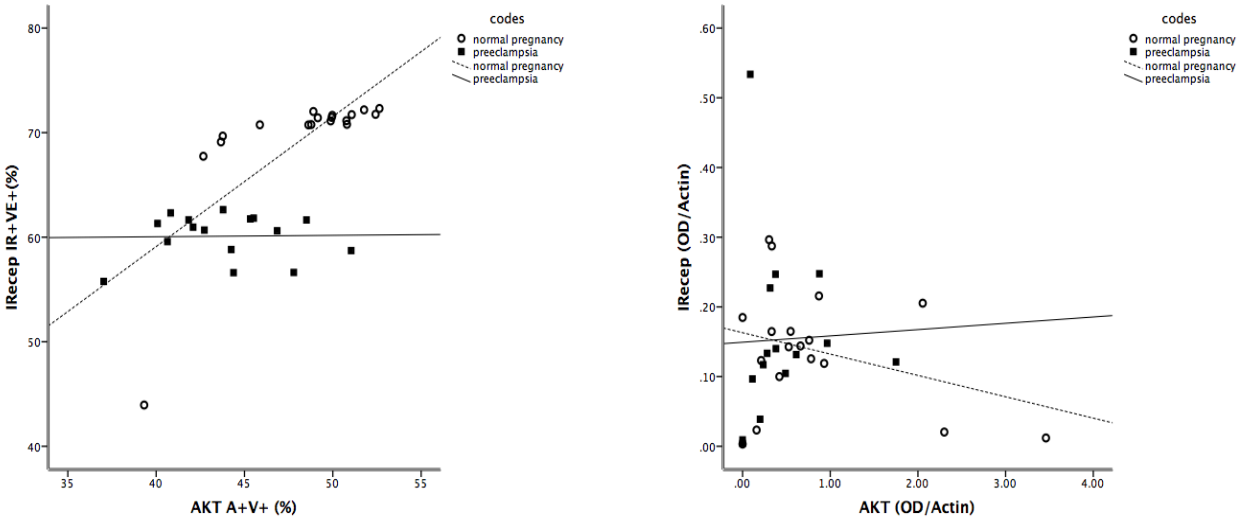


(a) (b)

Figure 3.8.2: Correlation of (a) intracellular active Akt receptors with microvascular blood flow, determined by Flow-cytometry (b) intracellular total Akt receptor protein with microvascular blood flow, determined by western blot.

In the normal pregnant group, there was no statistically significant correlation between endothelial cells Akt expression (as detected by Flow-cytometry), and microvascular tissue flow (r_s= 0.088, p= 0.729). Also, in the pre-eclamptic group, there was also no statistically significant correlation between endothelial cells active Akt expression with microvascular tissue flow (r_s= 0.465, p= 0.07) [Figure 3.8.2a]. In comparing the correlation of the total Akt proteins and microvascular blood flow in the endothelial cells, there is no significant correlation in either the normal pregnant group (r_s= 0.074, p= 0.787), or the pre-eclamptic group (r_s= 0.467, p= 0.108) [Figure 3.8.2b]. In the normal pregnant group, there was a significant correlation between the cell surface insulin receptor expression and the active Akt protein (r_s= 0.872, p< 0.001) [Figure 3.8.3a]. However, there was no correlation between the cell surface insulin receptors and the active Akt protein (r_s= -0.062, p= 0.82), in the pre-eclamptic group. In comparing the correlation of the total insulin receptor and Akt

proteins in the endothelial cells, there is no significant correlation in either the normal pregnant group (r_s= -0.232, p= 0.387), or the pre-eclamptic group (r_s= 0.148, p= 0.629) [Figure 3.8.3b]. [Appendix 3]



(a) (b)

Figure 3.8.3: Correlation of (a) intracellular active Akt receptors with surface insulin receptor, determined by Flow-cytometry (b) intracellular total Akt receptor protein with total insulin receptor, determined by western blot.

[Appendix 1B for FACs graph, and Appendix 2 for western blot pictures]

3.8.4. Discussion

This study evaluated the relationship between intracellular Akt receptor expressions, microvascular blood flow and insulin receptors in pre-eclampsia, compared to normal pregnancy. The hypothesis that altered endothelial insulin signalling pathway results in impaired microvascular perfusion in pre-eclampsia was tested.

The data showed no significant difference in total Akt protein (as determined by western blot) in between normal pregnancy and pre-eclampsia. However, there was a significant reduction in functional activity of the Akt protein in the pre-eclamptic group (as demonstrated by Flow-cytometry). There was no correlation between Akt expression and microvascular blood flow in normal pregnancy and pre-eclampsia. However, there was significant correlation between Akt

expression and endothelial cell surface insulin receptor expression, in the normal pregnant group. On the contrary, there was no correlation between Akt expression and endothelial cell surface insulin receptor expression, in the pre-eclamptic group. There was no correlation of total Akt protein and total insulin receptor protein in either group. These observations suggest that in pre-eclampsia, there is down-regulation of functional Akt protein in the endothelial cells. From the insulin signalling pathway, activation of the insulin receptor up-regulates Akt expression. In chapter 3.6 and 3.7, it is shown that there is down-regulation of insulin receptor protein expression in pre-eclampsia, compared to normal pregnancy, which in turn, down-regulates the active Akt, which results in impaired peripheral uptake of glucose and therefore insulin resistance.

Akt plays an important role in both the glucose metabolism, via the insulin pathway, and the tissue blood flow, via the eNOS pathway (Figure 1.2). Data from this study also shows that there is a direct correlation of cellular insulin receptor expression, and functionally active Akt receptors in the endothelial cells, in normal pregnancy. This correlation is not seen in pre-eclampsia. This shows that while in normal pregnancy, activation of the insulin receptor up-regulates Akt expression, this is lost in pre-eclampsia. Other unknown factor(s) controls Akt expression in endothelial cells in pre-eclampsia. This adds weight to the fact that pre-eclampsia is a multi-factorial disease.

Activation of the insulin-signalling pathway is expected to result in increased tissue blood flow via the activation of eNOS (Vincent *et al, 20*03). It is possible the increased serum insulin in pre-eclampsia is a compensatory response to overcome the insulin resistance, and decreased microvascular blood flow. However, this may be explained by other mechanism(s). Barrett *et al* (2011) demonstrated a dose dependent effect of insulin to increase leg blood flow in insulin sensitive subjects. Other studies demonstrated that hyperinsulinemia, increases cardiac output, without changing systolic blood pressure (Steinberg et al, 2000). It simultaneously increases blood flow to the leg (Steinberg et al, 2000). Capillary recruitment in response to insulin is more important than bulk flow to determine rates of tissue insulin-mediated glucose uptake (Vincent *et al, 20*03, Barrett *et al, 20*11).

Impaired insulin signalling in endothelial cells, leads to reduced insulin-induced eNOS, phosphorylation, leading to reduce NO formation (Kubota *et al, 20*11). NO is the most potent endogenous vasodilator known (Hellsten *et al, 20*12). It also inhibits platelet adherence and aggregation (Richey, 2013; Vanhoutte, 2016), reduces adherence of leukocytes to the endothelium (Vanhoutte 2016), and suppresses proliferation of vascular smooth muscle cells (Napoli *et al,* 2013). Several disorders are associated with reduced synthesis and/or increased degradation of vascular NO, like hypercholesterolemia (Esper *et al,* 2006; Brennan *et al,* 2014), diabetes mellitus (Kubota *et al,* 2013), hypertension (Richey 2013; Vanhoutte 2016), and tobacco use (Ranganath and Quon, 2007). Since, endothelial dysfunction causes attenuation of insulin-induced capillary recruitment and insulin delivery. This in turn reduced glucose uptake by skeletal muscles, increasing insulin resistance (Kabuto *et al, 20*11)

In addition to NO dependent vasodilatation of the arterioles, Insulin also exerts a vasoconstrictor effect via the peptide endothelin-1 (ET-1) (Ranganath and Quon, 2007; Eringa *et al,* 2002). Insulin stimulates ET-1 induced vasoconstriction of skeletal muscle arterioles, via PI3K/AKT pathway inhibition (Eringa *et al,* 2002). Unlike insulin-mediated vasodilatation, it remains functional during insulin resistance (Kim *et al,* 2006; Sarafidis and Bakris 2007). Insulin also induces vasoconstriction by activation of extracellular signal-regulated kinase 1/2 (ERK1/2) present on the endothelial cells. Removal of the arteriolar endothelium abolishes the insulin-induced vasoconstriction, suggesting that activation of endothelial ERK1/2 is required in acute insulin-induced vasoconstriction (Eringa *et al, 20*04). Insulin induces dose-dependent vasoconstriction of skeletal muscle arterioles during PI3-kinase inhibition (Eringa *et al,* 2002). In normal endothelium, insulin causes vasodilation via the the IR/PI3K/Akt pathway, and it suppresses the endothelial ERK1/2 pathway which causes vasoconstriction. In conditions, when the IR/PI3K/Akt pathway is suppressed or downregulated, the insulin causes vasoconstriction via the endothelial ERK1/2 pathway (Yu *et al,* 2011).

In summary, there is reduction of Akt in pre-eclampsia, compared to the normal pregnancy. This may result in decreased eNOS and subsequently NO in the pre-eclamptic arterioles. Thus, the

vasodilatory effect of insulin, via the IR/PI3K/Akt pathway, is reduced (Fig 1.3). On the other hand, Yu *et al* (2011) has shown that when there is down-regulation of IR/PI3K/Akt pathway, there is stimulation of ERK1/2 pathway, resulting in vasoconstriction and reduced microvascular blood flow, which is a feature of the disease. Furthermore, in pre-eclampsia, there is more insulin resistance, leading to increased serum insulin, which can predispose to more widespread vasoconstriction (Figure 3.4.3). As ERK1/2 was not part of this study, further research is needed.

Chapter 3.9

Endothelial Cell GLUT4 receptor and relationship with insulin Resistance in pre-eclampsia

3.9.1. Introduction

Pre-eclampsia is a multi-systemic disorder of the second half of pregnancy. Women whose pregnancies are complicated by pre-eclampsia have increased risk of diabetes and cardiovascular disease, later in life (Laivuori *et al* 1996; Wolf *et al, 20*04; Lykke *et al, 20*09). Although the cause of insulin resistance remains unclear, there is accumulating evidence that this might be related to the underlying endothelial dysfunction, which persists after the pre-eclamptic delivery (Sandvik *et al,* 2013).

Skeletal muscle is one of the major target organs of insulin and plays a vital role in insulin mediated glucose update. Insulin delivery to the muscles interstitium through the vascular endothelial cells is the rate-limiting step in insulin-stimulated glucose uptake (Kubota *et al, 20*13). Glucose uptake is mainly via GLUT4 in muscles (Govers, 2014). GLUT4 is regulated by its intracellular localization. In the absence of insulin, GLUT4 is retained in the intracellular storage compartments, unlike the other isomers of GLUT (Watson and Pessin, 2001). On stimulation by insulin or muscle contraction, GLUT4 translocate to the cell surface where it transports glucose into the cells (Brewer *et al, 20*14). Thus, GLUT4 is not only an important player in glucose homeostasis, but also a key element in insulin resistance and T2DM.

In pre-eclampsia, microvascular blood flow to tissues is reduced (Chapter 3.1). Insulin resistance is increased in pre-eclampsia (Chapter 3.4). Endothelial cell surface insulin receptor expression is down regulated without any change in total insulin receptor proteins, in pre-eclampsia (Chapter 3.6). Also, there is down-regulation of endothelial Akt receptor expression in pre-eclampsia (Chapter 3.8). There is evidence that GLUT4 expression is reduced in arteries of hypertension (Park *et al, 20*05; Atkins *et al, 20*01). In this chapter, it is hypothesized that changes in the endothelial GLUT4 receptor protein expression occur, and this might explain the insulin resistance in pre-eclampsia. To investigate this hypothesis, endothelial cells were incubated in the sera from

women with pre-eclampsia and normal pregnancy, and GLUT4 protein expression were correlated with insulin receptor expression and insulin resistance.

3.9.2. Method

Participants and Measurement of Insulin Resistance

In this study, I recruited women with pre-eclampsia (n=16), and normal pregnant controls (n=18) from the maternity department at Kingston Hospital, UK, as described in Chapter 2.1. Blood samples were obtained from the ante-cubital vein of each participant using aseptic techniques, as described in Chapter 2.2. Insulin resistance was calculated in each patient as described previously in Chapter 2.4.2, using serum free Insulin and fasting blood glucose.

Measurement of blood flow

As described previously in Chapter 2.3, blood flow was measured using a Filtrass strain-gauge plethysmography (Filtrass; DOMED, Munich, Germany).

Cell Culture

HDMEC were grown as per the supplier's guidance (Promo Cell, Germany) as already described in detail in Chapter 2.6.

Study of Insulin Signalling Pathway: Insulin receptors and GLUT4

Flow cytometry was used to analyse the insulin receptors and GLUT4 expression in the endothelial cells. The method is described in Chapter 2.7.3.

Statistical Analysis:

The demographic data were normally distributed and were summarised as mean and SEM [Table 3.1]. All the other data are presented as median and Inter-quartile range. The differences between the groups were calculated using Mann Whitney tests, as the clinical data was not normally distributed (P-P plots). The demographic data were compared using t-test. Correlation was done using the Spearman's formula. P-values of <0.05 were considered statistically significant.

3.9.3. Results

The clinical and demographic characteristics of the participants are shown in Table 3.1. There was no significant difference in age, BMI, gestational age or haematocrit between the two groups. Babies born to the pre-eclamptic women were smaller in weight than the normal pregnant group, which is statistically significant. (p = 0.023).

Previously, insulin resistance, measured by HOMA, was increased in pre-eclamptic group, compared to the normal pregnant group (Chapter 3.4). Insulin receptor expression was down regulated in pre-eclamptic group, compared to the normal pregnant group (Chapter 3.6). GLUT4 expression, as measured by Flow-cytometry, was not significantly different in between the two groups [46.61 (43.55 – 49.21) versus [51.97 (42.62 – 54.75); p= 0.326 for pre-eclampsia and normal pregnant groups respectively]. {Fig 3.9.1}

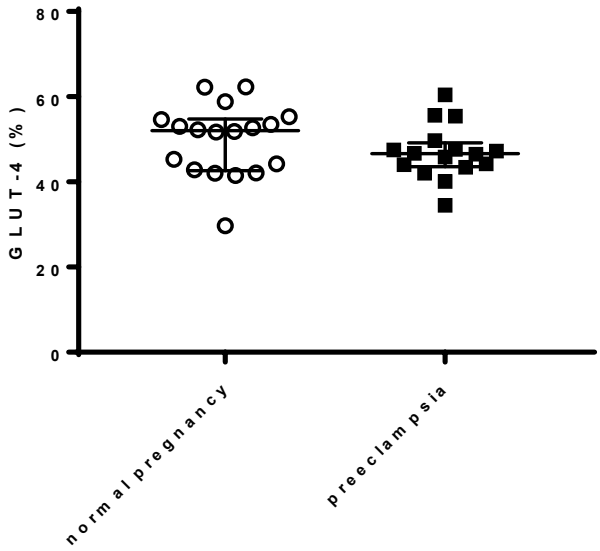


Figure 3.9.1: Comparison of Flow-cytometry of GLUT4 protein in normal pregnant controls and pre-eclamptic pregnancies.

In the normal pregnant group, no significant correlation exists between GLUT4 expression and HOMA (r_s= 0.044, p=0.861), serum insulin (r_s= -0.059, p=0.817), or endothelial cell surface insulin receptor expression (r_s= -0.197, p= 0.433) [Figure 3.9.2]. Furthermore, there was no significant correlation of GLUT4 expression with maternal age (r_s= 0.199, p= 0.428), gestational age (r_s=

0.032, p= 0.899), platelet count (r_s= 0.049, p= 0.848), haematocrit (r_s= -0.364, p= 0.138), MAP (r_s= 0.31, p= 0.21), BMI (r_s= -0.092, p= 0.717). and birth weight (r_s=-0.196, p= 0.435).

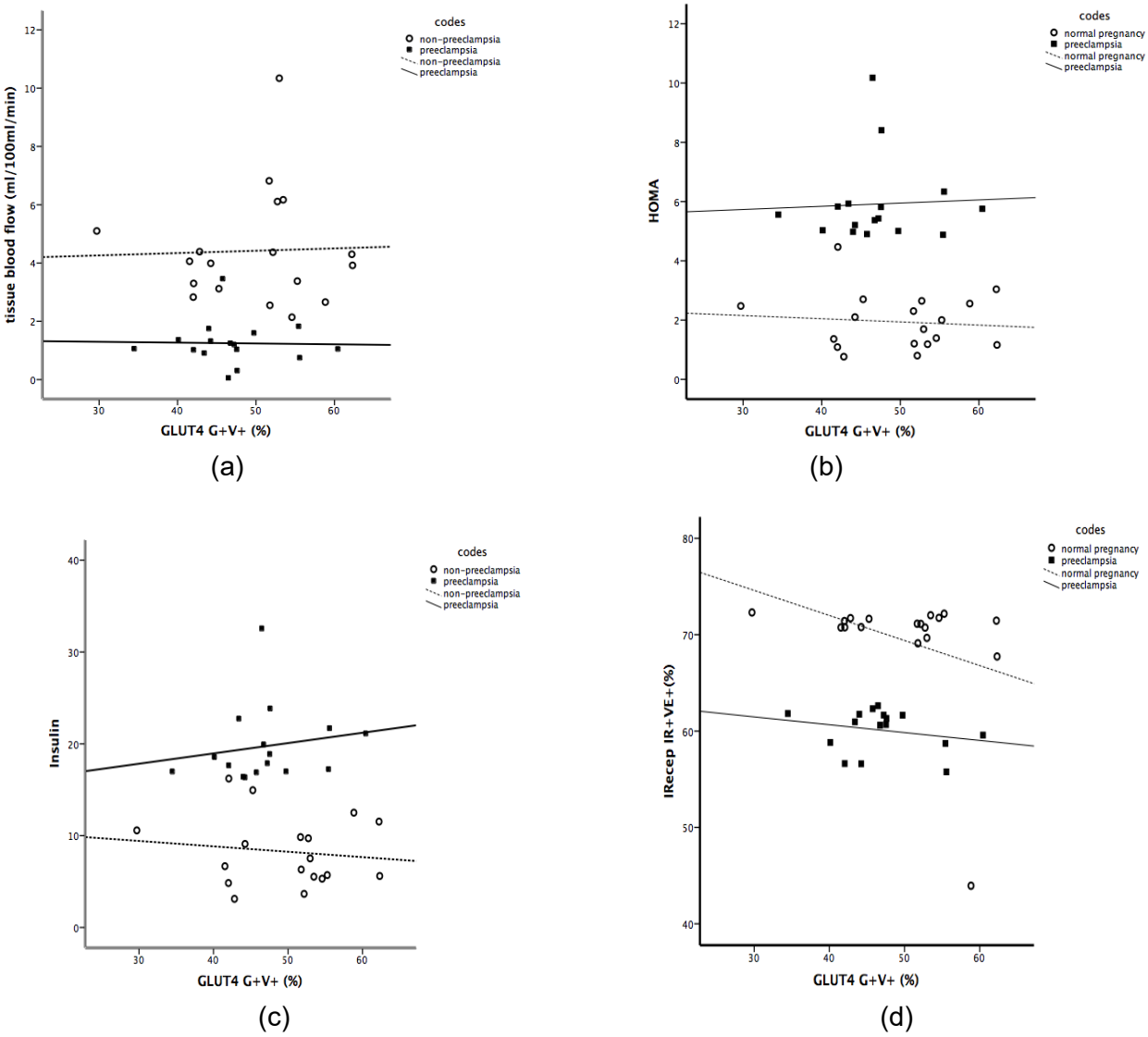


Figure 3.9.2: Correlation of GLUT4 receptor expression with (a) microvascular blood flow, (b) HOMA, (c) fasting Insulin level, and (d) cell surface Insulin receptors.

In the pre-eclamptic group, there was no significant correlation between GLUT4 expression and HOMA (r_s= 0.071, p=0.795), serum insulin (r_s= 0.332, p=0.208), or endothelial cell surface insulin receptor expression (r_s= -0.224, p= 0.405) [Figure 3.9.2]. Furthermore, there was no significant correlation of GLUT4 expression with maternal age (r_s= -0.373, p= 0.155), platelet count (r_s= -0.082, p= 0.762), haematocrit (r_s= 0.188, p= 0.485), MAP (r_s= 0.381, p= 0.145), BMI (r_s= -0.006, p= 0.983). and birth weight (r_s= 0.468, p= 0.068). However, GLUT4 has a statistically significant correlation with gestational age (r_s= 0.543, p= 0.03), in pre-eclampsia. [Appendix 3]

[Appendix 1C for FACs graph, and Appendix 2 for Western Blot pictures]

3.9.4. Discussion

In this chapter, endothelial cell total GLUT4 receptor, and its relationship with insulin resistance and insulin receptor expression were compared in pre-eclamptic pregnancies and normal pregnancies. This study tested the hypothesis, that altered endothelial insulin signalling pathway and changes in downstream expression of GLUT4, might be the possible mechanism for insulin resistance in pre-eclampsia. The study did not show a significant difference in endothelial cell GLUT4 between the two groups. Furthermore, there was no correlation between GLUT4 and insulin resistance, serum fasting Insulin, and surface expression of insulin receptors, in the two groups.

Human cells use glucose for the generation of ATP, by metabolism. The lipid bilayer of the cell membrane is impervious to carbohydrate. Glucose is transported from the blood across the cell membrane by saturable transport system, which is of two types; 1) firstly, SGLTs, which transport glucose against the concentration gradient, and 2) sodium independent glucose transporters (GLUTs), which transport glucose by facilitated diffusion along its concentration gradient (Jurcovicova, 2014). Currently, there are five established functional facilitative glucose transporter isoforms (GLUT1-4 and GLUTX1), with GLUT5 being a fructose transporter. The GLUT4 isoform is the major insulin-responsive transporter that is predominantly restricted to striated muscle and adipose tissue (Watson and Pessin, 2001). In the basal state, GLUT4 cycles slowly between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing in vesicular compartments within the cell interior (Govers 2014; Brewer *et al, 20*14). Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis and a smaller decrease in the rate of internalization by endocytosis (Huang and Czech 2005; Leto and Saltiel 2012).

Once insulin attaches to the cell, it stimulates GLUT4 via the PI3K/ Akt pathway. This results in mass exocytosis of the receptors to the cell surface facilitating glucose metabolism (Huang and

Czech 2005; Leto and Saltiel 2012). GLUT4 are constitutionally expressed in arteries and arterioles, where they participate in basal glucose uptake. Here it is utilised for VSMC contractions, thus maintaining the tone of the vascular tree (Park *et al,* 2005). They also are present by the muscles and adipose tissue, which helps insulin to maintain its function as anabolic and anti-catabolic hormone in humans (Govers 2014). Insulin resistance has been recognized as a main pathogenic factor in the development of type 2 diabetes, and has been associated with endothelial dysfunction, inflammation hypercoagulable state, dyslipidemia, and hypertension. The current thinking is that there is impaired insulin signalling pathway that leads to beta cells dysfunction, and its progression to Type 2 Diabetes (González-Sánchez *et al,* 2007). The key-step of this pathway is binding of insulin to its receptors, and subsequent activation of insulin receptor substrate proteins (González-Sánchez *et al,* 2007).

Although insulin dependent GLUT4 is the main transporter of glucose in endothelium, GLUT4 also participates in constitutive, noninsulin-dependent glucose uptake in arterial cells (Park *et al,* 2005; Atkins *et al,* 2001). Although Glut 4 mainly resides in intracellular vesicles in normal cells; in arterioles, they mainly reside on the cell surface only (Atkins *et al,* 2015). Also, there is evidence that GLUT4 expression is reduced in large arteries in hypertension (Park *et al, 20*05; Atkins *et al,* 2001). Arterial reactivity of GLUT4-knockout mice in increased, compared to other hypertensive animals (Park *et al,* 2005).

As previously discussed, GLUT4 resides on the surface and intracellular on the vessel wall. Once the cells are stimulated by insulin, the vesicles undergo exocytosis, and the surface GLUT4 receptors are increased, thus facilitating glucose uptake. It is likely that down-regulation of both insulin receptors (Chapter 3.6), and Akt protein (Chapter 3.8), in pre-eclampsia due to endothelial dysfunction does not make difference in the basal expression of GLUT 4. Therefore, changes in endothelial cell expression of GLUT 4 are unlikely to explain insulin resistance and impaired microvascular blood flow in pre-eclampsia. Prolonged exposure of cells to insulin, without enough glucose can lead to impaired GLUT4 translocation to the cell surface (Khalique *et al, 20*16).

In summary, this study failed to show a significant difference in endothelial cell GLUT4 receptors expression during normal pregnancy and pre-eclampsia. There was no correlation between GLUT 4 expression and both insulin resistance and endothelial surface insulin receptor expression. Therefore, altered endothelial expression of GLUT 4 is unlikely to explain insulin resistance in pregnancies complicated by pre-eclampsia.

Chapter 4: General Discussion

4.1 Summary of the study

Pre-eclampsia is a multisystemic disease of the second half of pregnancy whose cause(s) and effect(s) is still an enigma to modern science. It is associated with generalised endothelial dysfunction (Levine *et al, 20*04; Levine *et al, 20*06), and impaired tissue blood flow (Anim-Nyame *et al, 20*01, 2003). Women whose pregnancies are complicated by pre-eclampsia are more likely to develop diabetes and cardiovascular disease, later in life (Laivuori *et al,* 1996; Wolf *et al, 20*04; Lykke *et al, 20*09).

These studies were designed to investigate the hypothesis that changes in endothelial cell insulin signalling occur in pre-eclampsia, secondary to underlying endothelial dysfunction, resulting in insulin resistance. Impaired endothelial cell insulin signalling results in reduced tissue delivery of insulin and reduced GLUT-4 activation. This in turn might affect the altered microcirculation and insulin resistance seen in pre-eclampsia.

Microvascular blood flow

The cross-sectional study (chapter 3.1), showed that microvascular blood flow was reduced in the pre-eclamptic pregnancies, compared to normal pregnancies. Statistical correlation exists in the pre-eclamptic group with the gestational age, systolic blood pressure, mean arterial pressure and platelet count. Since SBP is an accepted index of disease severity, a decrease in microvascular blood flow is also an indicator of the disease severity.

Reduction in microcirculation seen in peripheral tissue, mirrors the reduction to vital organs, like liver, kidneys, uterus, etc. It precedes the alteration in the liver and renal blood tests. Also, our study confirms that the microvascular blood flow reduces with the gestational age in pre-eclampsia. It is like studies done previously (Anim-Nyame *et al, 20*01). Thus, it can be used as a marker to predict the severity of the disease.

Endothelial Dysfunction and angiogenic factors

Generalized endothelial dysfunction occurs in the disease (Chapter 3.2). It has already been shown previously that it is a known element to pre-eclampsia, secondary to yet unidentified factor(s) (Levine *et al,* 2004; Levine *et al,* 2006). Microvascular blood flow shows inverse correlation with the endothelial markers, like ICAM-1, VCAM-1, eSelectin, and Thrombomodulin, in the pre-eclamptic cohort. The endothelium plays an important role in control of smooth muscles tone through release of vasoconstrictor and vasodilatory substances, regulation of anticoagulation, antiplatelet, and fibrinolysis functions via release of different soluble factors (Roberts and Lain 2002; Mol *et al, 20*16). Since markers of endothelial dysfunction precede the clinical onset of the disease; it has been suggested to be the cause, and not the result, of pre-eclampsia.

Angiogenesis plays an important role in pregnancy. It is required for placentation, and pre-eclampsia is thought to be a disease due to defective placentation (Brennan *et al, 20*14). Angiogenic imbalance plays a pathogenic role in the aetiology of pre-eclampsia (Maynard *et al, 20*03; Venkatesha *et al, 20*06). This study has provided convincing evidence of an inverse correlation between anti-angiogenic factors and microvascular blood flow in pre-eclampsia (Chapter 3.3). Measurement of circulating angiogenic factors could be used to assess the severity of multisystem dysfunction in pre-eclampsia as reduced tissue perfusion precedes end organ dysfunction.

Insulin resistance and CEC

Insulin resistance is a feature of pre-eclampsia (Laivuori *et al* 1996; Anim-Nyame *et al, 20*15) and persists after delivery (Laivuori *et al* 1996). It has been attributed to endothelial dysfunction (Montagnani and Quon 2000; Ranganath and Quon 2007). Muscle is the main peripheral site of insulin action, where delivery process is the rate-limiting factor (Barrett *et al, 20*11). Changes in the microvascular environment, including endothelial dysfunction and tissue blood flow, may affect insulin delivery and insulin resistance, seen in pre-eclampsia (St-Pierre *et al, 20*10).

This study showed that insulin resistance is more pronounced in the pre-eclamptic group (Chapter 3.4). In the pre-eclamptic group, there is a statistically significant inverse correlation between microvascular tissue blood flow and insulin resistance, which was not demonstrated in the normal pregnant controls. Though there is some degree of insulin resistance in normal pregnancy, it is exacerbated in pre-eclampsia and becomes more severe as the gestational age progresses (Anim-Nyame *et al, 20*15). Mid-trimester insulin resistance may predict subsequent development of pre-eclampsia (Hauth *et al, 20*11).

Although the mechanism of increased insulin resistance in pre-eclampsia remains unexplained, this has been attributed to the underlying generalised endothelial dysfunction (Montagnani and Quon 2000; Ranganath and Quon 2007). Circulating endothelial cells (CEC) are sloughed endothelial cells (Woywodt *et al, 20*06). CEC count appears to correlate with the degree of endothelial dysfunction (Gignat-George *et al, 20*00), and inversely with endothelial repair (Fabbri-Arrigoni *et al, 20*12). This study has shown that insulin resistance and CEC counts are increased in pre-eclampsia (Chapter 3.5). Although, there is no correlation in between insulin resistance and CEC in pre-eclampsia (Chapter 3.5), insulin resistance showed positive correlation with the biochemical markers of endothelial dysfunction (Chapter 3.4).

Insulin receptor expression

Although muscle is the main peripheral site of insulin action (Saltiel and Kahn, 2001), insulin is delivered via both passive diffusion and trans-capillary transport mechanisms involving endothelial cell surface binding (Vincent *et al, 20*03; Posner 2017). The trans-capillary transport is initiated when insulin binds to its receptors on endothelial cells. The study showed that there is reduced expression of surface insulin receptors in pre-eclampsia compared to normal pregnancy; however, the total amount of insulin receptor protein in the cells in the two groups was equal (Chapter 3.6). This may be due to widespread endothelial cell dysfunction seen in pre-eclampsia despite lack of correlation between insulin receptor expression and markers of endothelial dysfunction. It is possible the effect of endothelial dysfunction on insulin receptor expression explains the long-term risk of cardiovascular disease in women whose pregnancies are complicated by pre-eclampsia.

Decreased insulin receptor expression in pre-eclampsia on vascular endothelial cells can explain the increased insulin resistance and reduced blood flow seen in this condition (Chapter 3.7). The association between insulin resistance and hypertension is well established but presently unexplained (Barrett *et al, 20*11). There is growing evidence that hyperinsulinemia is the link between diabetes and hypertension (Lykke *et al, 20*09), which can be secondary to decreased insulin receptor expression (Obisi *et al, 20*02).

Akt and GLUT4

Insulin increased tissue perfusion (Kubota *et al, 20*11, 2013). Insulin binding to its receptor activates both PI3K/AKT and the Ras-MAP kinase pathway, and the PI3K/AKT pathway mediates activation of eNOS (Kuboki *et al,* 2000; Zeng *et al,* 2000; Hermann *et al, 20*00). (Figure 1.3) Moreover, Insulin may also induce vasoconstriction by activation of extracellular signal-regulated kinase 1/2 (ERK1/2) present on the endothelial cells (Eringa *et al,* 2004). This study (Chapter 3.8), has demonstrated a reduction of Akt in pre-eclampsia, compared to the normal pregnancy, which can result in decreased eNOS and subsequently NO in the pre-eclamptic arterioles. Thus, the vasodilatory effect of insulin, via the IR/PI3K/Akt pathway, is reduced. On the other hand, there is stimulation of ERK1/2 pathway, resulting in vasoconstriction, which is evident in pre-eclampsia.

Once insulin attaches to its receptors, it activates GLUT4 via the PI3K/ Akt pathway, which then facilitates glucose metabolism (Huang and Czech 2005; Leto and Saltiel 2012). This study didn't show a significant difference in endothelial cell GLUT4 receptors expression during normal pregnancy and pre-eclampsia. There was no correlation between GLUT 4 expression and both insulin resistance and endothelial surface insulin receptor expression (Chapter 3.9). Therefore, altered endothelial expression of GLUT 4 is unlikely to explain insulin resistance in pregnancies complicated by pre-eclampsia.

4.2. Limitation of the study

There are some limitations to my study:

1. Firstly, the gastrocnemius muscle was studied, as it is presumed to have a high muscle to skin ration, meaning that the blood flow through it was more to support metabolism in the muscles, rather than temperature control by the skin (Anim-Nyame *et al, 20*01). Moreover, skeletal muscles lacked visible AV channels, so most of the bloods pass through the capillaries and microcirculation. An ankle occlusion cuff was not used to exclude arterio-venous shunts of the feet. This was done to prevent discomfort to the participants that might have had hemodynamic consequences and interfered with other aspects of the protocol.
2. Secondly, although the gastrocnemius muscle circumference between the two groups were similar, differences in adipose tissue composition could, by adding heterogeneity, influence the applicability of the findings more generally.
3. Ideally the flow-cytometry for GLUT4 should have been done on live whole cells, as GLUT4 receptors are expressed on the surface after stimulation of insulin receptors with Insulin via the PI3K/ Akt pathway. Also, the total GLUT4 protein should have been assessed by western blot.

4.3 Future work

4.3.1 Long-term effect of pre-eclampsia

I have incubated the endothelial cells in pre-eclamptic and normal pregnant sera for only 40 hours. This is because of cell senescence as detected by trypan assay. In future, the sera needed to be changed, and the cells passaged, to test the long-term effect on cell signalling. Following that, the cells should have been reverted to culture media to see if the effects were reversible or long lasting.

4.3.2 Qualitative assessment of the signalling pathway

In this study, only the quantity of the receptors was assessed, and the receptor proteins. It may be possible that the receptors are malfunctioning due to endothelial dysfunction. Further study is needed to look at the downstream effects of the receptors, once stimulated.

4.3.3 Postnatal effect of the pre-eclampsia

Ideally, the participants needed reassessment 6 months post- delivery. Their plethysmograph, ELISA for markers of endothelial dysfunction, and cell signalling pathway proteins, were repeated. Ideally, in pre-eclampsia all changes should revert, but evidence proves otherwise (Laivuori *et al,* 1996; Wolf *et al, 20*04; Lykke *et al, 20*09). It would have been ideal if the women were tested during their pregnancies (pre-eclampsia and normotensive cohorts), and after 6 months post-delivery of the same patients

Reference

- Abdul-Ghani MA, DeFronzo RA. Pathogenesis of insulin resistance in skeletal muscle. *J Biomed Biotechnol.* 2010; 476279: 1-19
- Abhari FR, Andarieh MG, Farokhfar A, Ahmady S. Estimating rate of insulin resistance in patients with pre-eclampsia using HOMA-IR Index and comparison with non-pre-eclampsia pregnant women. *Biomed Res Int* 2014; 2014: 140851.
- Aloizos S, Seretis C, Liakos N, *et al.* HELLP syndrome : Understanding and management of a pregnancy-specific disease. *J Obstet Gynecol.* 2013; 33(4): 331-7.
- Altenkirch HU, Fransson L, Koch G. Assessment of arterial and venous circulation in upper and lower extremities by venous occlusion strain gauge plethysmography. Normal values and reproducibility. *Vasa.* 1989; 18(2): 140-145.
- Anim-Nyame N, Gamble J, Sooranna SR, Johnson MR, Steer PJ. Evidence of impaired microvascular function in pre-eclampsia: a non-invasive study. *Clin Sci.* 2003; 104: 405-412.
- Anim-Nyame N, Gamble J, Sooranna SR, Johnson MR, Steer PJ. Relationship between insulin resistance and tissue blood flow in pre-eclampsia. *J Hypertens*. 2015a; 33(5): 1057-63.
- Anim-Nyame N, Ghosh A, Freestone N, Arrigoni FI. Relationship between insulin resistance and circulating endothelial cells in pre-eclampsia. *Gynecol Endocrinol.* 2015b; 31(10): 788-91.
- Anim-Nyame N, Hills FA, Sooranna SR, Steer PJ, Johnson MR. A longitudinal study of maternal plasma insulin-like growth factor binding protein-1 levels during normal pregnancy and pregnancies complicated by pre-eclampsia. *Hum Reprod*. 2000a; 15(10): 2215-2219.
- Anim-Nyame N, Sooranna PJ, Johnson MR, Gamble J, Steer PJ. Longitudinal changes in resting blood flow during normal pregnancy and pregnancies complicated by chronic hypertension and pre-eclampsia. *Cardiovasc Res*. 2001; 50: 603-609.
- Anim-Nyame N, Sooranna SR, Alaghband-Zadeh J, Steer PJ, Johnson MR. Insulin resistance and pre-eclampsia: a role for tumour necrosis factor-alpha? *Gynecol Endocrinol* 2004; 18:117–123.
- Anim-Nyame N, Sooranna SR, Johnson MR, Gamble J, Steer PJ. Resting blood flow in normal pregnancy and in pre-eclampsia. *Clin Sci*. 2000b; 99: 595-510.

- Anim-Nyame N, Sooranna SR, Steer PJ, Johnson MR. Longitudinal changes in maternal plasma levels of leptin during normal pregnancy and pre-eclampsia. *Hum Reprod* 2000c; 15: 2033–2036.
- Arcaro G, Cretti A, Balzano S, Lechi A, Muggeo M, Bonora E, *et al*. Insulin causes endothelial dysfunction in humans. Sites and mechanisms. *Circulation* 2002; 105:576–582.
- Atkins KB, Johns D, Watts S, Clinton-Webb R, Brosius FC 3rd. Decreased vascular glucose transporter expression and glucose uptake in DOCA-salt hypertension*. J Hypertens.* 2001; 19: 1581–1587.
- Atkins KB, Seki Y, Saha J, Eichinger F, Charron MJ, Brosius FC 3rd. Maintenance of GLUT4 expression in smooth muscle prevents hypertension-induced changes in vascular reactivity. *Physiol Rep* 2015; 3(2): e12299.
- Bakker W, Eringa EC, Sipkema P, van Hinsbergh VW. Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res.* 2009; 335(1):165-89.
- Barret EJ, Enggleton EM, Inyard AC, Wang H, Li G, Liu Z. The vascular actions of insulin control its delivery to muscle and regulate the rate limiting muscle insulin action. *Diabetologica* 2009; 52: 752–764.
- Barrett EJ, Wang H, Upchurch CT, Liu Z. Insulin regulates its own delivery to skeletal muscle by feed-forward actions on the vasculature. *Am J Physiol Endocrinol Metab*. 2011; 301(2): E252-E263.
- Bethel K, Luttgen MS, Damani S, *et al.* Fluid phase biopsy for detection and characterisation of circulating endothelial cells in myocardial infarction. *Phys Biol*. 2014; 11: 016002 (9pp).
- Bouïs D, Hospers GA, Meijer C *et al*. Endothelium in vitro: A review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis*. 2001; 4(2): 91-102.
- Bouthoorn SH, Gaillard H, Steegers EA, *et al.* Ethnic difference in blood pressure and hypertensive complications during pregnancy: the Generation R study. *Hypertension* 2012; 60: 198- 205.
- Brandão AH, Cabral MA, Leite HV, Cabral AC. Endothelial function, uterine perfusion and central flow in pregnancies complicated by pre-eclampsia*. Arq Bras Cardiol*. 2012; 99(4): 931-5.
- Bremme KA. Haemostatic changes in pregnancy. *Best Practise and Research Clinical Haematology.* 2003; 16 (2): 153-168.

- Brennan LJ, Morton JS, Davidge ST. Vascular dysfunction in pre-eclampsia. *Microcirculation.* 2014; 21(1): 4-14
- Brewer PD, Habtemichael EN, Romenskaia I, *et al*. Insulin-regulated GLUT4 translocation: membrane protein trafficking with six distinctive steps. *J Biol Chem.* 2014; 289(25): 17280-98.
- Brogan PA, Malik M, Shah N, *et al.* Systemic vasculitis: a cause of indeterminate intestinal inflammation. *Journal of pediatric gastroenterology and nutrition*. 2006 Apr 1;42(4):405-15.
- Brosens JJ, Pijenborg R, Brosens IA. The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: A review of literature. *Am J Obstet Gynecol* 2002; 187: 1416-23.
- Canbakan B, Keven K, Tutkak H, Danisman N, Ergun I, Nergizoglu G. Circulating endothelial cells in pre-eclampsia. *Journal of Human Hypertension*. 2007; 21: 558–563.
- Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: Implications for the pathophysiology of pre-eclampsia. *Placenta.* 2000; 21A: S25-S30.
- Carberry PA, Shepherd AMM, Johnson JM. Resting and maximal forearm skin blood flows are reduced in hypertension. *Hypertension.* 1992; 20: 349-355.
- Carling A, Alfirevic Z. Physiological changes of pregnancy and monitoring. *Best Practice and Research Clinical Obstetrics and Gynaecology.* 2008; 22(5): 801-823.
- Carmina E, Orio F, Palomba S, Longo RA, Cascella T, Colao A, Lombardi G, Rini GB, Lobo RA. Endothelial dysfunction in PCOS: role of obesity and adipose hormones. *The American journal of medicine*. 2006; 119(4): 356-e1.
- Carty DM, Anderson LA, Duncan CN, Baird DP, Rooney LK, Dominiczak AF, Delles C. Peripheral arterial tone: assessment of microcirculatory functions in pregnancy. *J Hypertens.* 2012; 30(1): 117-23.
- Cervos-Narvarro J, Matakas F. Electron microscopic evidence of for innervation of intracerebral arterioles in the cat. *Neurology*. 1974; 24: 282-286.
- Chaddha V, Viero S, Huppertz B, Kingdom J. Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med.* 2004; 9(5): 357-69.
- Chaiworapongsa T, Romero R, Korzeniewski SJ, *et al.* Maternal plasma concentrations of angiogenic/antiangiogenic factors in the third trimester of pregnancy to identify the patient at risk

for stillbirth at or near term and severe late pre-eclampsia. *Am J Obstet Gynecol* 2013; 208: 287.e1-15.

- Chaiworapongsa T, Romero R, Yoshimatsu J, *et al.* Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. *J Matern Fetal Neonatal Med*. 2002; 12(1): 19-27.
- Chavarría ME, Lara-González L, García-Paleta Y, Vital-Reyes VS, Reyes A. Adhesion molecules changes at 20 gestation weeks in pregnancies complicated by pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol.* 2008; 137(2): 157-64.
- Chavarría ME, Lara-González L, García-Paleta Y, Vital-Reyes VS, Reyes A. Adhesion molecules changes at 20 gestation weeks in pregnancies complicated by pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol.* 2008; 137(2): 157-64.
- Chen X, Scholl TO. Maternal biomarkers of endothelial dysfunction and preterm delivery. PLoS One. 2014; 9(1): e85716.
- Christ F, Bauer A, Brugger D, Niklas M, Gartside IB, Gamble J. Description and validation of a novel liquid metal-free device for venous congestion plethysmography. *J Appl Physiol.* 2000a; 89(4): 1577-1583.
- Christ F, Gamble J, Gartside IB, Kox WJ. Increased microvascular water permeability in patients with septic shock, assessed with venous congestion plethysmography (VCP). *Intensive Care Medicine*. 1998; 24: 18 - 27.
- Christ F. Bauer A. Brugger D, Niklas M, Gartside IB, Gamble J. A novel automatically calibrating, liquid free measuring device for venous congestion plethysmography. *J Applied Physiol.* 2000b; 89: 1577-1583.
- Clancy R, Marder G, Martin V, Belmont HM, Abrahamson SB, Buyon J. Circulating activated endothelial cells in systemic lupus erythromatosis: Further evidence for diffuse vasculopathy. *Arthitis Rheum*. 2001; 44: 1203-1208.
- Clarke LA, Hong Y, Eleftheriou D, *et al.* Endothelial injury and repair in systemic vasculitis of the young. *Arthritis Rheum* 2010; 62: 1770–80.
- Coelho TM, Sass N, Camano L, Moron AF, Mattar R, Stávale JN, Silva MR, Martins Mda G, Nogueira Neto J. Micro vessel density in the placental bed among pre-eclampsia patients. *Sao*

Paulo Med J. 2006; 124(2): 96-100.

- Conley KE, Orgway GA, Richardson RS. Deciphering the mysteries of myoglobin in striated muscle. *Acta Physiol Scand*. 2000; 168: 623-634.
- Csordas A, Bernhard D. The biology behind the atherothrombotic effects of cigarette smoke. *Nature Reviews Cardiology*. 2013; 10: 219-230.
- Cvitic S, Desoye G, Hiden U. Glucose, insulin and oxygen interplay in placental hypervascularisation in diabetes mellitus. *Biomed Res Int.* 2014; 2014: 145846
- Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nat Clin Pract Cardiovasc Med.* 2009; 6(1): 16-26
- DeFronzo RA. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing link. The Claude Bernard Lecture 2009. *Diabetologia*. 2010; 53(7): 1270-1287.
- Dogan E, Demir SC, Gulek UK. Maternal soluble vascular cytoplasmic adhesion molecule-1 and fibronectin levels in early and late onset pre-eclamptic pregnancy. *Clin Exp Obstet Gynecol.* 2014; 41(6): 681-4.
- Dong Y, Desneux N, Lei C, Niu CY. Transcriptome Characterization Analysis of Bactrocera minax and New Insights into Its Pupal Diapause Development with Gene Expression Analysis. *Int J Biol Sc* 2014; 10(9): 1051-63.
- Dzau VJ. Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension* 2001; 37: 1047–1052.
- Edmunds N, Marshall JM. Oxygen delivery and oxygen consumption in rat hind limb during systemic hypoxia: role of adenosine. *J Physiol*. 2001; 536: 927-935.
- Edmunds N, Moncado S, Marshall JM. Does nitric oxide allow endothelial cells to sense hypoxia and mediate hypoxic dilatation? In vivo and in vitro studies. *J Physiol*. 2003; 546(2): 521-527.
- Erdbruegger U, Dhaygude A, Haubitz M, Woywodt A. Circulating endothelial cells: markers and mediators of vascular damage. *Curr Stem Cell Res Ther.* 2010; 5(4): 294-302.
- Eringa EC, Stehouwer CD, Merlijn T, Westerhof N, and Sipkema P. Physiological concentrations of insulin induce endothelin-mediated vasoconstriction during inhibition of NOS or PI3-kinase in skeletal muscle arterioles. *Cardiovasc Res* 2002; 56: 464–471.

- Eringa, EC, Stehouwer CDA, van Nieuw Amerongen GP, Ouwehand L, Westerhof N, Sipkema P. Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium. *Am J Physiol Heart Circ Physiol* 2004; 287: H2043–H2048.
- Esper RJ, Nordaby RA, Vilariño JO, *et al.* Endothelial dysfunction: a comprehensive appraisal. *Cardiovascular Diabetology*. 2006; 5(4): 1-18
- Fabbri-Arrigoni FI, Clarke L, Wang G, *et al.* Levels of circulating endothelial cells and colony-forming units are influenced by age and dyslipidaemia. *Pediatr Res* 2012; 72: 299–304.
- Feig DS, Shah BR, Lipscombe LL, Wu CF, Ray JG, Lowe J, Hwee J, Booth GL. Pre-eclampsia as a risk factor for diabetes: a population- based cohort study. *PLoS Med.* 2013; 10(4): e1001425.
- Félétou M. Endothelium-dependent hyperpolarization and endothelial dysfunction. *J cardiovasc Pharmacol.* 2016 ; 67(5) : 373-87.
- Ferreira GD, Orcy RB, Martins-Costa SH, Ramos JGL, Brum IS, von Eye Corleta H, Capp E. Insulin stimulation of Akt/PKB phosphorylation in the placenta of pre-eclampsia patients [English;Portuguese] Estimulacao com insulina da fosforilacao da Akt/PKB em placenta de pacientes com pre-eclampsia. *Sao Paulo Med J*. 2011; 129(6): 387- 391.
- Fisher G, Windham ST, Griffin P, *et al.* Associations of human skeletal muscle fibre type and insulin sensitivity, blood lipids and vascular haemodynamic in a cohort of of pre-menopausal women. *Eur J Appl Physiol*. 2017; 117(7): 1413-22
- Fisher SJ. Why is placentation abnormal in pre-eclampsia? *Am J Obstet Gynecol.* 2015; 213(4 Suppl): S115-22.
- Freeman DJ, McManus F, Brown EA, Cherry L, Norrie J, Ramsay JE, Clark P, Walker ID, Sattar N, Greer IA. Short- and long-term changes in plasma inflammatory markers associated with pre-eclampsia. *Hypertension.* 2004; 44(5): 708-14.
- Frusca T, Soregaroli M, Platto C, Enterri L, Lojacono A, Valcamonico A. Uterine artery velocimetry in patients with gestational hypertension. *Obstet Gynecol.* 2003; 102(1): 136-40.
- Fu Q, Levine BD. Autonomic circulatory control during pregnancy in humans. *Semin Reprod Med.* 2009; 27(4): 330-337.
- Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. "Regulation of the

vascular extracellular superoxide dismutase by nitric oxide and exercise training". *The Journal of Clinical Investigation*. 2000; 105 (11): 1631–9.

- Funakoshi Y, Ichiki T, Shimokawa H, *et al.* Rho-kinase mediates Angiotensin II- induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension*. 2001; 38(1): 100-104.
- Gaillard R, Bakker R, Willemsen SP, Hofman A, Steegers EA, Jaddoe VW. Blood pressure tracking during pregnancy and the risk of gestational hypertensive disorders: the Generation R Study. *Eur Heart J.* 2011; 32: 3088-97.
- Gamble J, Christ F. Gartside IB. Human pre-capillary resistance decreases in response to small cumulative increases in venous congestion pressure. *J Physiol* 1998; 507(2): 611- 617.
- Ganong WF. Endocrine functions of the pancreas and regulation of carbohydrate metabolism. In: Ganong WF. *Reviews of Medical physiology*. 2005; 25th edition. USA; Appleton/Lange. 333-356.
- Gaugler-Senden IP, Tamsma JT, van der Bent C, Kusters R, Steegers EA, de Groot CJ. Angiogenic factors in women ten years after severe very early onset pre-eclampsia*. PLoS One.* 2012; 7(8): e43637
- Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW. Systemic inflammatory priming in normal pregnancy and pre-eclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol.* 2007; 178(9): 5949-56.
- Ghosh A, Freestone N, Anim-Nyame N, *et al.* Microvascular function in pre-eclampsia is influenced by insulin resistance and an imbalance of angiogenic factors. *Physiol Rep*. 2017; 5(8): e13185
- Gignat-George F, Sampol J. Circulating endothelail cells in vascular: new insight into old concept. *Eur J Haematol*. 2000; 65: 215-220.
- Gilert JS, Ryan MJ, LaMarca BB, Sedeek M, Murphy SR, Granger JP. Pathophysiology of hypertension during pre-eclampsia: linking placental ischemia with endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 2008. 294: H541–H550.
- Godo S, Shimokawa H. Endothelial functions. *Arterioscler Throb Vasc Biol*. 2017; 37(9): e108-e114.
- González-Sánchez JL, Serrano-Ríos M. Molecular basis of insulin action. *Drug News Perspect.*

2007; 20(8): 527-31.

- GOPEC consortium. Disentangling fetal and maternal susceptibility for pre-eclampsia: A British multicentre candidate-gene study. *Am J Hum Genet*. 2005; 77(1): 127-131.
- Govers R. Cellular regulation of glucose uptake by glucose transporter GLUT4. *Adv Clin Chem*. 2014; 66: 173-240.
- Granner DK. Hormones of the Pancreas and Gastrointestinal Tract. In: Murray R.K, Granner D.K, Mayes P.A, Rodwell VW. *Harper's Biochemistry*. 2000; 25th edition. London: McGraw-Hill. 610-626.
- Grindheim G, Estansen ME, Langesaeter E, Rosseland LA, Toska K. Changes in blood pressure during healthy pregnancy: a longitudinal cohort study. *J Hypertens*. 2012; 30: 342-50.
- Groothuis JT, van Vliet L, Kooijman M, Hopman MTE. Venous cuff pressures from 30mm Hg to diastolic pressure are recommended to measure arterial inflow by plethysmography. *J Appl Physiol.* 2003; 95(1): 342-47
- Grundmann M, Woywodt A, Kirsch T, Hollwitz B, Oehler K, Erdbruegger U, Haller H, Haubitz M. Circulating endothelial cells: a marker of vascular damage in patients with pre-eclampsia. *Am J Obstet Gynecol.* 2008; 198(3): 317.e1-5.
- Gutterman DD, Chabowski DS, Kadlec AO, *et al.* The human microcirculation : Regulation of flow and beyond. *Circ Res*. 2016; 118(1): 157-72.
- Hale LJ, Coward RJM. Insulin signalling to the kidney in health and disease. *Cli Sc.* 2013; 124(6): 351-370.
- Hammer LW, Ligon AL, Hester RL. Differential inhibition of functional dilatation of small arterioles by indomethacin and glibenclamide. *Hypertension.* 2001; 37: 599-603.
- Hauth JC, Clifton RG, Roberts JM, Myatt L, Spong CY, Leveno KJ, Varner MW, Wapner RJ, Thorp Jr JM, Mercer BM, Peaceman AM, Ramin SM, Carpenter MW, Samuels P, Sciscione A, Tolosa JE, Saade G, Sorokin Y, Anderson GD. Eunice Kennedy Shriver National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. Maternal Insulin resistance and pre-eclampsia. *Am J Obstet Gynecol* 2011; 204: 327e1–6.
- Hellsten Y, Nyberg M, Jensen LG, Mortensen SP. Vasodilator interactions in skeletal muscle blood flow regulation. *J Physiol*. 2012; 590(24): 6297- 6305

- Hermann C, Assmus B, Urbich C, Zeiher AM, Dimmeler S. Insulin-mediated stimulation of protein kinase Akt: A potent survival-signalling cascade for endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 2000; 20: 402-409.
- Herse FN, Fain JN, Janke J, *et al.* Adipose tissue-derived soluble fms-like tyrosine kinase-1 is an obesity-relevant endogenous paracrine adipokine. *Hypertension*. 2011; 58: 37-42.
- Heyl W, Handt S, Reister F, Gehlen J, Mittermayer C, Rath W. The role of soluble adhesion molecules in evaluating endothelial cell activation in pre-eclampsia. *Am J Obstet Gynecol.* 1999; 180(1): 68-72.
- Hibbard JU, Shroff SG, Cunningham FG. Cardiovascular alterations in normal and pre-eclamptic pregnancies. Chapter 14 in *Chesley's Hypertensive disorders if pregnancy*. 4th ed. 2015: 291-313
- Hiden U, Lang I, Ghaffari-Tabrizi N, Gauster M, Lang U, Desoye G. Insulin action on the human placental endothelium in normal and diabetic pregnancy. *Current Vascular Pharmacology.* 2009; 7(4): 460-467.
- Himsworth HP. The syndrome of Diabetes Mellitus and its causes. *Lancet*. 1949; 253 (6551): 465-473.
- Hodson K, Man CD, Smith FE, Thelwall PE, Cobelli C, Robson SC, Taylor R. Mechanism of Insulin resistance in normal pregnancy. *Horm Metab Res*. 2013; 45(8): 567-71
- Huang S, Czech MP. The GLUT4 glucose transporter. *Cell Metabolism.* 2005; 5(4) : 237-252.
- Hudlicka O. Microcirculation in skeletal muscle. *Muscles Ligaments Tendons J.* 2011; 1(1): 3-11.
- Humar R, Zimmerli L, Battegay E. Angiogenesis and hypertension: an update. *J Hum Hypertens.* 2009; 23(12): 773-82.
- Hung TH, Skepper JN, Chernock-Jones DS, Burton GJ. Hypoxia- reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in pre-eclampsia. *Circ Res* 2002; 90: 1274-81.
- Hyman C, Winsor T. History of plethysmography. *The Journal of cardiovascular surgery.* 1961; 2: 506-18.
- Ince C, Mayeux PR, Nguyen T, *et al.* Endothelium in sepsis. *Shock* 2016 ; 45(3) : 259-70.
- Irving RJ, Walker BR, Noon JP, Watt GCM, Webb DJ, Shore AC. Microvascular correlates of blood

pressure, plasma glucose and insulin resistance in health. *Cardiovasc Res* 2002; 53:271–276.

- Jacob M, Chappell D, Becker BF. Regulation of blood flow and volume exchange across the microcirculation. *Critical Care*. 2016; 20: 319.
- Joyner MJ, Dietz NM, Shepherd JT. From Belfast to Mayo and beyond: the use and future of plethysmography to study blood flow in human limbs. *J Appl Physiol*. 2001; 91(6): 2431-41.
- Jurcovicova J. Glucose transport in brain- effect of inflammation. *Endocr Regul*. 2014; 48(1): 35-48.
- Karanam VL, Page NM, Anim-Nyame N. Maternal tissue blood flow and oxygen saturation in pre-eclampsia and intrauterine growth restriction*. Eur J Obstet Gynecol Reprod Biol.* 2014; 178: 148-52.
- Karthikeyan VJ, Lip GY. Endothelial damage/ dysfunction and hypertension in pregnancy. *Front Biosci (Elite Ed)*. 2011; 1(3): 1100-8.
- Karumanchi SA, Lindheimer MD. Pre-eclampsia pathogenesis: 'Triple A rating' autoantibodies and anti-angiogenic factors. *Hypertension.* 2008; 51: 991-992.
- Katz A, Nambi SS, Mather K, *et al*. Quantitative insulin sensitivity check index: A simple, accurate method for assessing Insulin sensitivity in Humans. *Journal of Clinical Endocrinology and Metabolism.* 2000; 85(7):2402-2410.
- Khalique A, Sarwade RD, Pandey PR, *et al.* Prolonged exposure to insulin with insufficient glucose leads to impaired GLUT4 translocation. *Biochemical & Biophysical Research Communications.* 2016; 474(1): 64-70.
- Kim J, Montagnani M, Koh KK, Quon MJ. Reciprocal relationship between insulin Resistance and endothelial dysfunction. *Circulation* 2006; 113: 1888-1904.
- Knight M, Kenyon S, Brocklehurst P, Neilson J, Shakespeare J, Kurinczuk JJ (Eds.) on behalf of MBRRACE- UK. Saving Lives, Improving Mothers' Care - Lessons learned to inform future maternity care from the UK and Ireland. *Confidential Enquiries into Maternal Deaths and Morbidity 2009–12.* Oxford: National Perinatal Epidemiology Unit, University of Oxford 2014.
- Konukoglu D, Uzun H. Endothelial Dysfunction and Hypertension. *Adv Exp Med Biol.* 2017; 956: 511-540.

- Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, *et al.* Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo: a specific vascular action of insulin. *Circulation* 2000; 101:676–681.
- Kubota T, Kubota N, Kadowaki T. The role of endothelial insulin signalling in the regulation of glucose metabolism. *Rev Endocr Metab Disord. 2013*; 14(2): 207-16.
- Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, Kawai T, Hashimoto S, Kobayashi T, Sato M, Tokuyama K, Nishimura S, Tsunoda M, Ide T, Murakami K, Yamazaki T, Ezaki O, Kawamura K, Masuda H, Moroi M, Sugi K, Oike Y, Shimokawa H, Yanagihara N, Tsutsui M, Terauchi Y, Tobe K, Nagai R, Kamata K, Inoue K, Kodama T, Ueki K, Kadowaki T. Impaired insulin signalling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. *Cell Metab*. 2011; 13(3): 294-307.
- Laganowsky A, Reading E, Allison TM, *et al*. Membrane proteins binds lipids selectively to modulate their structure and function. *Nature.* 2014; 510(7503): 172-175.
- Laight DW, Carrier MJ, Anggard EE. Antioxidants, diabetes and endothelial dysfunction. *Cardiovasc Res* 2000; 47: 457–464.
- Laivuori H, Tikkanen MJ, Ylikorkala O. Hyperinsulinaemia 17 years after pre-eclamptic first pregnancy. *J Clin Endocrinol Metabol*. 1996; 81(8): 2908-11.
- Lassance L, Miedl H, Absenger M, Diaz-Perez F, Lang U, Desoye G, Hiden U. Hyperinsulinemia stimulates angiogenesis of human fetoplacental endothelial cells: A possible role of insulin in placental hypervascularization in diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism.* 2013; 98(9): E1438- E1447.
- Latroche C, Gitiaux C, Chretien F, *et al*. Skeletal muscle microvasculature : A highly dynamic lifeline. *Physiology*. 2015; 30(6): 417-27.
- Leto D, Saltiel AR. Regulation of glucose transport by insulin: traffic control of GLUT4. *Nature Reviews Molecular cell biology.* 2012; 13: 383-396.
- Levine RJ, Maynard SE, Qian C, *et al.* Circulating angiogenic factors and the risk of pre-eclampsia. *N Engl J Med.* 2004; 350: 672– 683.
- Levine RJ, Qian C, Maynard SE, Yu KF, Epstein FH, Karumanchi SA. Serum sFlt1 concentration

during pre-eclampsia and mid trimester blood pressure in healthy nulliparous women. *Am J Obstet Gynecol.* 2006; 194:1034–1041

- Liang H, Vårtun Å, Acharya G. Agreement between preload reserve measured by impedance cardiography and echocardiography during pregnancy. *Arch Gynecol Obstet.* 2018; 298(1): 59-66.
- Liu LX, Arany Z. Maternal Cardiac Metabolism in pregnancy. *Cardiovascular Research*. 2014, 101 (4): 545-53.
- Loukas M, Groat C, Khangura R, Owens DG, Anderson RH. The normal and abnormal anatomy of the coronary arteries. *Clin Anat*. 2009; 22(1): 114-28.
- Lykke JA, Langhoff-Roos J, Sabai BM, Funai EF, Triche EW, Paidas MJ. Hypertensive Pregnancy Disorders and Subsequent Cardiovascular Morbidity and Type 2 Diabetes Mellitus in the Mother. *Hypertension*. 2009; 53: 944-951
- Mahendru AA, Everett TR, Wilkinson IB, Lees CC, McEniery CM. A longitudinal study of maternal cardiovascular function from preconception to the postpartum period. *J Hypertension*. 2014; 32: 849-856.
- Masoura S, Kalogiannidis I, Makedou K, Theodoridis T, Koiou K, Gerou S, Athanasiadis A, Agorastos T. Biomarkers of endothelial dysfunction in pre-eclampsia and neonatal morbidity: a case-control study. *Eur J Obstet Gynecol Reprod Biol.* 2014; 175: 119-23.
- Matsubare K, Higaki T, Matsubara Y, Nawa A. Nitric Oxide and reactive oxygen species in the pathogenesis of pre-eclampsia. *Int J Mol Sci* 2015; 16(3): 4600-14.
- Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985; 28(7): 412-9.
- May WY. Physiological Changes in pregnancy (Chaper 5). In Arulkumaran S, Sivanesaratnam V, Chatterjee A, Kumar P. *Essentials of Obstetrics (1st edition).* Jaypee Brothers. 2004; 41-46.
- Maynard SE, Min JY, Merchan J, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in pre-eclampsia. *J Clin Invest.* 2003; 111: 649–658

- McClung JA, Nasser N, Saleem M, Rossi GP, Weiss MB, Abraham NG, Kappas A. Circulating endothelial cells are elevated in patients with type II diabetes mellitus independent of HbA_{1C}. *Diabetologia*. 2005; 48: 345-350.
- McVeigh GE, Cohn JN. Endothelial dysfunction and the metabolic syndrome. *Curr Diab Rep*. 2003; 3: 87-92.
- Mol BWJ, Roberts CT, Thangaratinam S, *et al*. Pre-eclampsia. *The Lancet*. 2016; 387(10022): 999-1011.
- Montagnani M, Quon MJ. Insulin action in vascular endothelium: potential mechanisms linking insulin resistance with hypertension. *Diabetes, Obesity & Metabolism*. 2000; 2(5): 285-292.
- Moore LE, Pereira N. Physiological Changes in pregnancy (Chapter 10). In Van de Velde M, Scholefield H, Plante LA. *Maternal Critical Care: A Multidisciplinary Approach* (1st Edition). Cambridge University Press. 2013; 107-119.
- Mourad JJ, des Guetz G, Debbabi H, Levy BI. Blood pressure rise following angiogenesis inhibition by bevacizumab. A crucial role for the microcirculation. *Ann Onco.* 2008; 19(5): 927-934.
- Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood*. 1999; 93: 2951-2958.
- Mutter WP, Karumanchi SA. Molecular mechanisms of pre-eclampsia. *Microvas Res.* 2008; 75: 1–8.
- Myatt L. Role of placenta in pre-eclampsia. *Endocrine* 2002; 19(1): 103-11.
- Myers J, Mires G, Macleod M, Baker P. In pre-eclampsia, the circulating factors capable of altering in vitro endothelial function precede clinical disease. *Hypertension.* 2005; 45: 258-263.
- Nama V, Antonius TF, Onwude J, Manyonda IT. Mid-trimester blood pressure drops in normal pregnancy: Myth or reality? *J Hypertens*. 2011; 29: 763-768.
- Nama V, Manyonda IT, Onwude J, Antonious TF. Structural capillary rarefaction and the onset of pre-eclampsia. *Obstet Gynecol* 2012; 119 (5): 967-974.
- Napoli C, Paolisso G, Casamassimi A, Al-Omran M, Barbieri M, Sommese L, Infante T, Ignarro LJ. Effects of nitric oxide on cell proliferation: novel insights. *J Am Coll Cardiol.* 2013; 62(2): 89-95.

- Nevo O, Soustiel JF, Thaler I. Maternal cerebral blood Flow during normal pregnancy. *Am J Obstet Gynecol.* 2010; 203(5): 475, e1-6.
- Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ. Prospective study of placental angiogenic factors and maternal vascular function before and after pre-eclampsia and gestational hypertension. *Circulation.* 2010; 122(5): 478-487.
- Nyberg M, Mortensen SP, Thaning P, Saltin B, Hellsten Y. Interstitial and plasma Adenosine stimulates Nitric Oxide and Prostacyclin formation in human skeletal muscle. *Hypertension.* 2010; 56(6): 1102-1108
- Obici S, Feng Z, Karkanias G, Baskin DG, Rossetti L. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nature Neuroscience*. 2002; 5: 566-572.
- Ormazabal V, Nair S, Elfeky O, *et al.* Association between insulin resistance and the development of cardiovascular disease. *Cardiovasc Diabetol.* 2018; 17(1): 122.
- Ospina-Tascón GA, Nieto Calvache AJ, Quinones E, *et al.* Microcirculatory blood Flow derangements during severe pre-eclampsia and HELLP syndrome. *Pregnancy Hypertens*. 2017; 10: 124-130.
- Ouzounian JG, Elkayam U. Physiologic changes during normal pregnancy and delivery. *Cardiology Clinics*. 2012; 30(3): 317-329.
- Oyama-Kato M, Ohmichi M, Takahashi K*, et al.* Change in pulse wave velocity throughout normal pregnancy and its value in predicting pregnancy-induced hypertension: a longitudinal study. *Am J Obstet Gynecol*. 2006; 195(2): 464-69.
- Ozaki K, Hori T, Ishibashi T, Nishio M, Aizawa Y. Effect of chronic cigarette smoking on Endothelial function in young men. *J Cardio.* 2010; 56(3): 307- 313.
- Ozkor M, Auyyumi AA. Endothelium derived hyperpolarizing factor and vascular function. *Cardiol Res Pract*. 2011; 2011: 156146.
- Palaniappan L, Carnethon MR, Wang Y, *et al*. Predictors of the incident metabolic syndrome in adults: The Insulin Resistance Atherosclerosis study. *Diabetes Care.* 2004; 27(3): 788-793.
- Pani LN, Korenda L, Meigs JB, Driver C, Chamany S, Fox CS, Sullivan L, D'Agostino RB, Nathan DM. Effect of aging on A1C levels in individuals without diabetes: evidence from the Framingham

Offspring Study and the National Health and Nutrition Examination Survey 2001-2004. *Diabetes Care*. 2008; 31(10): 1991-6.

- Papageorghiou AT, Yu CK, Cicero S, Bower S, Nicolaides KH. Second-trimester uterine artery Doppler screening in unselected populations: a review. *J Matern Fetal Neonatal Med.* 2002; 12(2): 78-88.
- Paradisi G, Steinberg HO, Hempfling A, Cronin J, Hook G, Shepard MK, Baron AD. Polycystic ovary syndrome is associated with endothelial dysfunction. *Circulation*. 2001; 103(10): 1410-5.
- Park JB, Charbonneau F, Schiffrin EL. Correlation of endothelial function in large and small arteries in human essential hypertension. *J Hypertens*. 2001; 19(3): 415-20.
- Park JL, Loberg RD, Duquaine D, Zhang H, Deo BK, Ardanaz N, Coyle J, Atkins KB, Schin M, Charron MJ, Kumagai AK, Pagano PJ, Brosius FC 3rd. GLUT4 facilitative glucose transporter specifically and differentially contributes to agonist-induced vascular reactivity in mouse aorta. *Arterioscler Thromb Vasc Biol.* 2005; 25(8): 1596-602.
- Peach CJ, Mignone VW, Arruda MA, *et al.* Molecular pharmacology of VEGF-A isoforms: binding and signalling at VEGFR2. *Int J Mol Sci.* 2018; 19(4): E1264.
- Petrák O, Widimský J Jr, Zelinka T, Kvasnicka J, Strauch B, Holaj R, Stulc T, Kvasnicka T, Bílková J, Skrha J. Biochemical markers of endothelial dysfunction in patients with endocrine and essential hypertension. *Physiol Res*. 2006; 55(6): 597-602
- Pignatelli P, Menichelli D, Pastori D, Violi F. Oxidative stress and cardiovascular disease: new insights. *Kardiol Pol*. 2018; 76(4): 713-722
- Pipp F, Heil M, Issbrucker K, *et al.* VEGFR-1–selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res.* 2003; 92: 378–385.
- Posner BI. Insulin Signalling: The inside story. *Can J Diabetes*. 2017; 41(1): 108-113.
- Powe CE, Levine RJ, Karumanchi SA. Pre-eclampsia: A Disease of the Maternal Endothelium. *Circulation*. 2011; 123: 2856-2869.
- Powers RW, Roberts JM, Plymire DA, *et al*. Low placental growth factor across pregnancy identifies a subset of women with preterm pre-eclampsia: type 1 versus type 2 pre-eclampsia? *Hypertension* 2012; 60: 239–46.

- Pozrikidis C. Numerical simulation of blood flow through microvascular capillary networks. *Bull Math Biol.* 2009; 71(6): 1520-41
- Radziuk J. Insulin sensitivity and its measurement: structural commonalities among the methods. *J Clin Endocrinol Metab*. 2000; 85(12): 4426-33.
- Ranganath M, Quon MJ. Insulin action and insulin resistance in vascular endothelium. *Current Opinion in Clinical Nutrition and Metabolic Care.* 2007; 10(4): 523-530.
- Ray CJ, Abbas MR, Coney AM, Marshall JM. Interactions of adenosine, prostaglandins and nitric oxide in hypoxia-induced vasodilatation: in vivo and in vitro studies. *J Physiol*. 2002; 544: 195-209.
- Reaven GM. Insulin resistance: from bit player to centre stage. *CMAJ.* 2011 Mar 22; 183(5): 536-7.
- Regazzetti C, Peraldi P, Grémeaux T, *et al*. Hypoxia decreases insulin signaling pathways in adipocytes. *Diabetes.* 2009; 58(1): 95-103.
- Reimann M, Schutte AE, Schwarz PE. Insulin resistance- the role of ethnicity: evidence from Caucasian and African cohorts. *Horm Metab Res.* 2007; 39(12): 853-57.
- Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy*. Am J Obstet Gynecol*. 2000; 183(1): S1-S22.
- Richey JM. The vascular endothelium, a benign restrictive barrier? NO! The role of nitric oxide in regulating insulin action. *Diabetes.* 2013; 62(12): 4006-8.
- Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14,719 initially healthy American women. *Circulation.* 2003; 107(3): 391-7.
- Roberts JM, Cooper DW. Pathogenesis and genesis of Pre-eclampsia. *Lancet* 2001; 357: 53-56. (use}
- Roberts JM, Gammill H. Insulin resistance in pre-eclampsia. *Hypertension* 2006; 37: 341–2.
- Roberts JM, Hubel CA. The two-stage model of pre-eclampsia: variation on the theme. *Placenta.* 2009; 30: S32-37.
- Roberts JM, Lain KY. Recent insights into the pathogenesis of pre-eclampsia. *Placenta* 2002; 23: 359-72.
- Robillard PY, Dekker G, Chaouat G*, et al.* Epidemiological studies on primipaternity and

immunology in pre-eclampsia- a statement after twelve years of workshops. *J Reprod Immunol.* 2011; 89(2): 104-117.

- Rosenfeld L. Insulin: discovery and controversy. *Clin Chem*. 2002; 48(12): 2270-88.
- Salamalekis E, Vitoratos N, Makrakis E, Mastorakas G, Eleftheriadis M, Creatsas G. No association between Insulin resistance and pre-eclampsia. *J Matern Fetal Neonatal Med.* 2005; 18(2): 113-5.
- Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature.* 2001; 414: 799-806.
- Sandvik MK1, Leirgul E, Nygård O, Ueland PM, Berg A, Svarstad E, Vikse BE. Pre-eclampsia in healthy women and endothelial dysfunction 10 years later. *Am J Obstet Gynecol.* 2013; 209(6): 569.e1-569.e10.
- Sanghavi M, Rutherford JD. Cardiovascular physiology of pregnancy. *Circulation.* 2014; 130: 1003-1008.
- Sarafidis PA, Bakris GL. Insulin and endothelin: an interplay contributing to hypertension development? *J Clin Endocrinol Metab*. 2007; 92(2): 379-385.
- Sarafidis PA, Lasaridis AN, Nilsson PM, Pikilidou MI, Stafilas PC, Kanaki A, Kazakos K, Yovos J, Bakris GL. Validity and reproducibility of HOMA-IR, 1/HOMA-IR, QUICKI and McAuley's indices in patients with hypertension and type II diabetes. *J Hum Hypertens.* 2007; 21(9): 709-16.
- Sarelius IH. Coupling of muscle metabolism and muscle blood flow in capillary units during contraction. *Acta. Physiol. Scand*. 2000; 168(4), 531-541.
- Savvidou M, Noori M, Anderson J, Hingorani A, Nicolaides K. Maternal endothelial function and serum concentrations of placental growth factor and soluble endoglin in women with abnormal placentation. *Ultrasound in Obstetrics & Gynecology.* 2008; 32: 871-876.
- Scioscia M, Gumaa K, Kunjara S, Paine MA, Selvaggi LE, Rodeck CH, Rademacher TW. Insulin resistance in human pre-eclamptic placenta is mediated by serine phosphorylation of insulin receptor substrate-1 and -2. *J Clin Endocrinol Metab*. 2006; 91(2): 709- 717.
- Scioscia M, Gumaa K, Rademacher TW. The link between insulin resistance and pre-eclampsia: new perspective. *J Reprod Immunol* 2009; 82: 100–5.

- Secomb TW. Theoretical models for regulation of blood flow. *Microcirculation.* 2008; 15(8): 765-75.
- Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem.* 2013; 153(1): 13-19.
- Shoemaker JK, Badrov MB, Al-Khazraji BK, *et al*. Neural control of vascular function in skeletal muscle. *Compr Physiol.* 2015; 6(1): 303-29.
- Siauve N, Chalouhi GE, Deloison B, Alison M, Clement O, Ville Y, Salomon LJ. Functional imaging of the human placenta with magnetic resonance. *Am J Obstet Gynecol.* 2015; 213(4 Suppl): S103-14.
- Sibai BM, Caritis S, Hauth J. What we learned about pre-eclampsia? *Semin Perinatol.* 2003; 27: 239- 246.
- Simonsen U, Aalkjaer C. Small artery structure and function: a dual interaction with many players. *Basic Clin Pharmacol Toxicol.* 2012; 110(1): 2-4.
- Sinha S, Sinha GP, Gupta K, Kumar S, Gupta A. Effect of pre-eclampsia on Insulin sensitivity. *Int J Appl Basic Med Res*. 2014; 4(1): 7-10.
- Skjærven R, Vatten LJ, Wilcox AJ, *et al.* Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population-based cohort. *Br Med J.* 2005; 331: 877.
- Skrha J, Haas T, Sindelka G, Prázný M, Widimský J, Cibula D, Svacina S. Comparison of the insulin action parameters from hyperinsulinemic clamps with homeostasis model assessment and QUICKI indexes in subjects with different endocrine disorders. *J Clin Endocrinol Metab.* 2004; 89(1): 135-41.
- Snijders T, Nederveen JP, Verdijk LB, *et al.* Muscle fibre capillarisation as determining factor on indices of insulin sensitivity in humans. *Physiol Rep*. 2017; 5(10): e13278
- Sohlberg S, Mulic-Lutvica A, Lindgren P, Ortiz-Nieto F, Wikström AK, Wikström J. Placental perfusion in normal pregnancy and early and late pre-eclampsia: a magnetic resonance imaging study. *Placenta.* 2014; 35(3): 202-6.
- Spaan JJ, Houben AJHM, Musella A, Ekhart T, Spaanderman MEA, Peeters LLH. Insulin resistance relates to microvascular reactivity 23years after pre-eclampsia. *Microvascular*

Research. 2010; 80 (3): 417-421.

- Steeghs N, Gelderblom H, Roodt JO, *et al.* Hypertension and rarefaction during treatment with telatinib, a small molecule angiogenesis inhibitor. *Clin Cancer Res.* 2008; 14(11): 3470-3476.
- Stefanovic´ M, Vukomanovic´ P, Milosavljevic´ M, *et al* . Insulin resistance and C-reactive protein in pre-eclampsia. *Bosnian J Basic Med Sc* 2009; 9: 235–8.
- Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. *Diabetologia* 2000; 45: 623-34.
- St-Pierre P, Genders AJ, Keske MA, Richards SM, Rattigan S. Loss of insulin-mediated microvascular perfusion in skeletal muscle is associated with the development of insulin resistance. *Diabetes Obes Metab* 2010; 12:798–805.
- Strijbos MH, Snijder CA, Kraan J, Lamers CH ,Gratama JW, Duvekot JJ. Levels of circulating endothelial cells in normotensive and severe pre-eclamptic pregnancies. *Cytometry B Clin Cytom.* 2010; 78(6): 382-3866.
- Szarka A, Rigó J Jr, Lázár L, Beko G, Molvarec A. Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and pre-eclampsia determined by multiplex suspension array. *BMC Immunol.* 2010;11: 59.
- Tanentsapf I, Heitmann BL, Adegboye AR. Systematic review of clinical trials on dietary interventions to prevent excessive weight gain during pregnancy among normal weight, overweight and obese women. *BMC Pregnancy Childbirth.* 2011; 26: 11-81.
- Taylor RN, Roberts JM. Endothelial cell dysfunction. In: *Chelsey's Hypertensive Disorders of Pregnancy,* edited by Lindheimer M, Roberts JM, and Cunningham FG. Stanford, CT: Appleton & Lange, 2007, p. 395–429.
- Thadhani R, Ecker JL, Mutter WP, Wolf M, Smirnakis KV, Sukhatme VP, Levine RJ, Karumanchi SA. Insulin resistance and alterations in angiogenesis: additive insults that may lead to pre-eclampsia. *Hypertension*. 2004; 43: 988–992.
- Thomas GD. Neural control of the circulation. *Adv Physiol Edu*. 2011; 35(1): 28-32.
- Thong FSL, Dugani CB, Klip A. Turning signals on and off: GLUT4 traffic in the insulin-signalling highway. *Physiology (Bethesda).* 2005; 20: 271-84

- Tsai AG, Friesenecker B, Cabrales P, *et al*. The vascular wall as a regulator of tissue oxygenation. *Current Opinion in Nephrology and Hypertension*. 2006; 15(1): 67-71.
- Tsai AG, Johnson PC, Intaglietta M. Oxygen gradients in the microcirculation. *Physiol Rev*. 2003; 83(3): 933-963.
- Tsioufis C, Dimitriadis K, Katsiki N, Tousoulis D. Microcirculation in Hypertension: An Update on Clinical Significance and Therapy. *Curr Vasc Pharmacol.* 2015; 13(3): 413-7.
- Tuzcu ZB, Asicioglu E, Sunbul M, Ozben B, Arikan H, Koc M. Circulating endothelial cell number and markers of endothelial dysfunction in previously pre-eclamptic women. *Am J Obstet Gynecol.* 2015; 213(4): 533. E1-7.
- Tykochi R, Boerman EM, Jackson WF. Smooth muscle ion channels and regulation of vascular tone in resistance arteries and arterioles*. Compr Physiol*. 2017; 7(2): 485-581.
- van Veer TR, Panerai RB, Haeri S, *et al.* Cerebral autoregulation in different hypertensive disorders of pregnancy. *Am J Obstet Gynecol.* 2015; 212(4): 513. e1-7.
- Vanhoutte PM. Regenerated Endothelium and Its Senescent Response to Aggregating Platelets. *Circ J.* 2016; 80(4): 783-90
- VanWijk MJ, Kublickiene K, Boer K, VanBavel E. Vascular Function in pre-eclampsia. *Cardiovasc Res* 2000; 47: 38-48.
- Venkatesha S, Toporsian M, Lam C, *et al*. Soluble endoglin contributes to the pathogenesis of pre-eclampsia*. Nat Med.* 2006; 12: 642– 649.
- Verlohren S, Herraiz I, Lapaire O, *et a* . The sFlt-1/PlGF ratio in different types of hypertensive pregnancy disorders and its prognostic potential in pre-eclamptic patients. *Am J Obstet Gynecol* 2012a; 206: 58. e1-8.
- Verlohren S, Stepan H, Dechend R. Angiogenic growth factors in the diagnosis and prediction of pre-eclampsia. *Clin Sci* 2012b; 122(2): 43-52.
- Villa PM, Laivouri H, Kajantie E, Kaaja R. Free fatty acid profiles in pre-eclampsia. *Prostaglandins Leukot Essent Fatty Acids.* 2009; 81(1): 17-21.
- Vincent D, Ilany J, Kondo T, *et al.* The role of endothelial insulin signalling in the regulation of vascular tone and insulin resistance. *J Clin Invest*. 2003; 111(9): 1373-80.

- Vincent MA, Clerk LH, Lindler JR, Kilbanov AL, Clark MG. Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* 2004; 53: 1418–1423.
- Virtanen KA, Lönnroth P, Parkkola R, Peltoniemi P, Asola M, Viljanen T, Tolvanen T, Knuuti J, Rönnemaa T, Huupponen R, Nuutila P. Glucose uptake and perfusion in subcutaneous and visceral adipose tissue during insulin stimulation in nonobese and obese humans. *The Journal of Clinical Endocrinology & Metabolism*. 2002; 87(8): 3902-10.
- Visintin C, Mugglestone MA, Almerie MQ, Nherera LM, James D, Walkinshaw S, Guideline Development Group. Management of hypertensive disorders during pregnancy: summary of NICE guidance. *BMJ.* 2010; 341: c2207.
- von Versen-Hoeynck FM, Powers RW. Maternal-fetal metabolism in normal pregnancy and pre-eclampsia*. Frontiers in Bioscience*. 2007; 12(2457-2470): 1093-4715.
- Wagatsuma A. Effect of aging on expression of angiogenesis-related factors in mouse skeletal muscle. *Exp Gerontol.* 2006; 41:49-54.
- Walford G, Loscalzo J. "Nitric oxide in vascular biology". *Journal of Thrombosis and Haemostasis*. 2003; 1(10): 2112–8.
- Wallace TM, Matthews DR. The assessment of insulin resistance in man. *Diabet Med*. 2002; 19(7): 527-34.
- Wallis MG, Wheatley CM, Rattigan S, Barrett EJ, Clark AD, Clark MG. Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. *Diabetes*. 2002; 51(12): 3492-8.
- Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K, Tokuda K. Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. *Clinical chemistry.* 1986; 32(8): 1551-4.
- Watson RT, Pessin JE. Intercellular organisation of Insulin signalling and GLUT4 translocation. *Recent Prog Horm Res.* 2001; 56: 175-93.
- Wautier MP, Chappey O, corda S, Stein DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE, links oxidative stress to altered gene expression via RAGE. *Am J Physiol.* 2001; 280; E685-E694.

- Williams PJ, Pipkin FB. The genetics of pre-eclampsia and other hypertensive disorders of pregnancy. *Best Practice & Research Clinical Obstetrics & Gynaecology*. 2011; 25(4): 405-17.
- Wolf M, Hubel CA, Lam C, Sampson M, Ecker JL, Ness RB, Rajakumar A, Daftary A, Shakir ASM, Seely EW, Roberts JM, Sukhatme VP, Karumanchi SA, Thadani R. Pre-eclampsia and future cardiovascular disease: Potential role of altered angiogenesis and Insulin resistance. *J Clin Endocrin Met* 2004; 89(12): 6239-43.
- Woywodt A, Blann AD, Kirsch T, Erdbruegger U, Banzet N, Haubitz M, Dignat-George F. Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. *J Thromb Haemost.* 2006; 4(3): 671-7.
- Yamamoto T, Suzuki Y, Kojima K, Suzumori K. Reduced flow-mediated vasodilation is not due to decrease in production of nitric oxide in pre-eclampsia. *Am J Obstet Gynaecol* 2005; 192(2): 558-63.
- Yannoutsos A, Levy BI, Safar ME, *et al.* Pathophysiology of hypertension: interactions between macro and microvascular alterations through endothelial dysfunction. *J Hypertens.* 2014; 32(2): 216-24.
- Yu Q, Gao F, Ma XL. Insulin says NO to cardiovascular disease. *Cardiovascular research.* 2011; 89(3): 516-524.
- Zeng G, Nystrom FH, Ravichandran LV, Cong L, Kirby M, Mostowski H, Quon MJ. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signalling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation.* 2000; 101: 1539-1545.

Contribution to existing body of knowledge

Publication

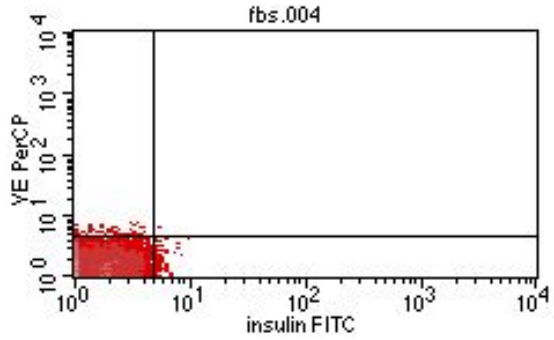
- Anim-Nyame N, Ghosh A, Freestone N, Arrigoni FI. Relationship between insulin resistance and circulating endothelial cells in pre-eclampsia. *Gynecol Endocrinol.* 2015; 31(10): 788-91.
- Ghosh A, Freestone N, et al . Microvascular function in pre-eclampsia is influenced by insulin resistance and an imbalance of angiogenic mediators. *Physio Reports*. 2017 (Accepted for publication)

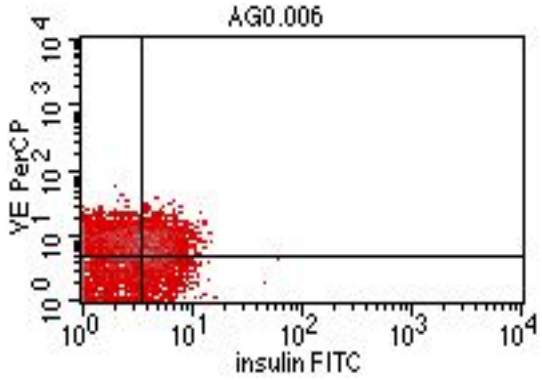
Presentation to learned society

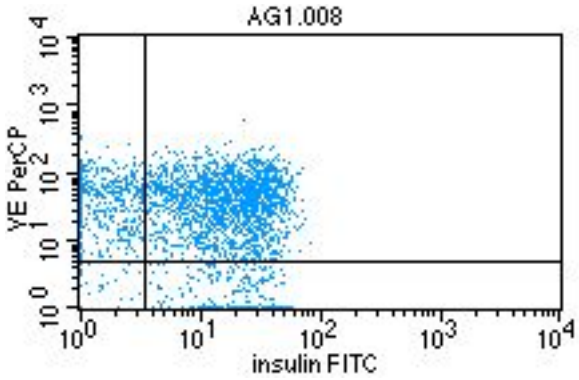
- **The World Congress of Microcirculation:-** A Ghosh, Anim-Nyame N, et al 'Relationship between microvascular blood flow and angiogenic factors in pre-eclampsia.' Oral and poster presentation at Kyoto, Japan in September 2015.
- **European ISSHP**: A Ghosh et al . 'Relationship of Pre-eclampsia, Microcirculation and CEC':- poster presentation at London, U.K. in 2009.

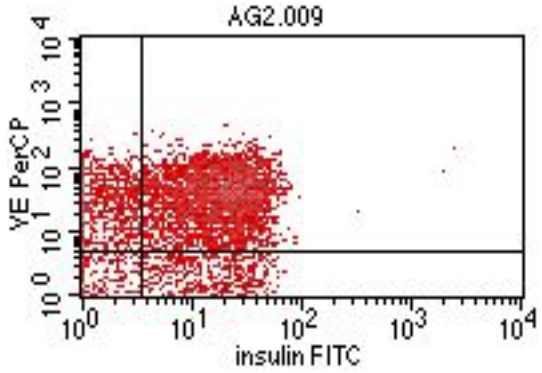
Appendix 1.

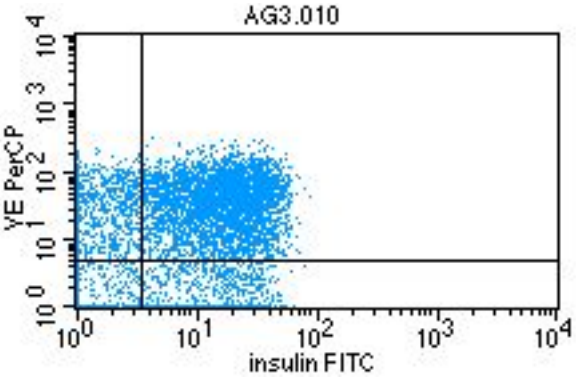
A. Flow-cytometry pictures of insulin receptors

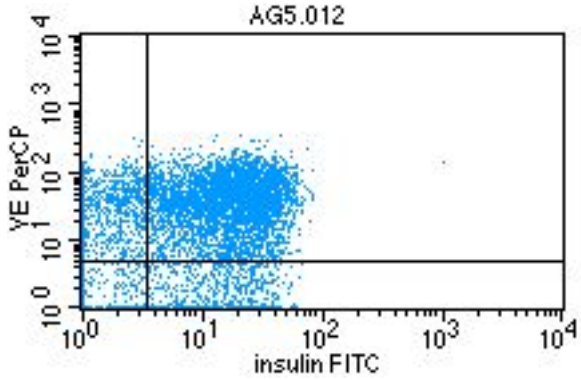


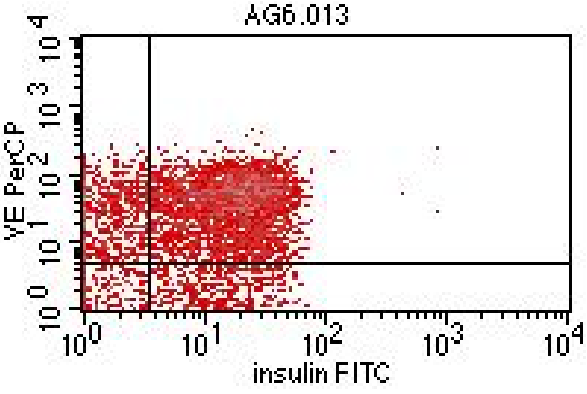


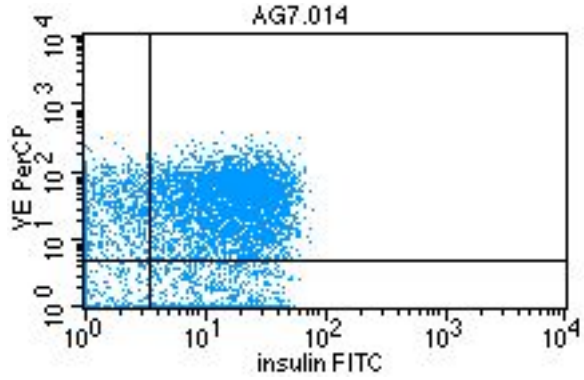


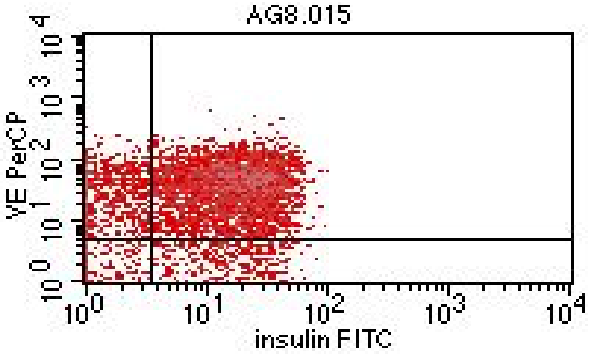


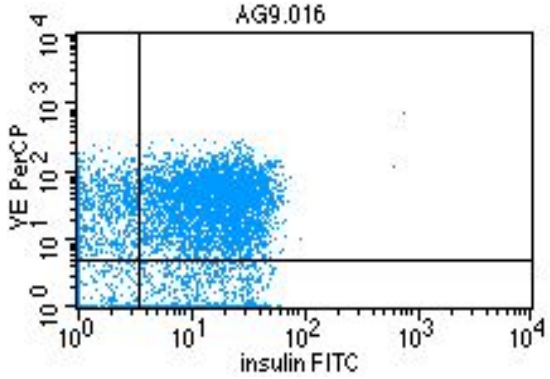


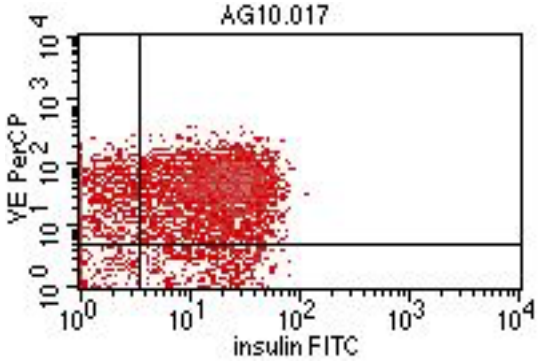


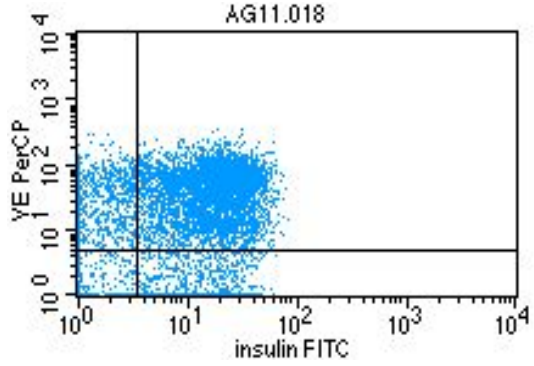


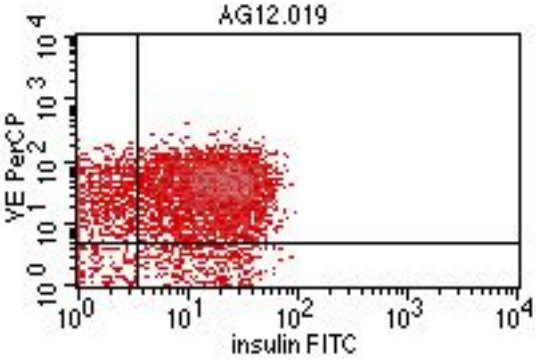

AG7.014
YE PerCP
insulin FITC

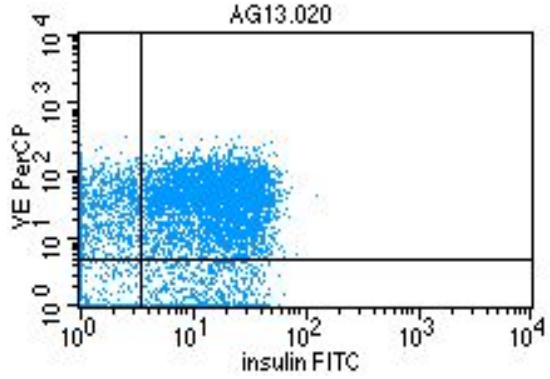

AG8.015
YE PerCP
insulin FITC

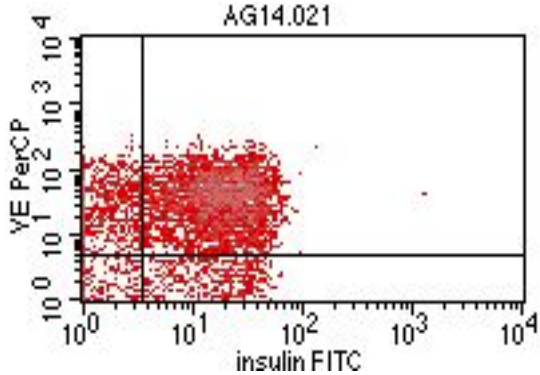

AG9.016
YE PerCP
insulin FITC

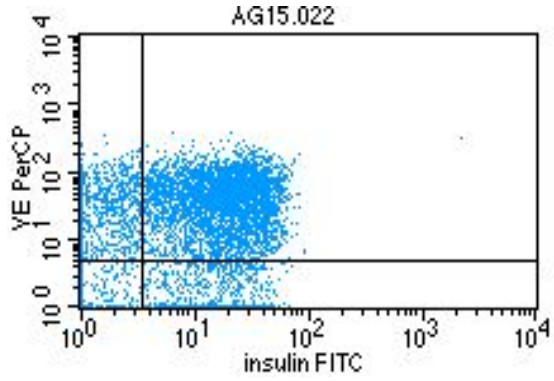

AG10.017
YE PerCP
insulin FITC

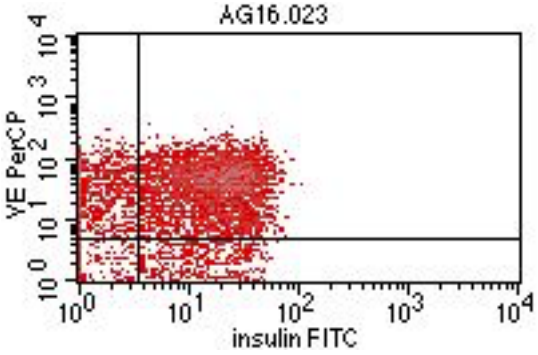

AG11.018
YE PerCP
insulin FITC

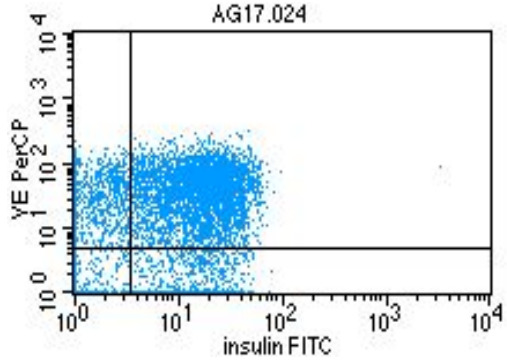

AG12.019
YE PerCP
insulin FITC

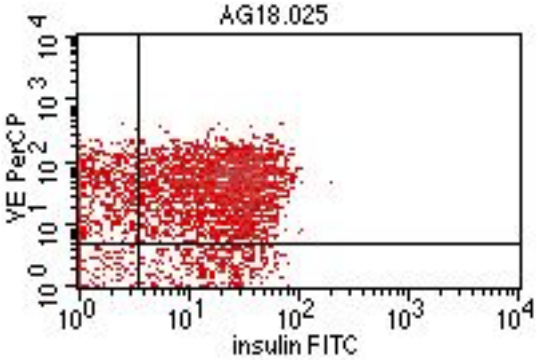

AG13.020
YE PerCP
insulin FITC

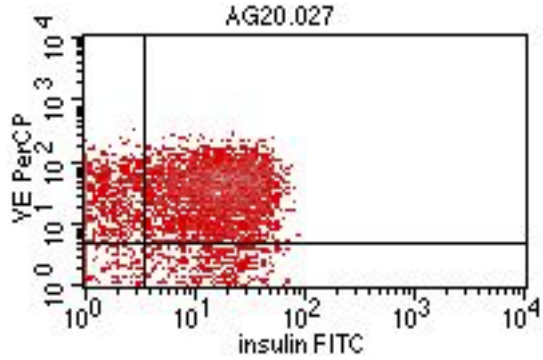

AG14.021
YE PerCP
insulin FITC

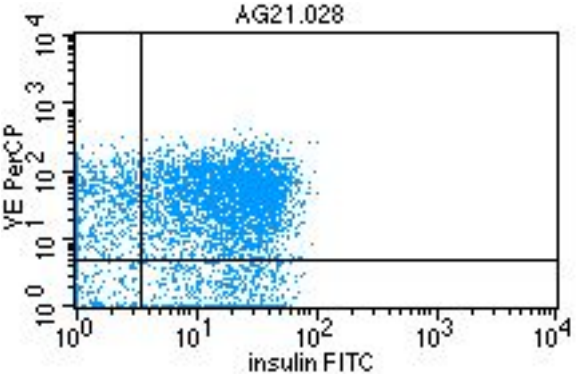

AG15.022
YE PerCP
insulin FITC

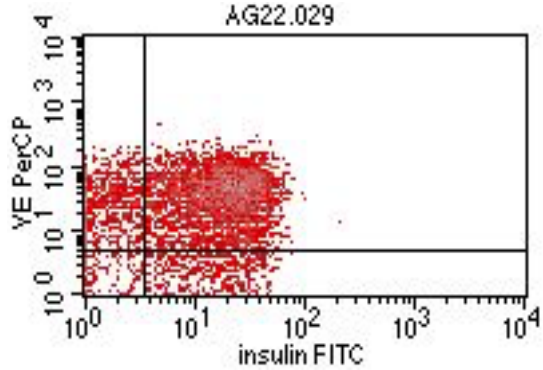

AG16.023
YE PerCP
insulin FITC

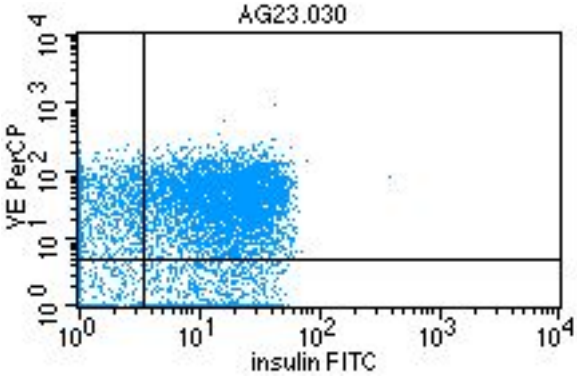

AG17.024
YE PerCP
insulin FITC

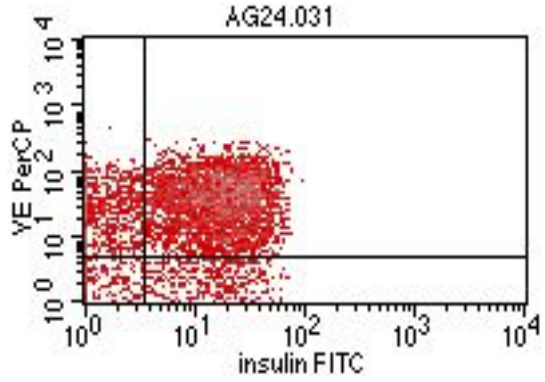

AG18.025
YE PerCP
insulin FITC

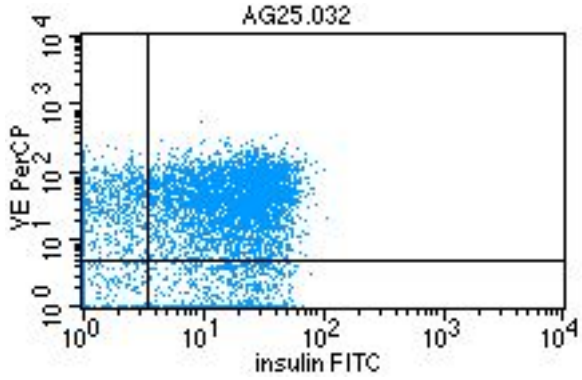

AG20.027
YE PerCP
insulin FITC

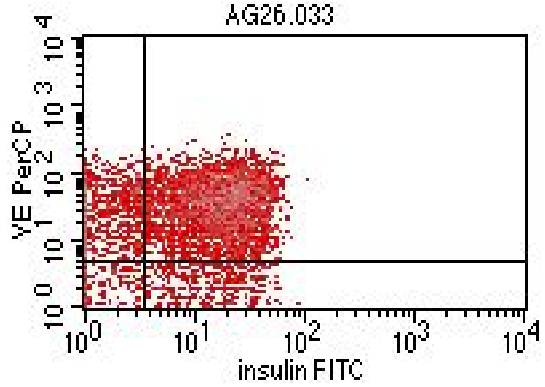

AG21.028
YE PerCP
insulin FITC

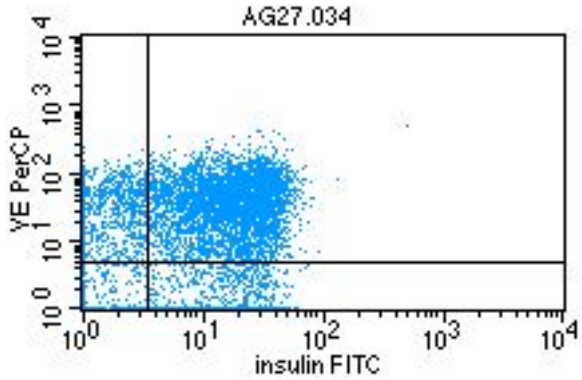

AG22.029
YE PerCP
insulin FITC

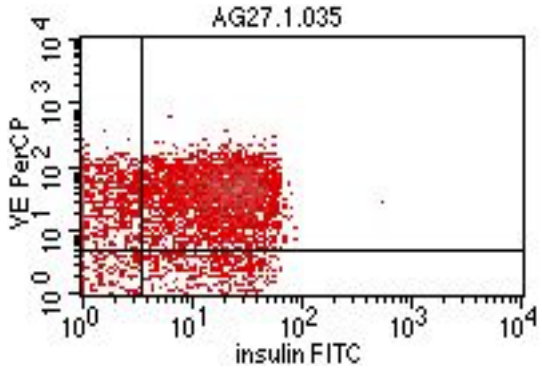

AG23.030
YE PerCP
insulin FITC

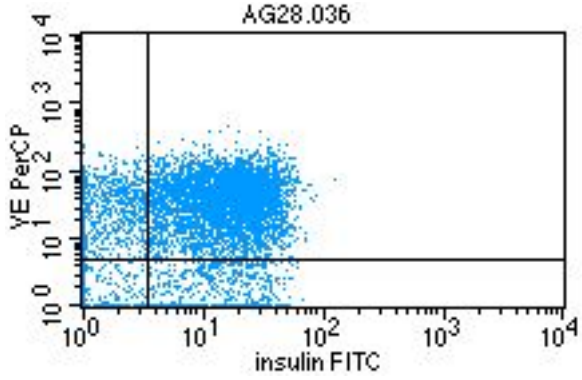

AG24.031
YE PerCP
insulin FITC

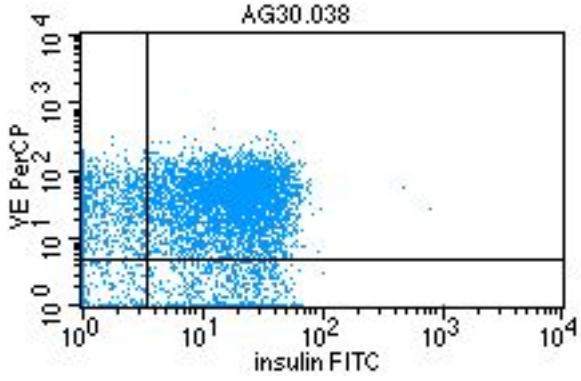

AG25.032
YE PerCP
insulin FITC

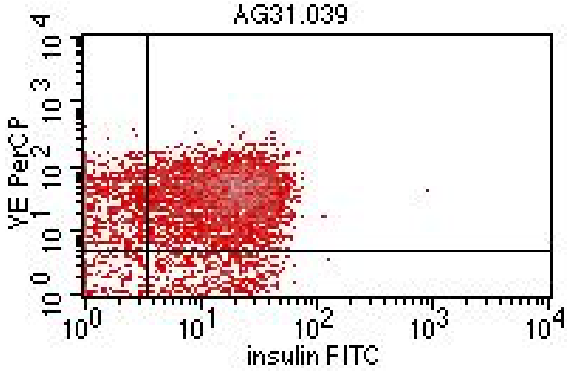

AG26.033
YE PerCP
insulin FITC

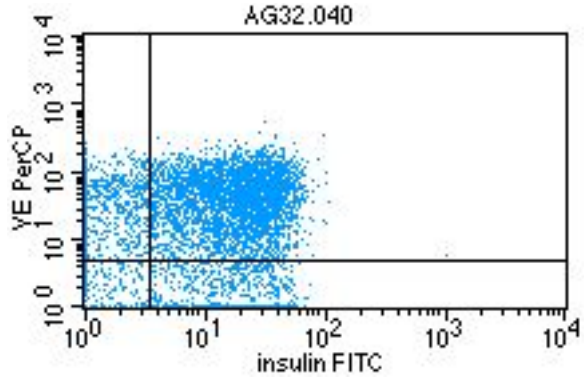

AG27.034
YE PerCP
insulin FITC

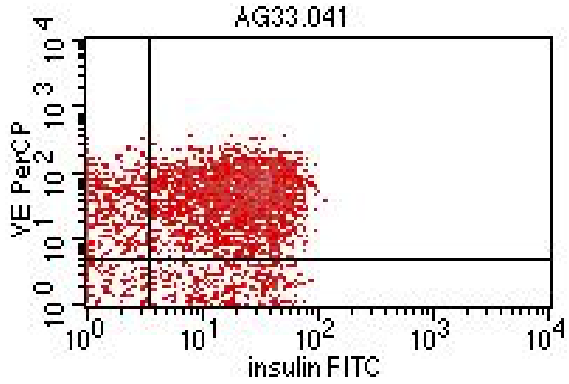

AG27.1.035
YE PerCP
insulin FITC

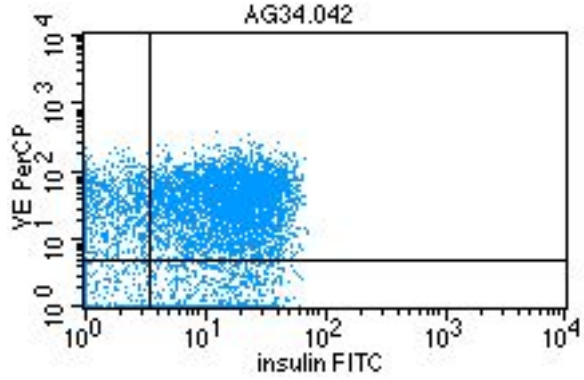

AG28.036
YE PerCP
insulin FITC

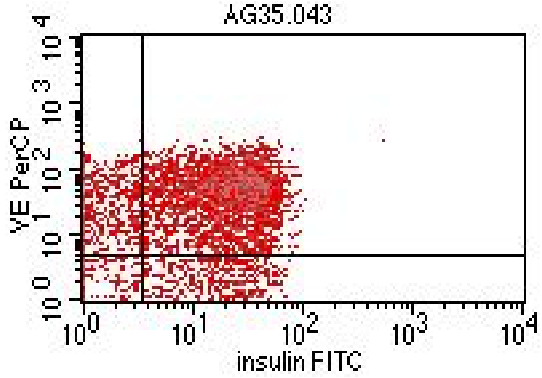

AG30.038
YE PerCP
insulin FITC


AG31.039
YE PerCP
insulin FITC

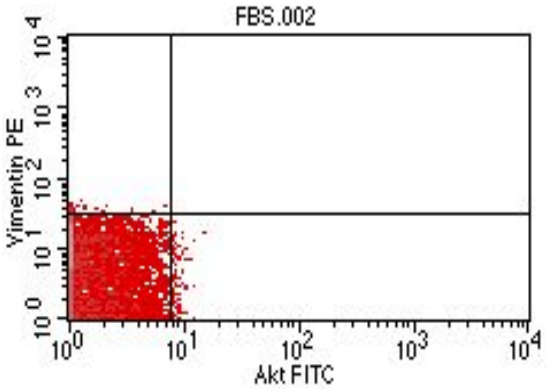

AG32.040
YE PerCP
insulin FITC

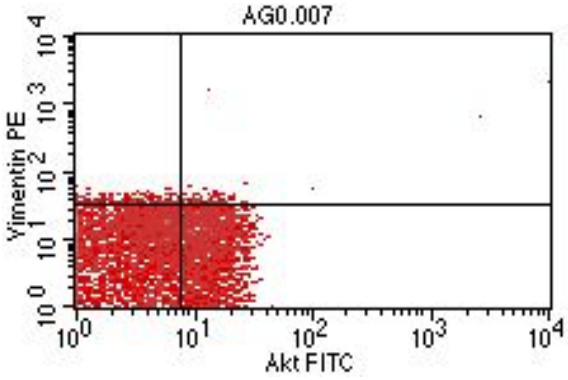

AG33.041
YE PerCP
insulin FITC

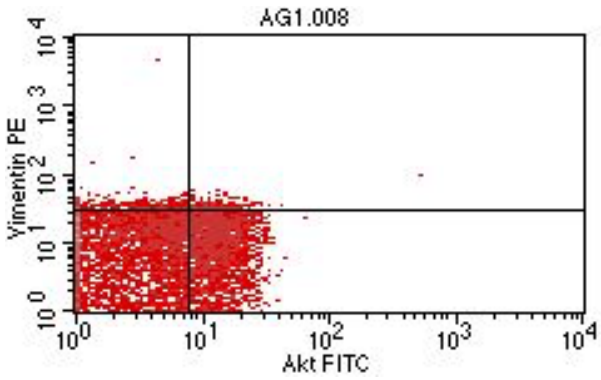

AG34.042
YE PerCP
insulin FITC

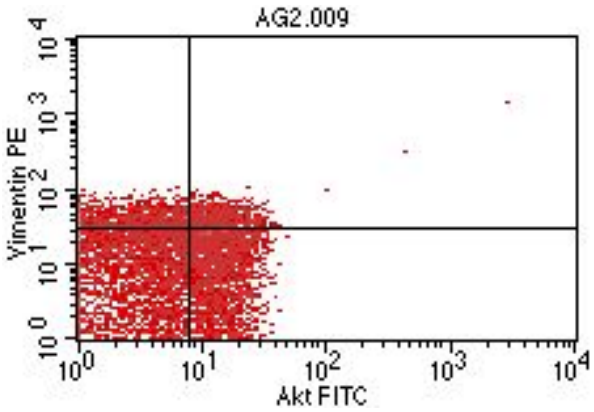

AG35.043
YE PerCP
insulin FITC

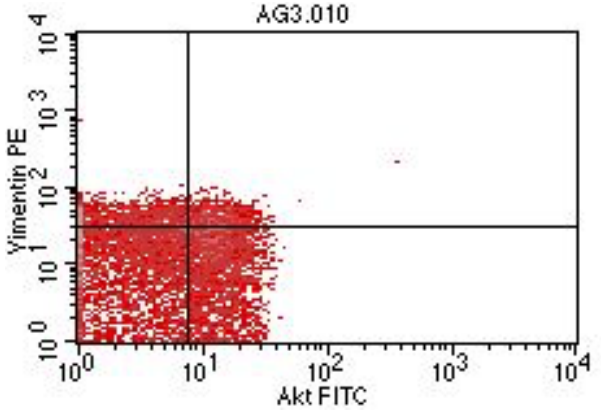
B. Flow-cytometry pictures of Akt receptors

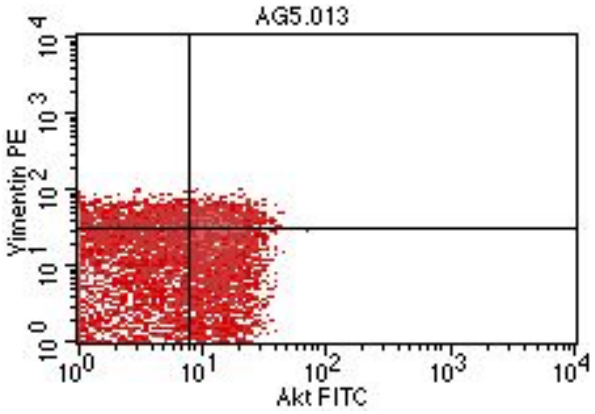


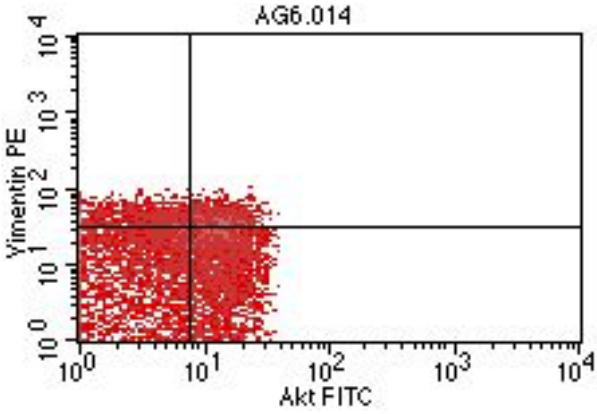


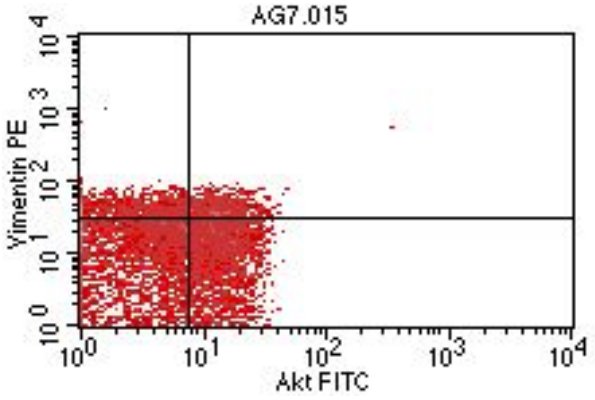


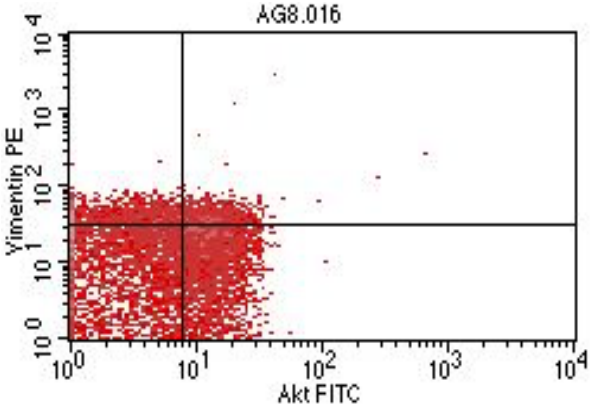


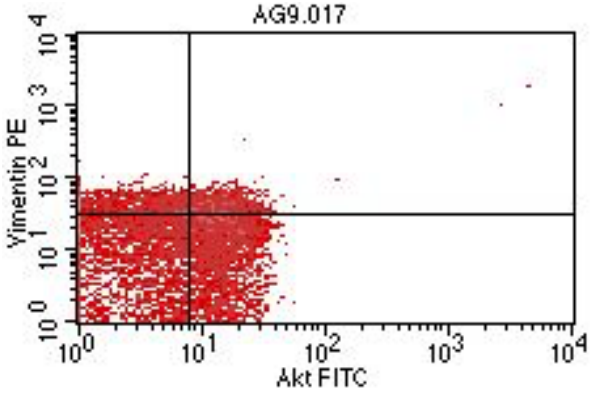


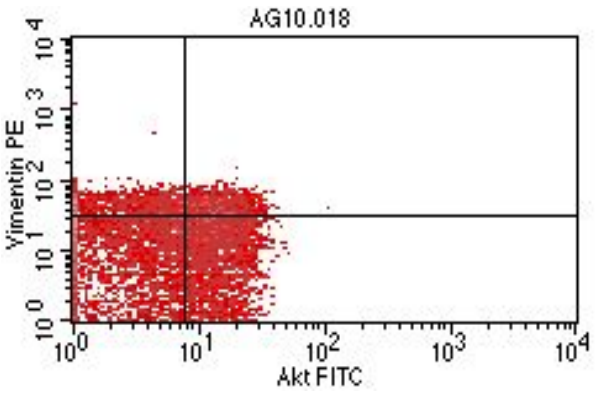

AG5.013
Yimentin PE
Akt FITC

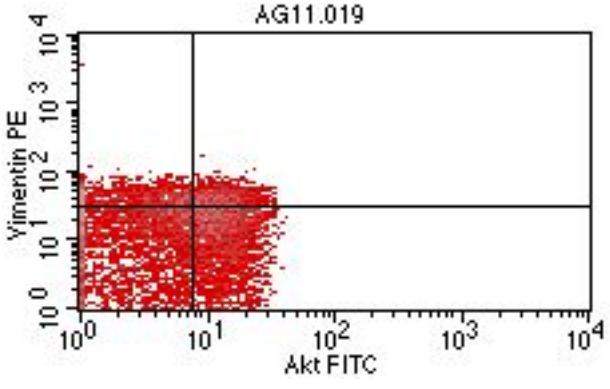

AG6.014
Yimentin PE
Akt FITC

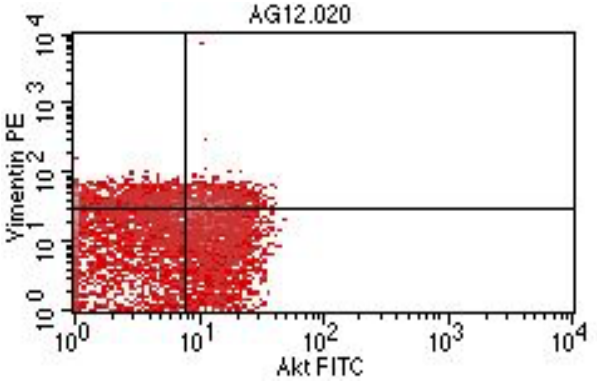

AG7.015
Yimentin PE
Akt FITC

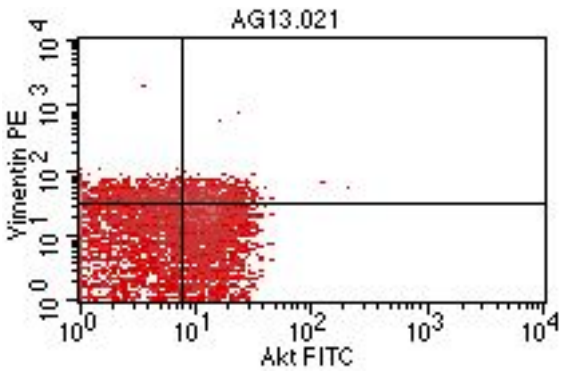

AG8.016
Yimentin PE
Akt FITC

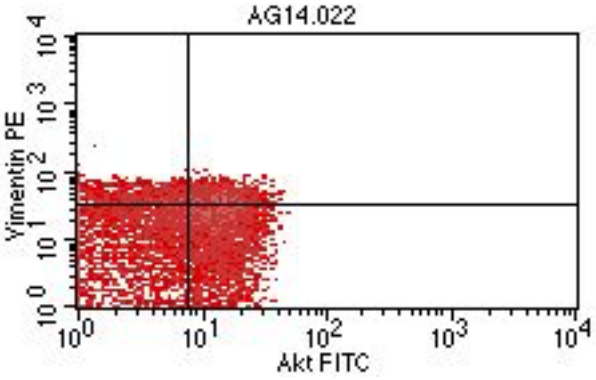

AG9.017
Yimentin PE
Akt FITC

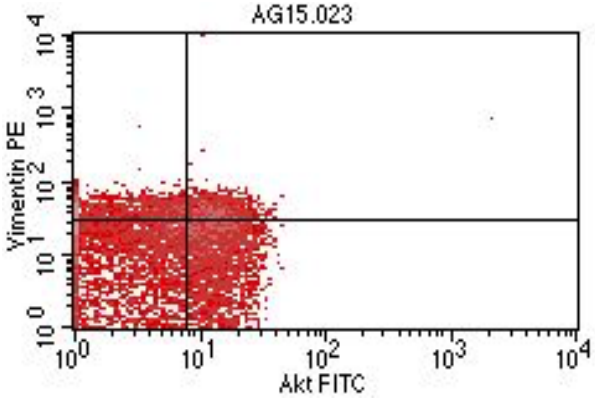

AG10.018
Yimentin PE
Akt FITC

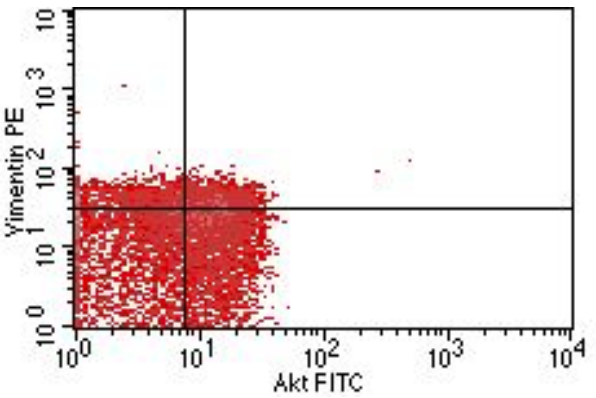

AG11.019
Yimentin PE
Akt FITC

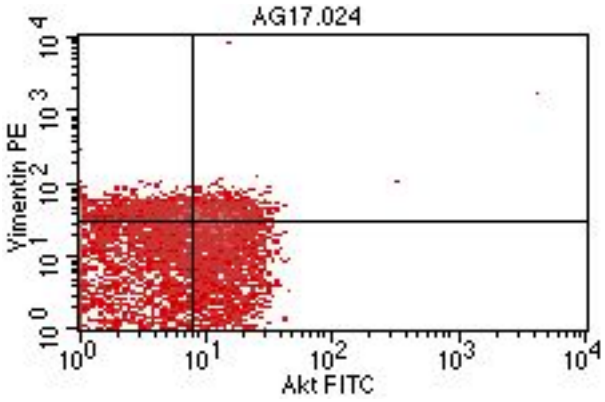

AG12.020
Yimentin PE
Akt FITC

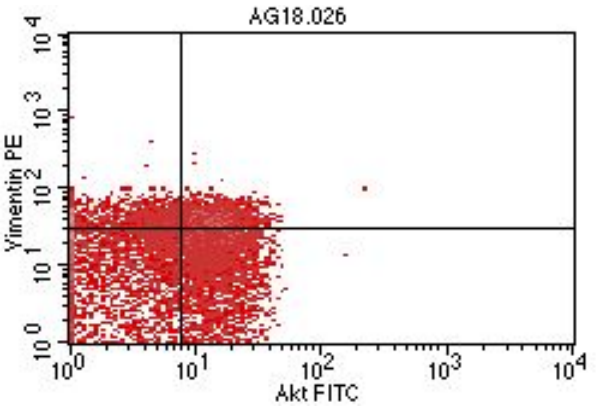

AG13.021
Vimentin PE
Akt FITC

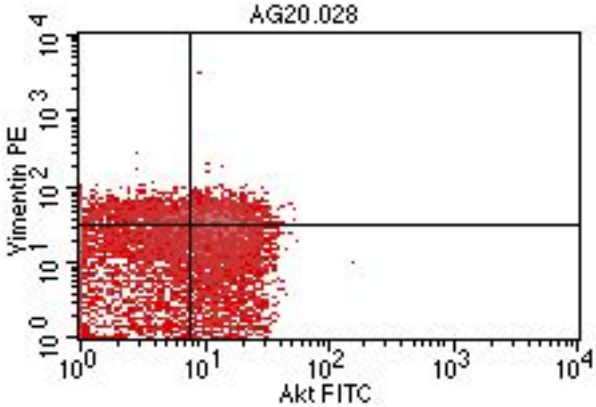

AG14.022
Vimentin PE
Akt FITC

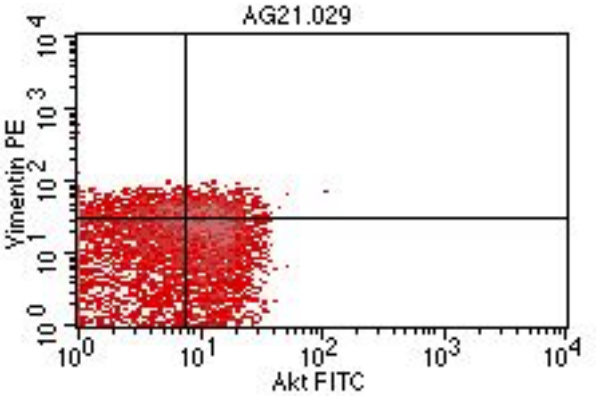

AG15.023
Vimentin PE
Akt FITC

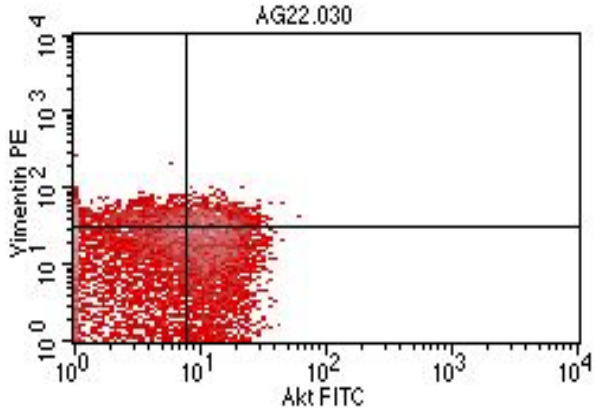

Vimentin PE
Akt FITC

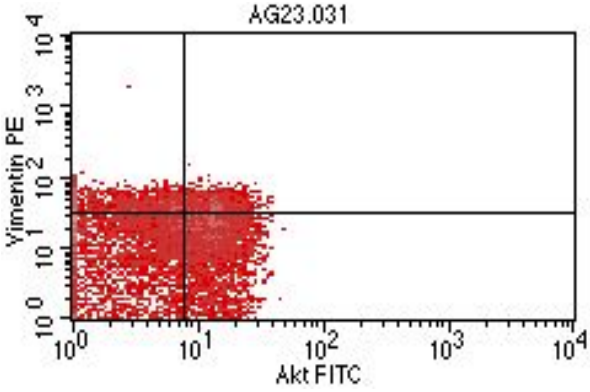

AG17.024
Vimentin PE
Akt FITC

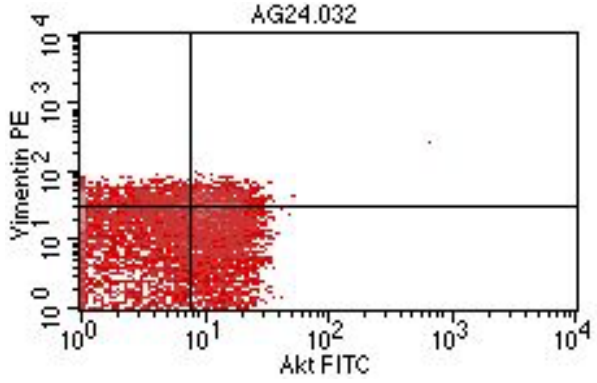

AG18.026
Vimentin PE
Akt FITC

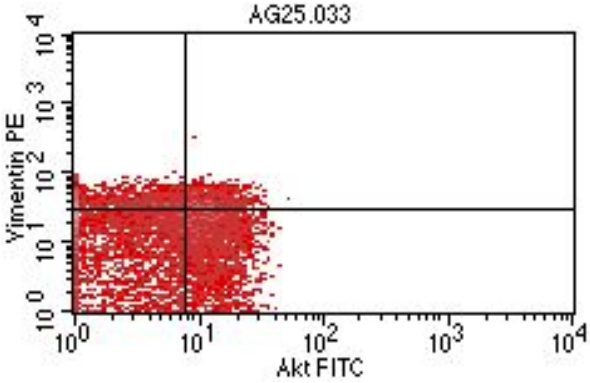

AG20.028
Vimentin PE
Akt FITC

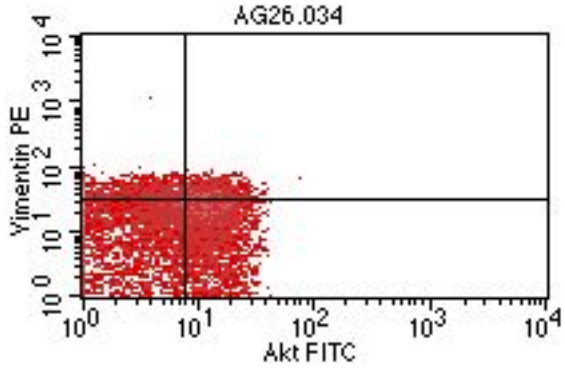

AG21.029
Vimentin PE
Akt FITC

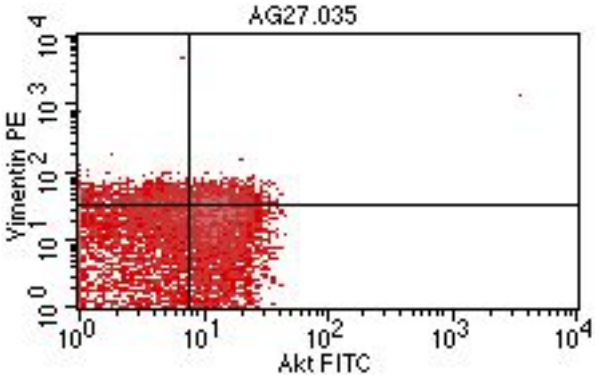

AG22.030
Vimentin PE
Akt FITC

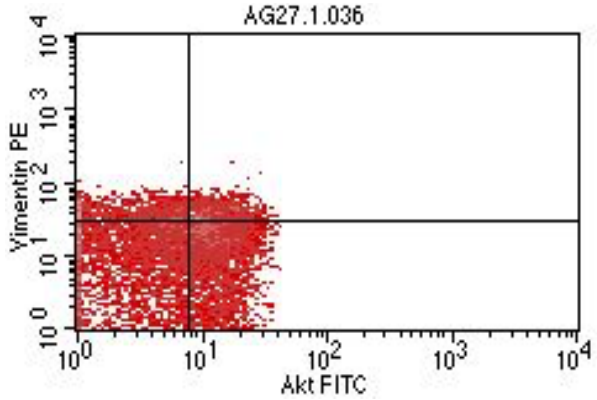

AG23.031
Vimentin PE
Akt FITC

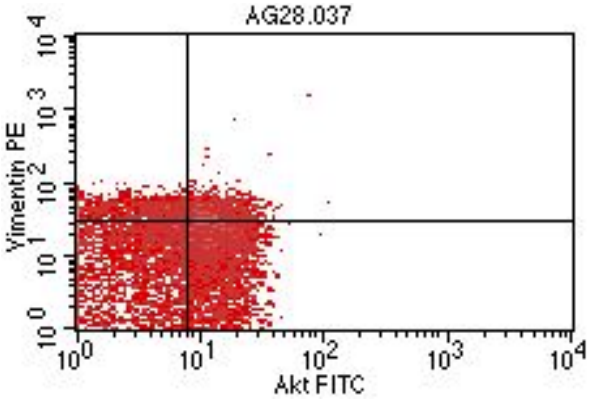

AG24.032
Vimentin PE
Akt FITC

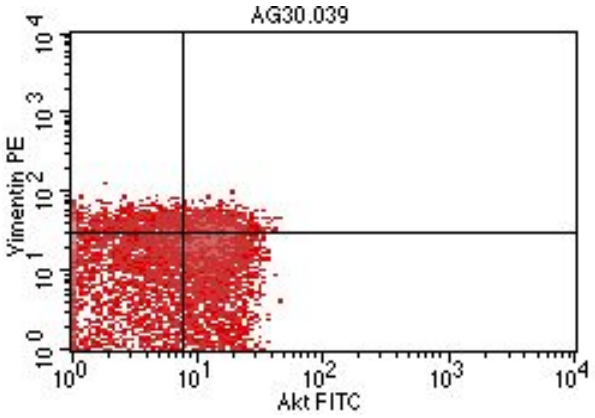

AG25.033
Vimentin PE
Akt FITC

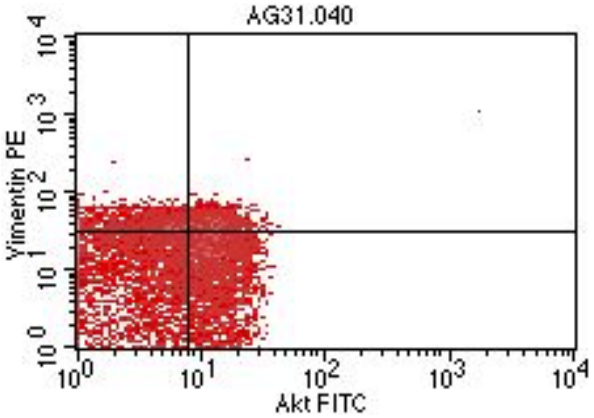

AG26.034
Vimentin PE
Akt FITC

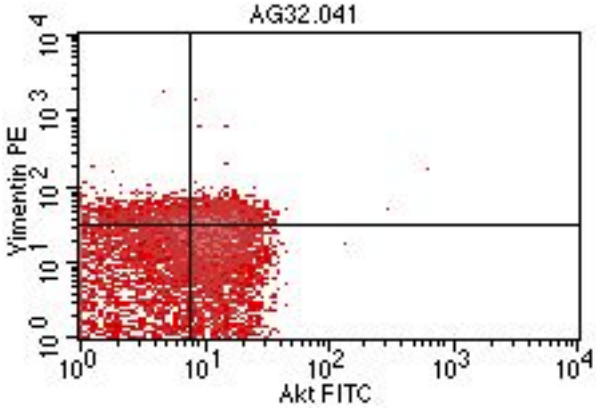

AG27.035
Vimentin PE
Akt FITC

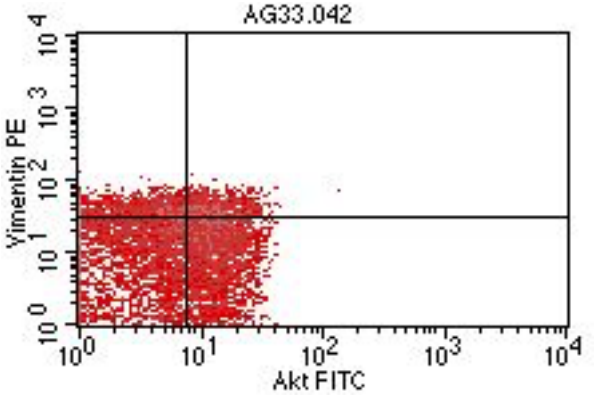

AG27.1.036
Vimentin PE
Akt FITC

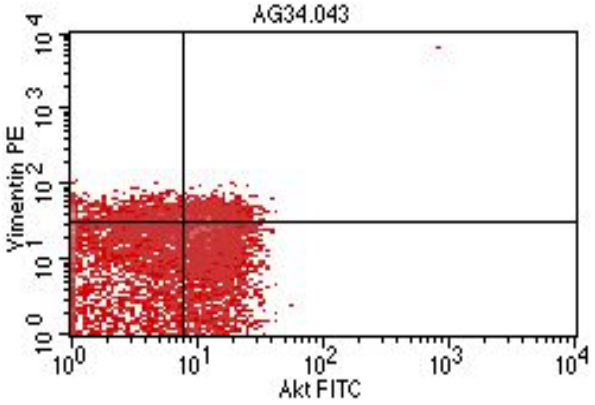

AG28.037
Vimentin PE
Akt FITC

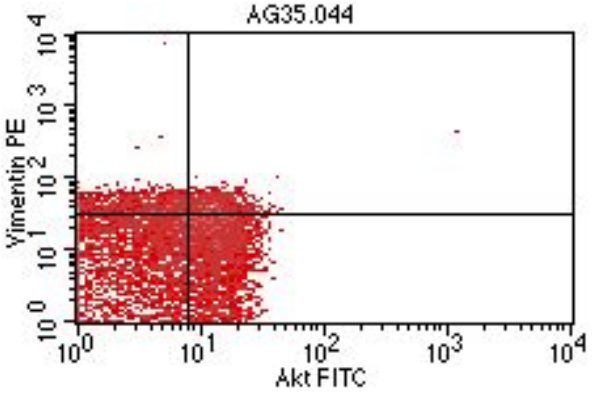

AG30.039
Yimentin PE
Akt FITC


AG31.040
Yimentin PE
Akt FITC

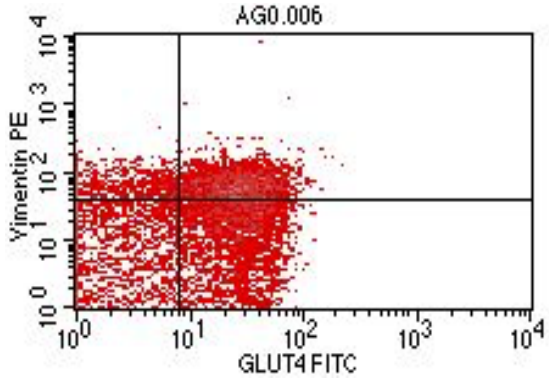

AG32.041
Yimentin PE
Akt FITC

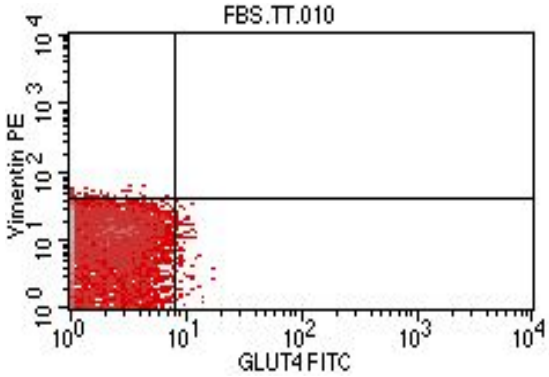

AG33.042
Yimentin PE
Akt FITC

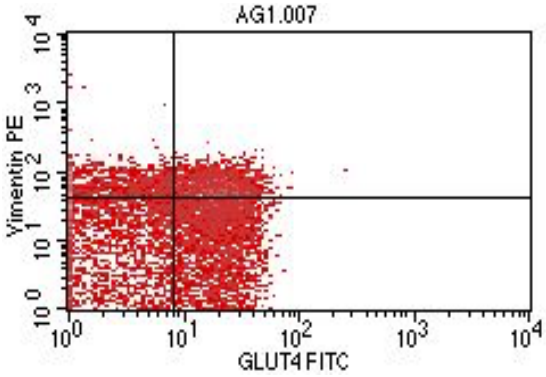

AG34.043
Yimentin PE
Akt FITC

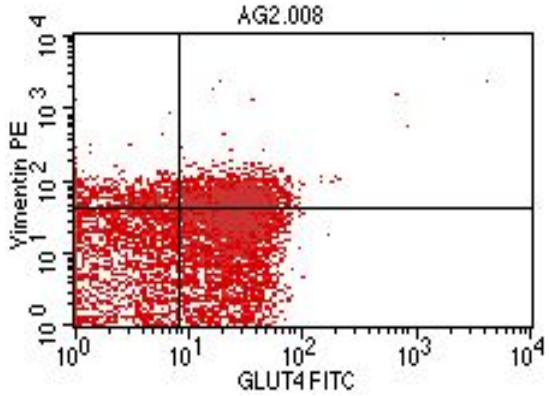

AG35.044
Yimentin PE
Akt FITC

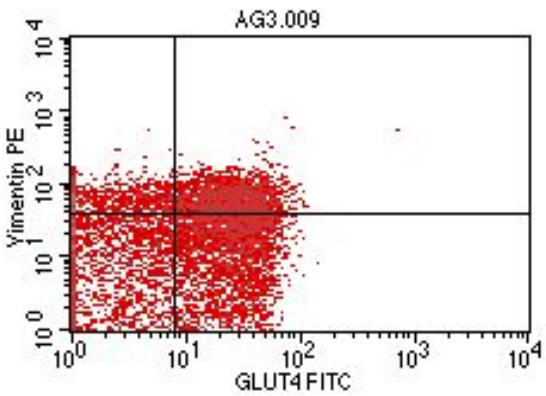
C. Flow-cytometry pictures of GLUT4 receptors

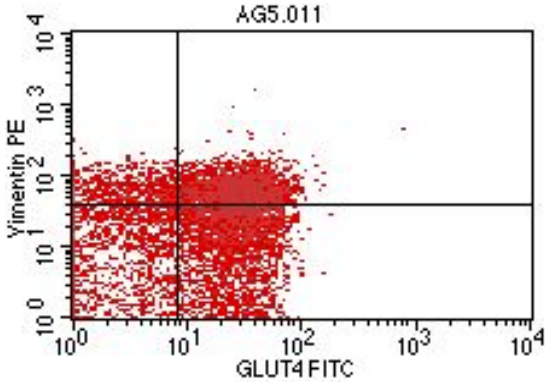


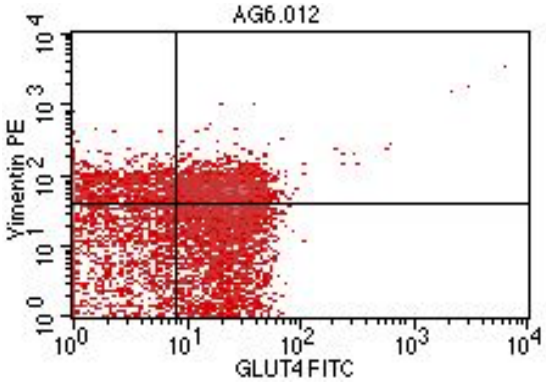


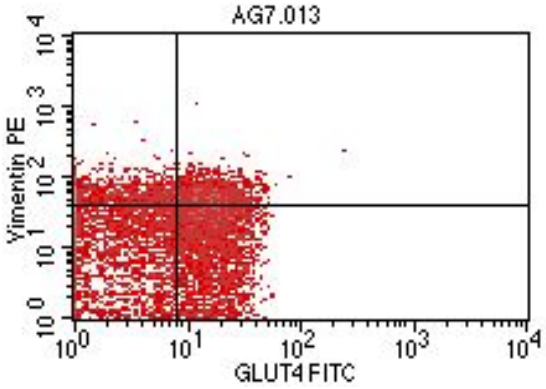


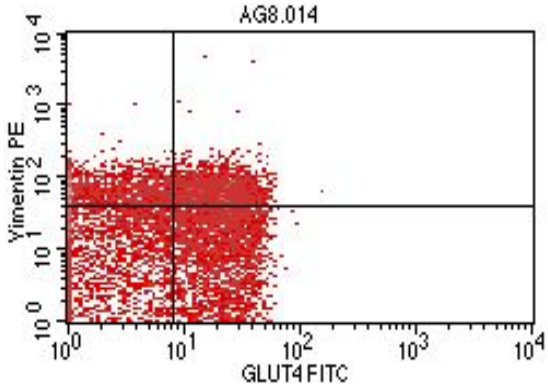


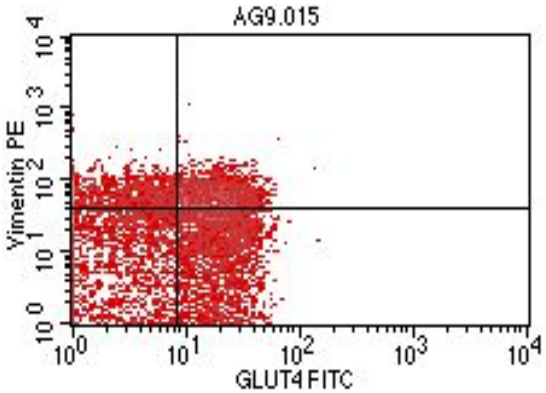


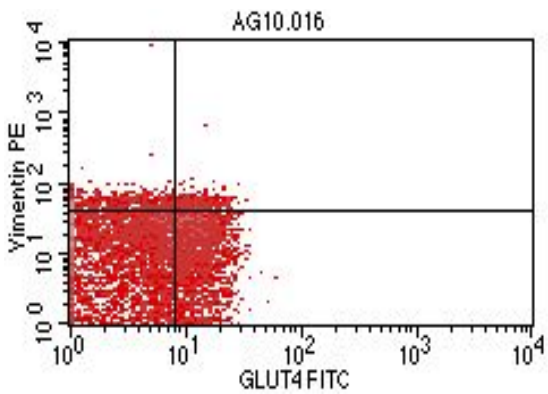


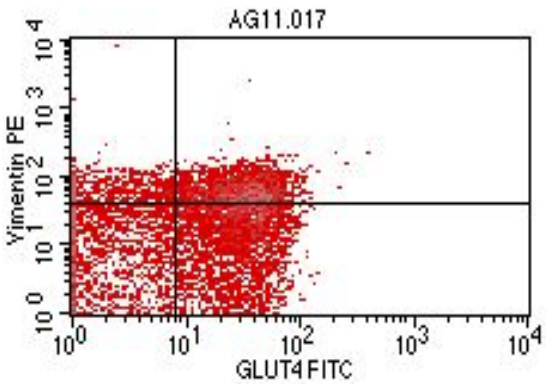


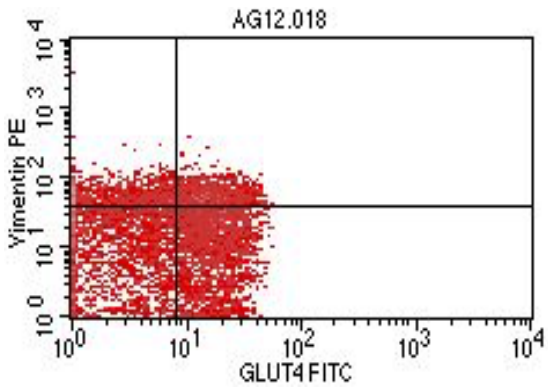

AG7.013
Vimentin PE
GLUT4 FITC

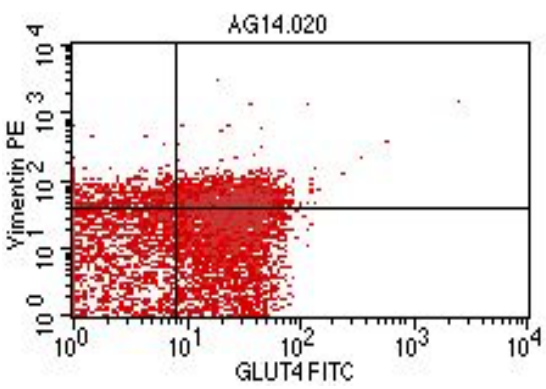

AG8.014
Vimentin PE
GLUT4 FITC

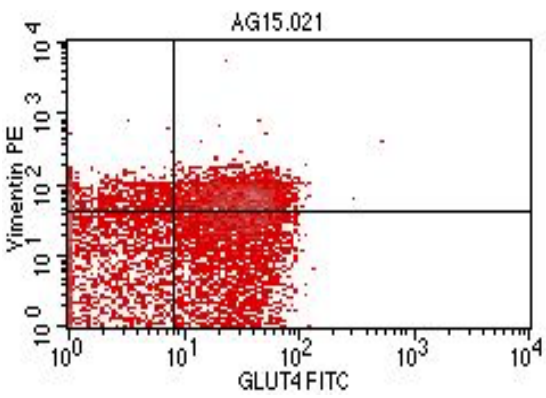

AG9.015
Vimentin PE
GLUT4 FITC

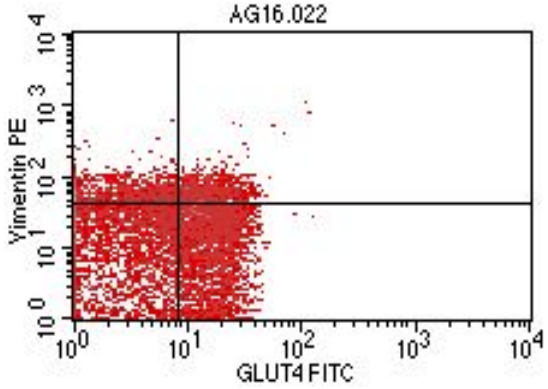

AG10.016
Vimentin PE
GLUT4 FITC

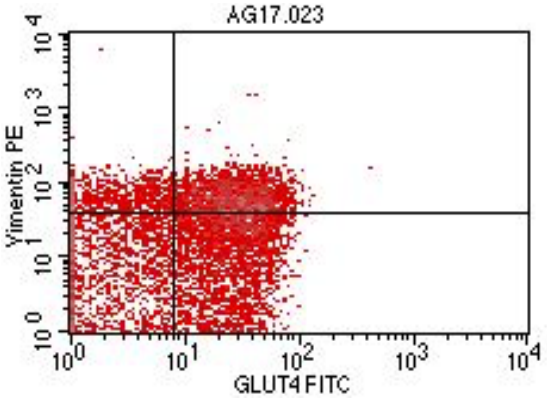

AG11.017
Vimentin PE
GLUT4 FITC

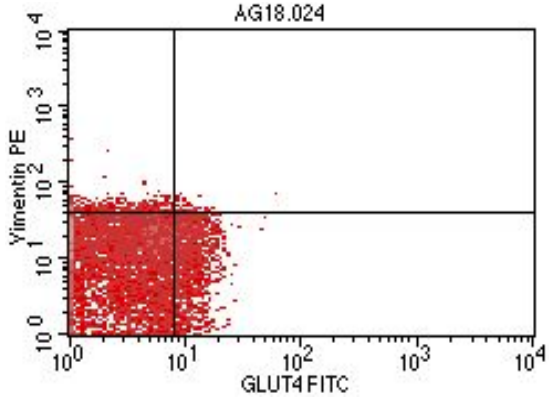

AG12.018
Vimentin PE
GLUT4 FITC

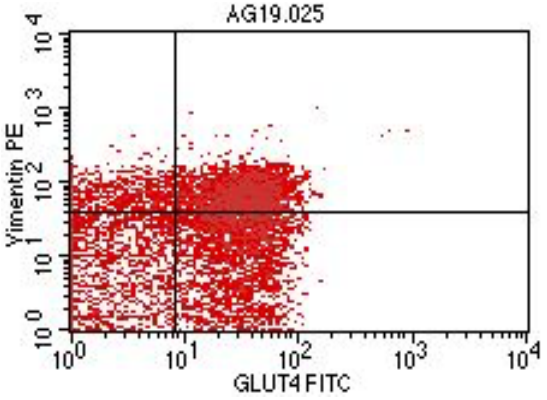

AG14.020
Vimentin PE
GLUT4 FITC

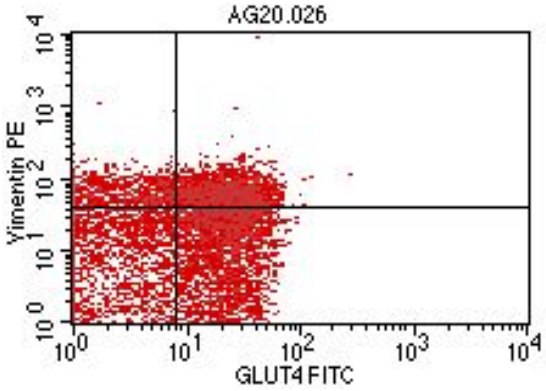

AG15.021
Vimentin PE
GLUT4 FITC

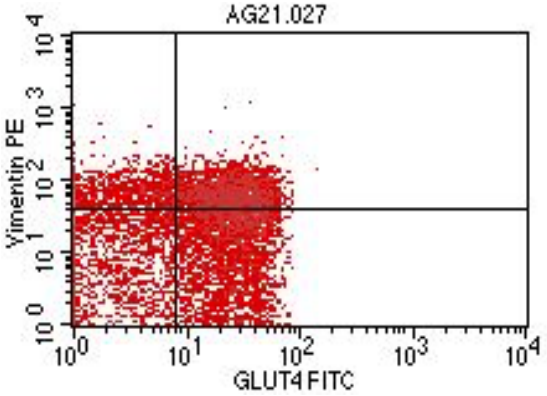

AG16.022
Vimentin PE
GLUT4 FITC

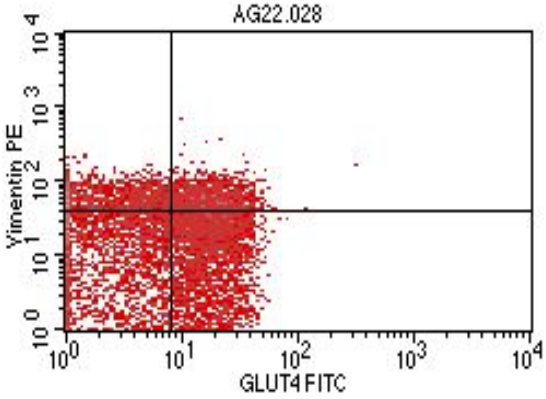

AG17.023
Vimentin PE
GLUT4 FITC

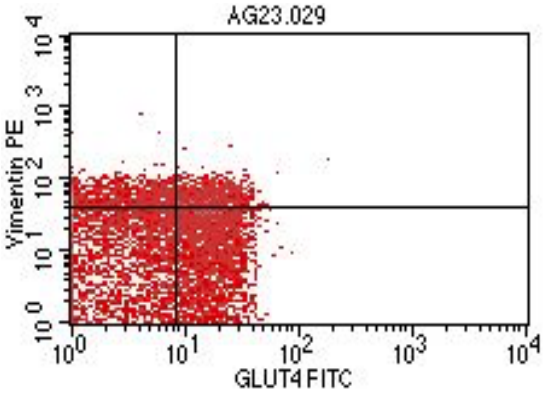

AG18.024
Vimentin PE
GLUT4 FITC

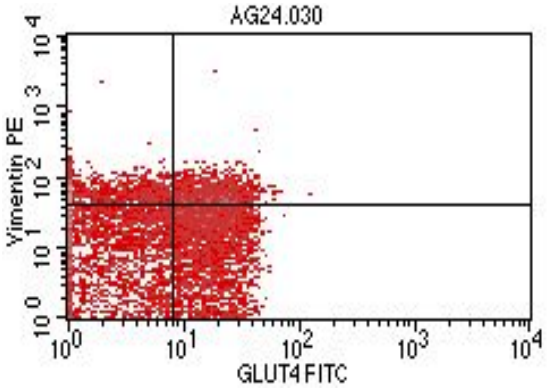

AG19.025
Vimentin PE
GLUT4 FITC

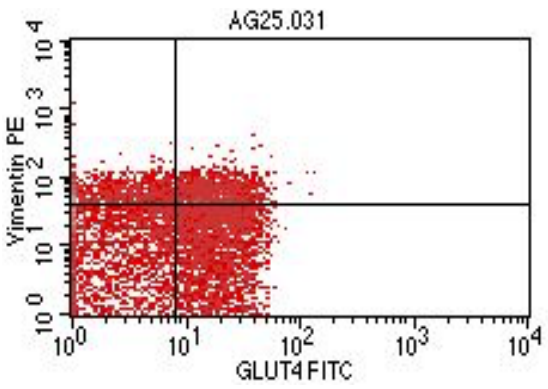

AG20.026
Vimentin PE
GLUT4 FITC

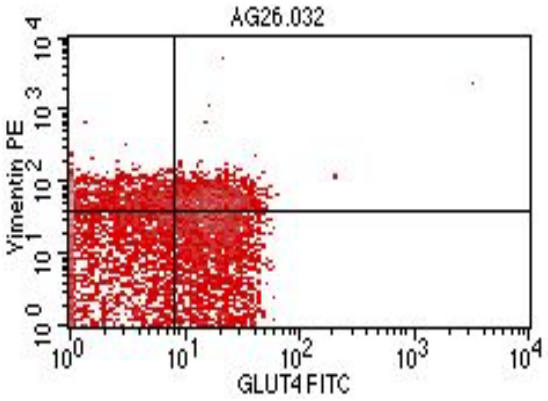

AG21.027
Vimentin PE
GLUT4 FITC

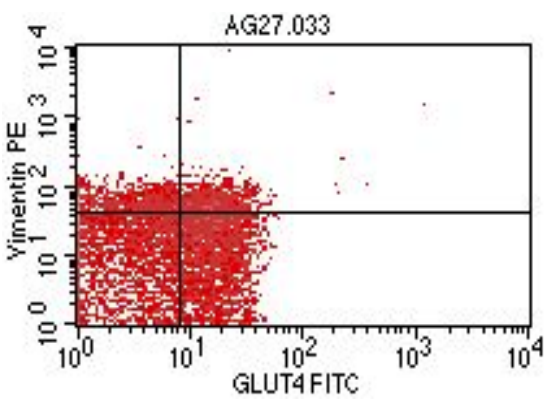

AG22.028
Vimentin PE
GLUT4 FITC

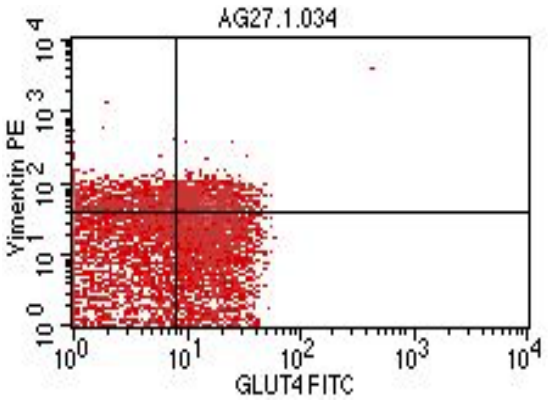

AG23.029
Vimentin PE
GLUT4 FITC

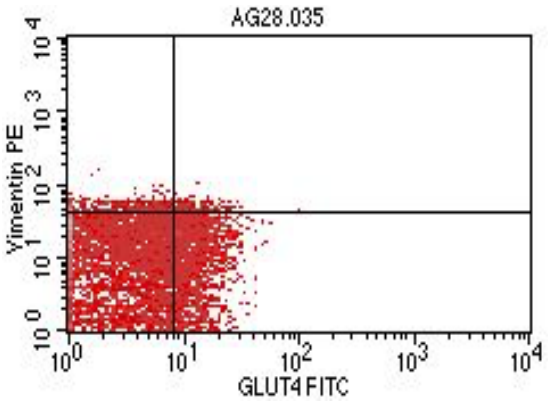

AG24.030
Vimentin PE
GLUT4 FITC

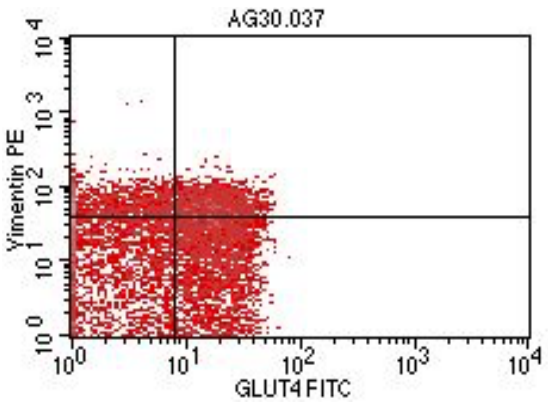

AG25.031
Vimentin PE
GLUT4 FITC

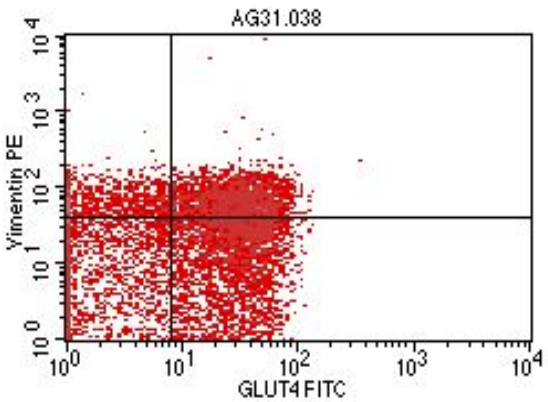

AG26.032
Vimentin PE
GLUT4 FITC

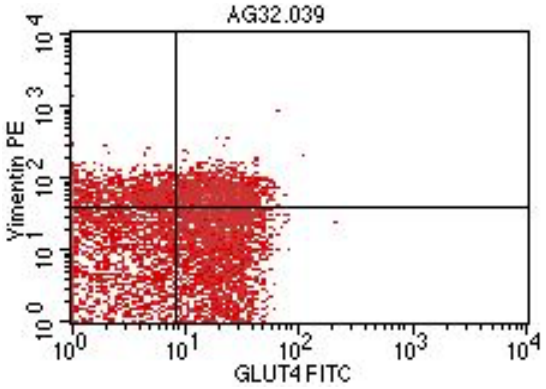

AG27.033
Vimentin PE
GLUT4 FITC

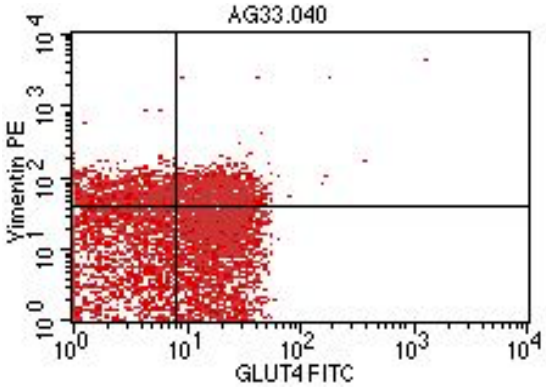

AG27.1.034
Vimentin PE
GLUT4 FITC

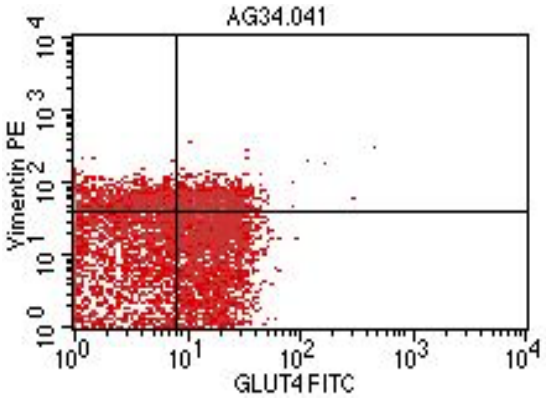

AG28.035
Vimentin PE
GLUT4 FITC

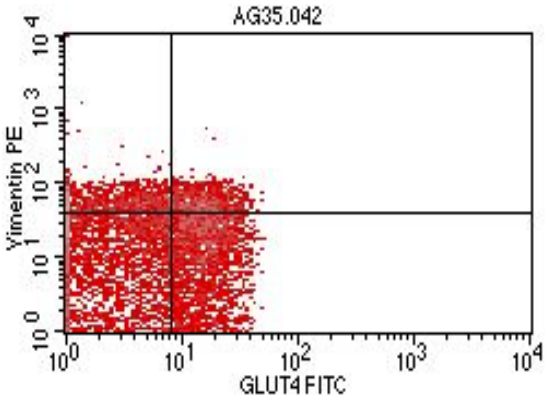

AG30.037
Vimentin PE
GLUT4 FITC


AG31.038
Vimentin PE
GLUT4 FITC


AG32.039
Yimentin PE
GLUT4 FITC


AG33.040
Yimentin PE
GLUT4 FITC

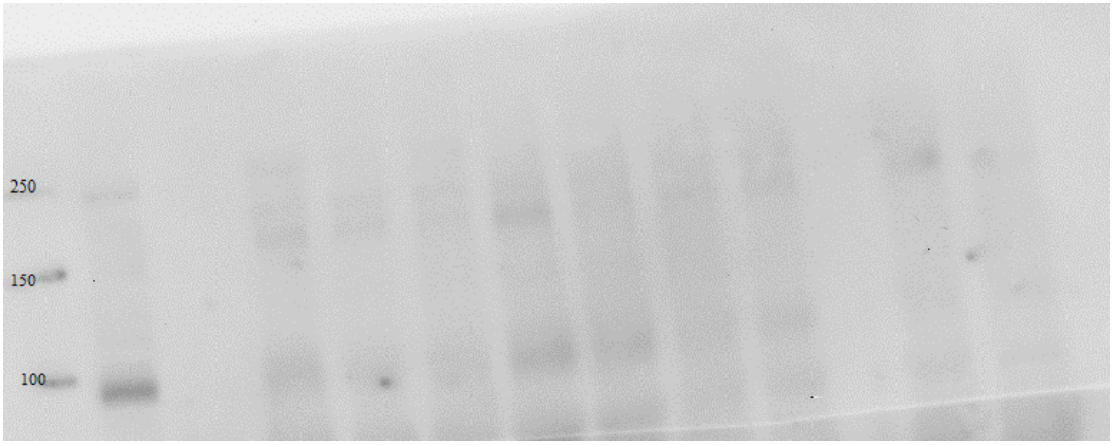

AG34.041
Yimentin PE
GLUT4 FITC


AG35.042
Yimentin PE
GLUT4 FITC

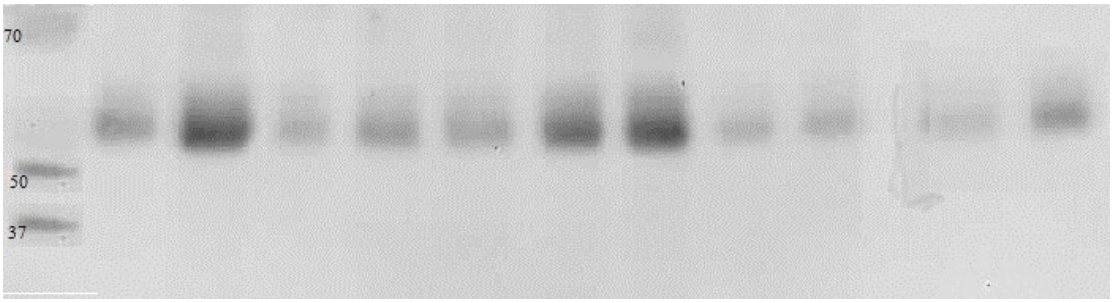
Appendix 2

Western block of (a) Insulin Receptors, (b)Akt, and (c) Actin

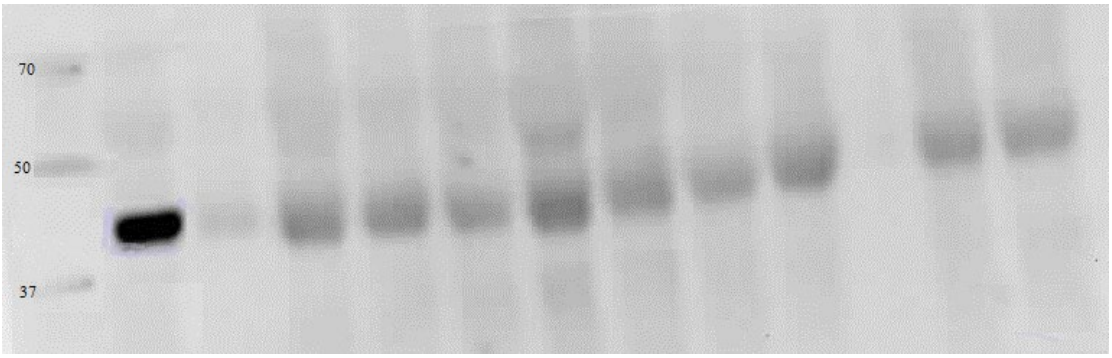
A: Patients AG0-AG9



(a)

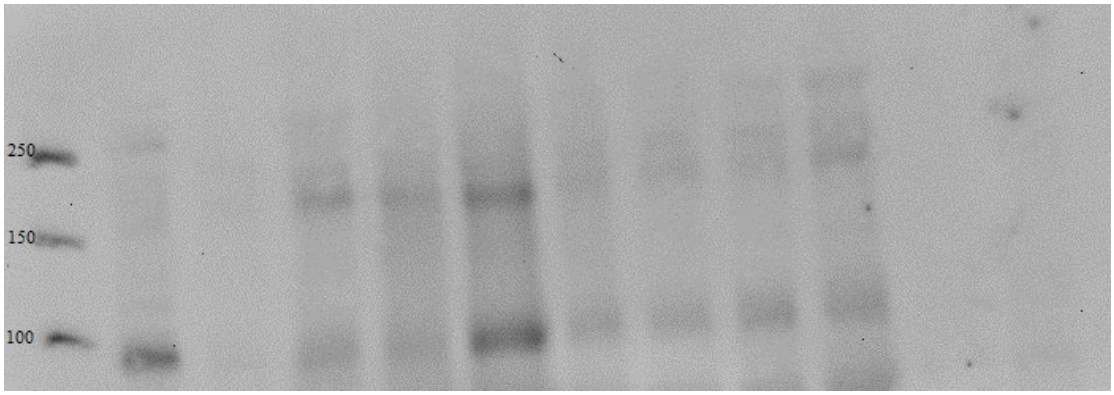


(b)

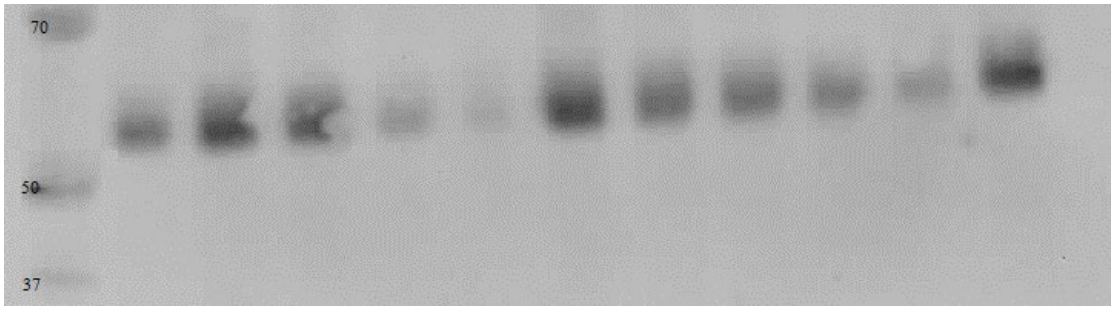


(c)

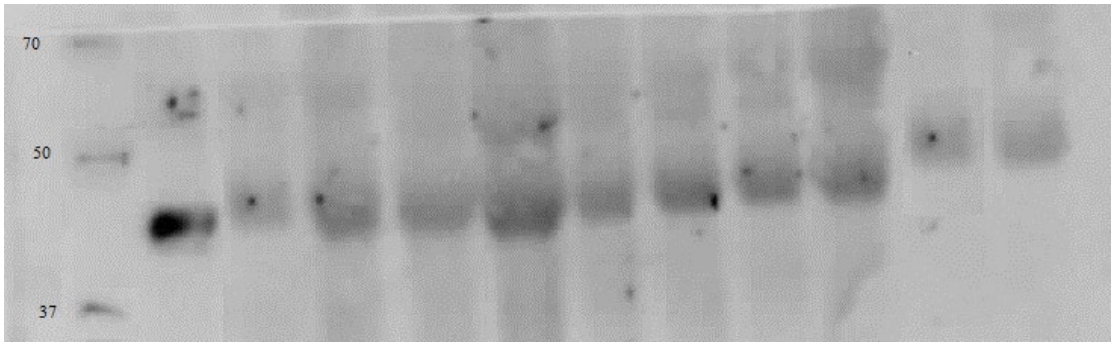
B: Patients AG10-AG19



(a)

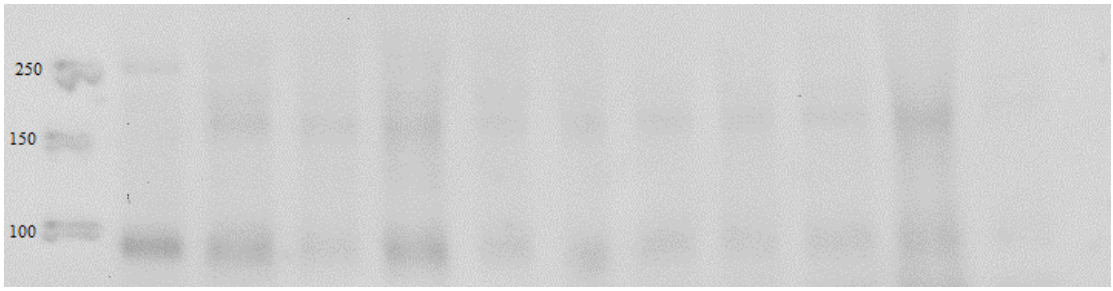


(b)

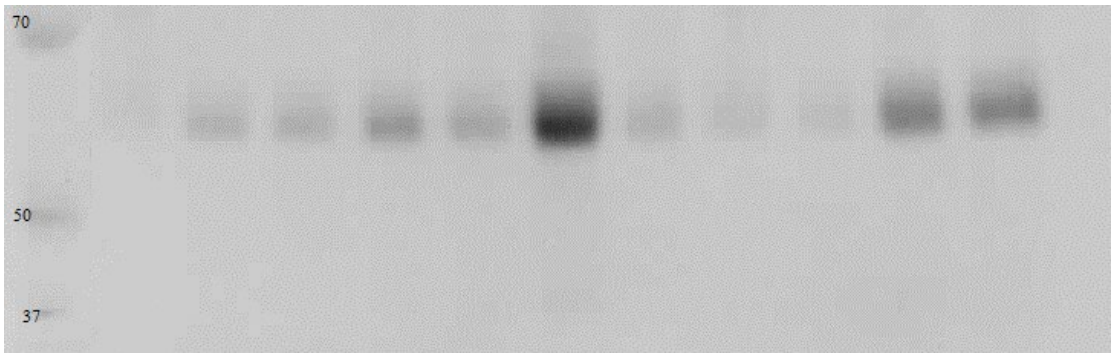


(c)

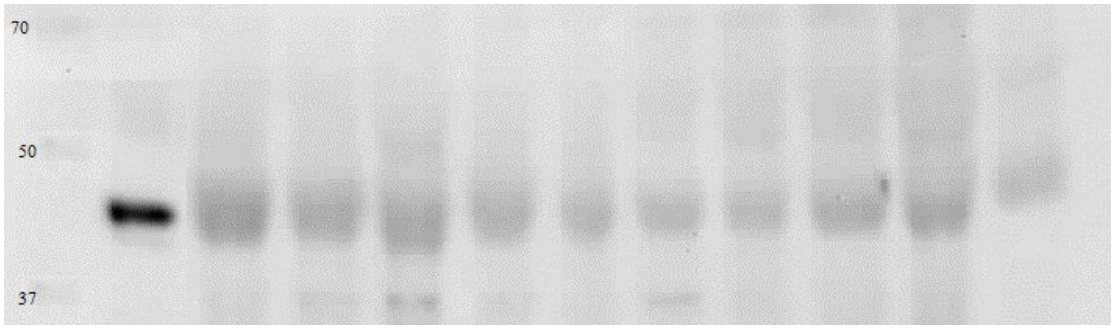
C: Patients AG20-AG27.1



(a)

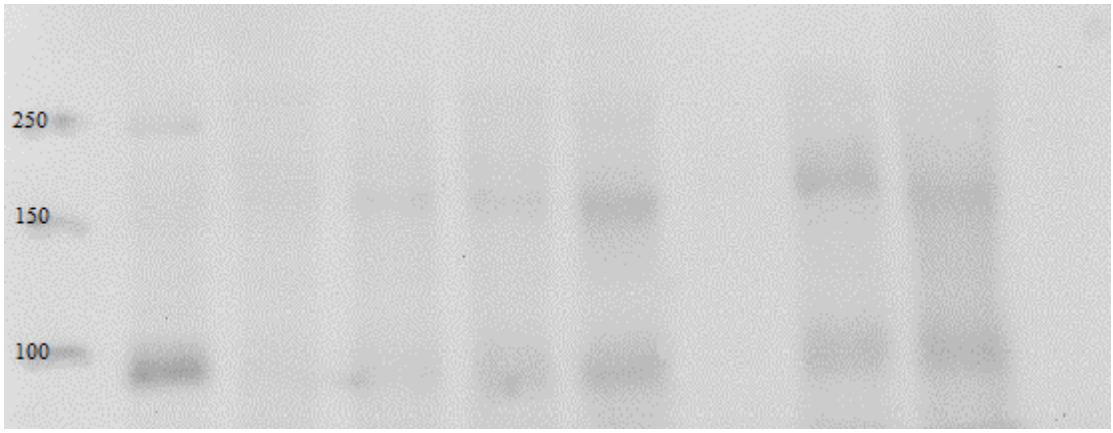


(b)

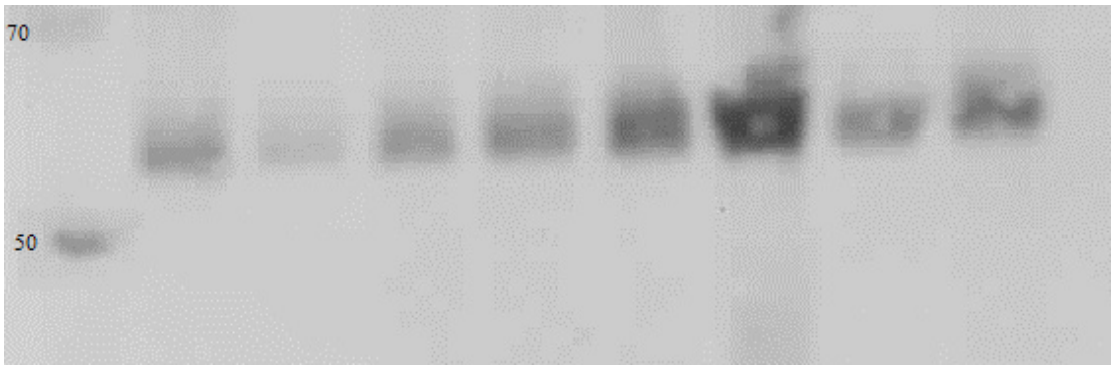


(c)

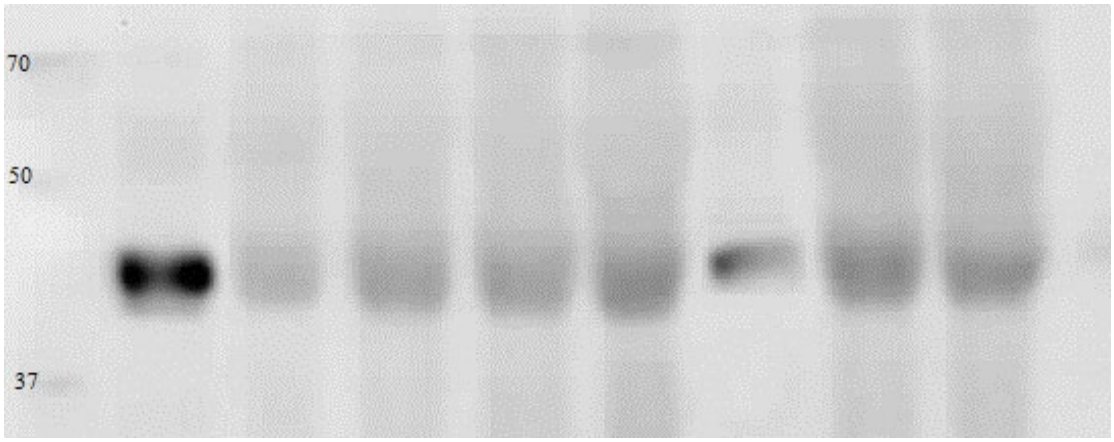
D: Patients AG28-AG35



(a)

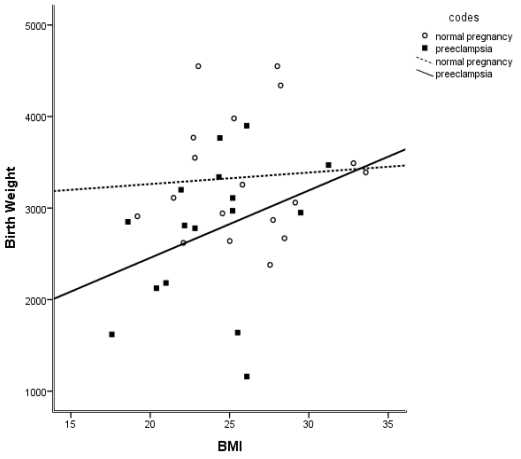


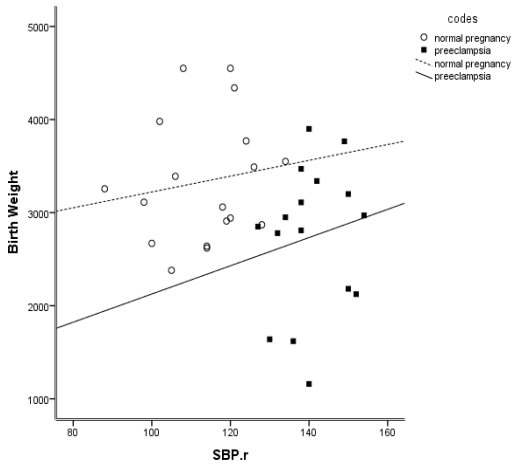
(b)



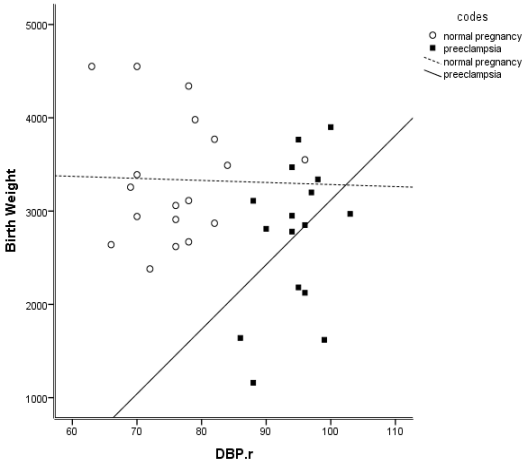
(c)

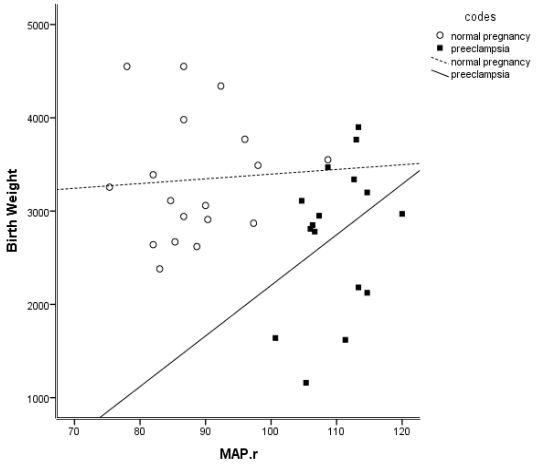
Appendix 3



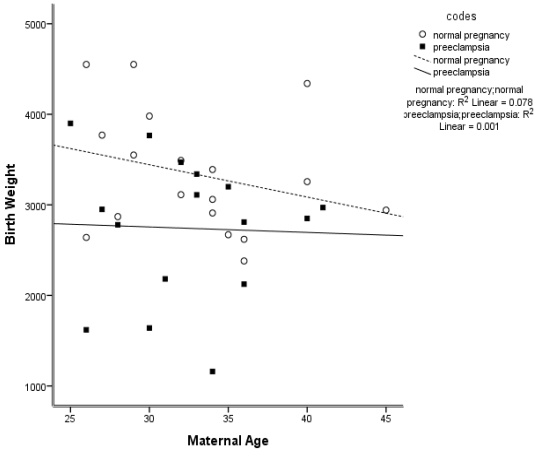


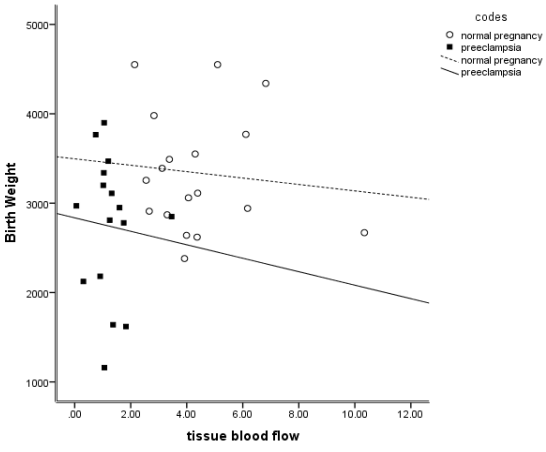
Comparison of foetal birth weight with BMI and Systolic blood pressure



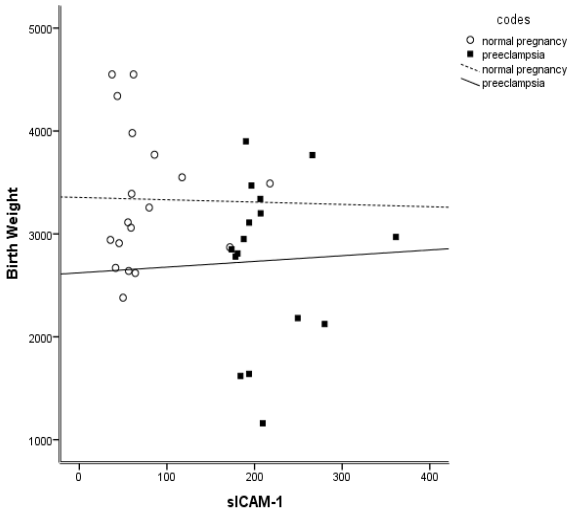


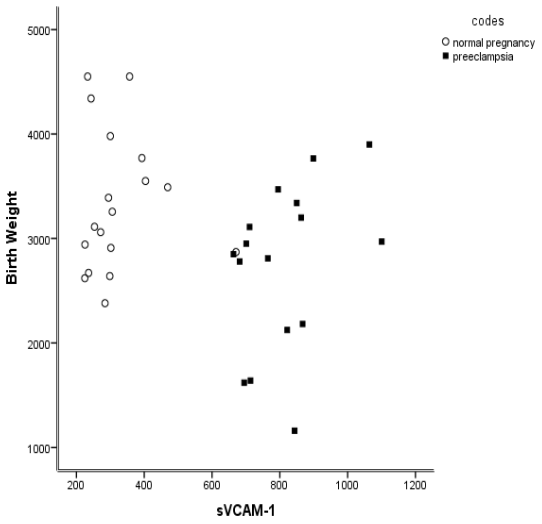
Comparison of foetal birth weight with diastolic blood pressure and mean arterial pressure



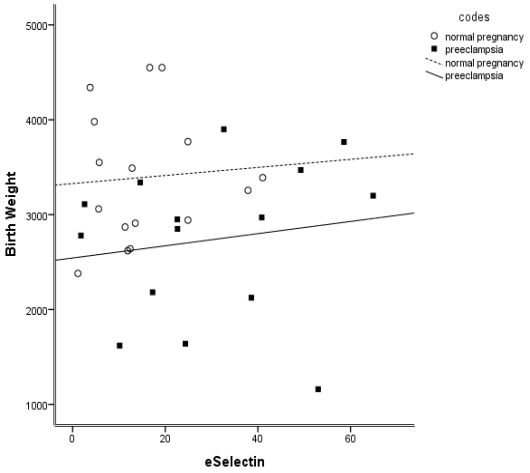


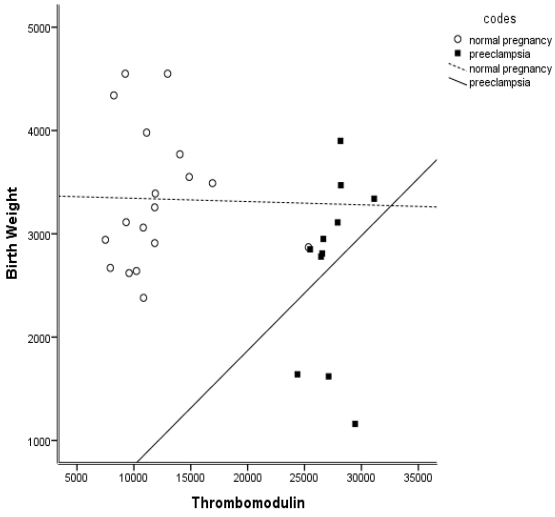
Comparison of foetal birth weight with maternal age and tissue blood flow



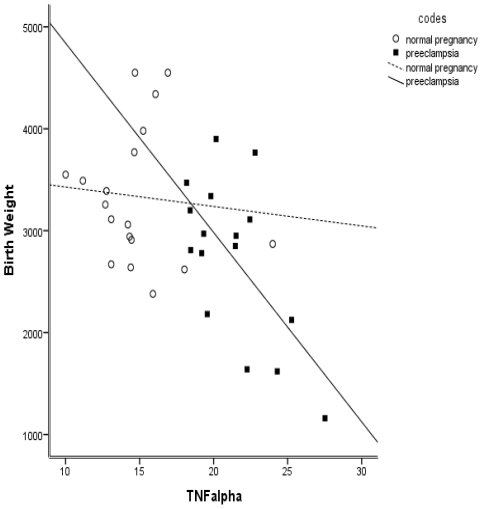


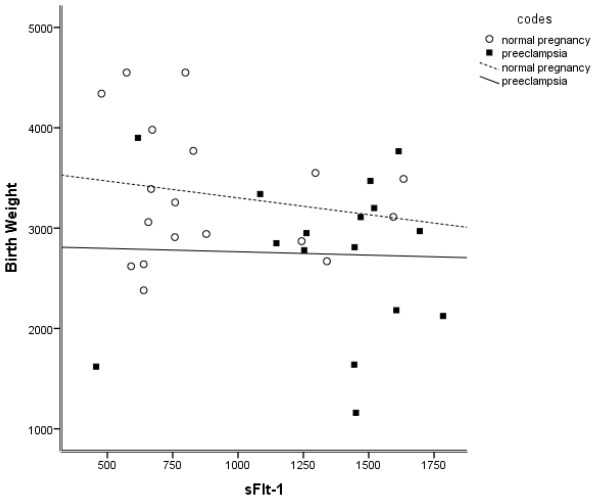
Comparison of foetal birth weight with sICAM-1 and sVCAM-1



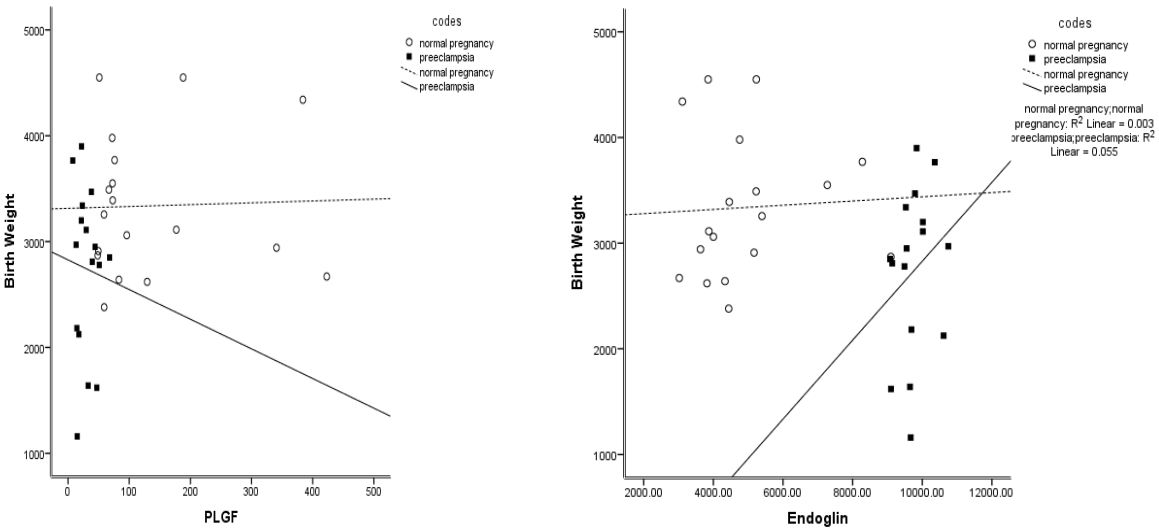


Comparison of foetal birth weight with eSelectin and Thrombomodulin

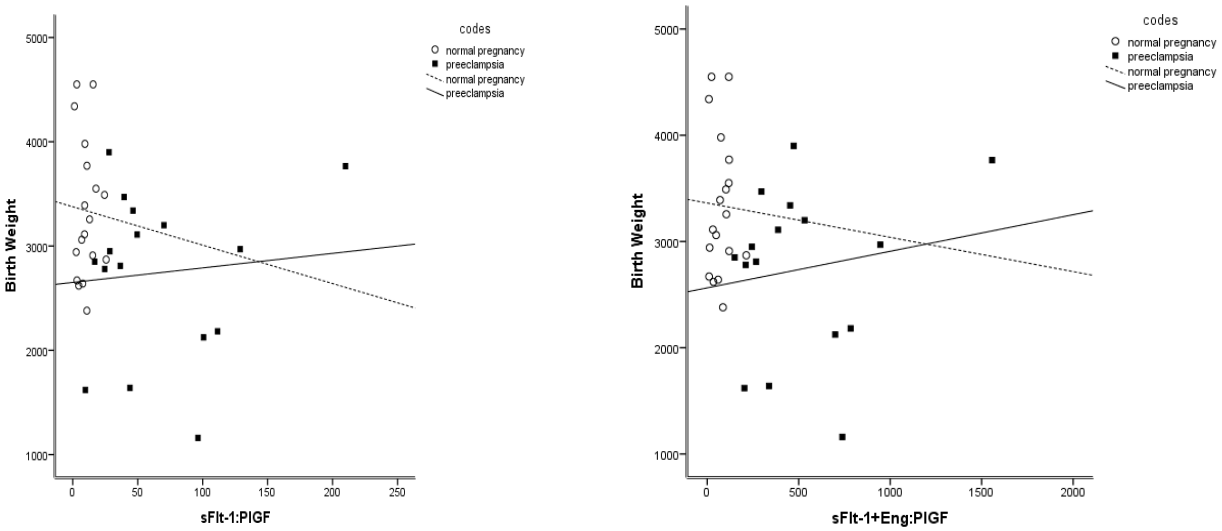




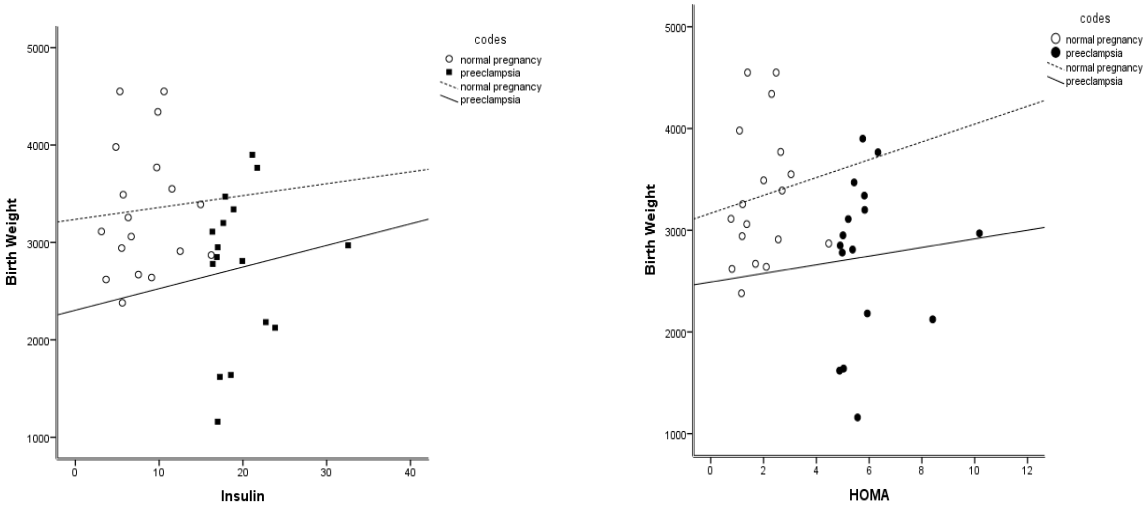
Comparison of foetal birth weight with TNF-α and sFlt-1



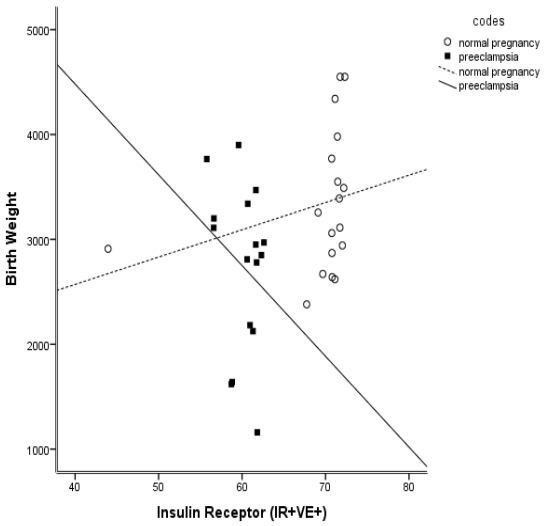
Comparison of foetal birth weight with PlGF and Endoglin

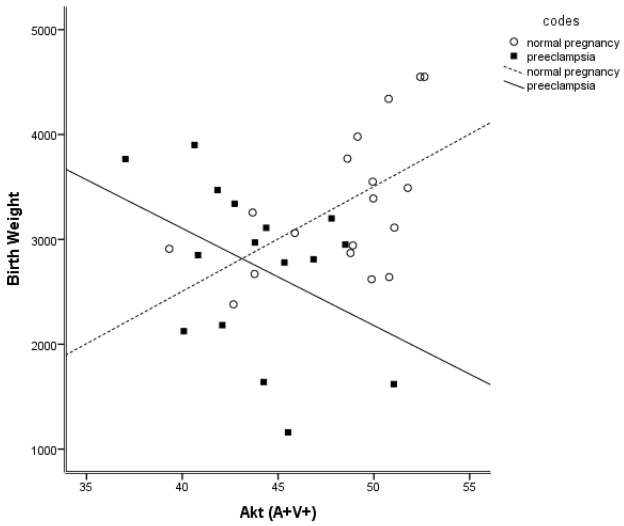


Comparison of foetal birth weight with sFlt-1: PlGF and sFlt-1+Eng: PlGF

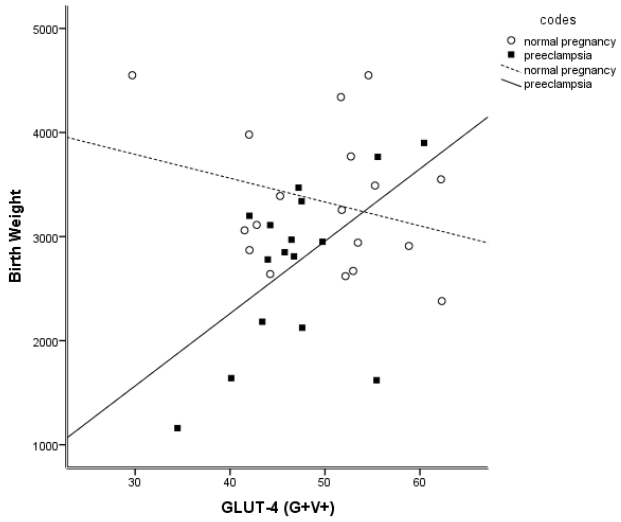


Comparison of foetal birth weight with serum Insulin and HOMA

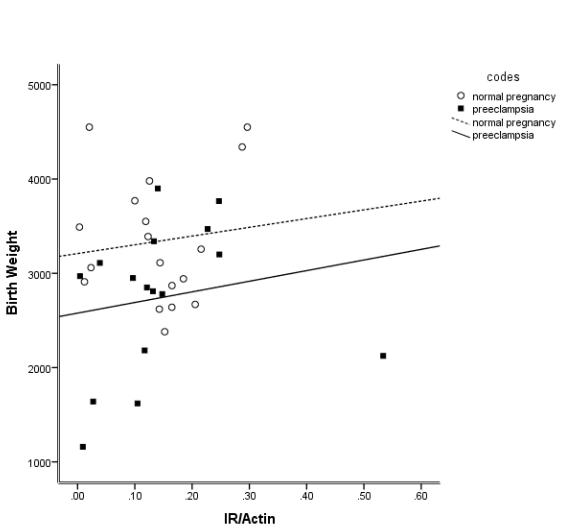


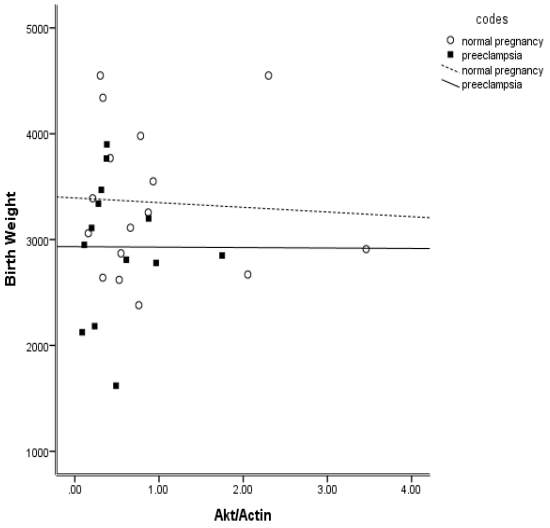


Comparison of foetal birth weight with insulin receptor and activated Akt (estimated by FACs)



Comparison of foetal birth weight with activated GLUT4 (estimated by FACs)





Comparison of foetal birth weight with Insulin receptor: Actin ration and Akt: Actin (estimated by WB)