A STUDY OF COAGULATION POTENTIAL IN PRE-ECLAMPSIA

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Submitted in fulfilment of the requirements for the degree of Master of Science (Medical Science) by research

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June 2010

Abstract

Pre-eclampsia is a multisystem obstetric disease of unknown aetiology that is associated with enhanced coagulation, endothelial activation and reduced placental function. A two-stage model for pre-eclampsia has been proposed involving abnormal placentation and the maternal response to this.

In this thesis, I have identified a possible relationship between annexin V expressed on the surface of syncytiotrophoblasts and fibrin deposition, suggesting that annexin V may serve to protect the surface of placental villi from excessive coagulation. A prothrombinase assay was developed for the purpose of measuring maternal plasma microparticle procoagulant activity between pre-eclamptic women and healthy pregnant control women. No significant difference was found in microparticle procoagulant activity between these two groups. However, quantitation of fetal Corticotrophin-Releasing Hormone mRNA (CRH mRNA) in maternal plasma as a measure of placental cell debris was undertaken between pre-eclamptic and healthy control groups. There were four-fold higher levels of placental cell debris in the maternal blood of pre-eclamptic patients compared to healthy pregnant controls and the mean fetal CRH mRNA level was greater in the group of pre-eclamptic patients over 36 weeks' gestation compared to pre-eclamptic patients under 36 weeks' gestation. Factor VII coagulant activity was also positively correlated with placental cell debris in maternal circulation in pre-eclampsia which suggests that placental cell debris may have procoagulant potential. Measures of coagulation activation, endothelial activation and placental function in maternal plasma were in keeping with the pattern expected for pre-eclamptic patients. Maternal erythrocyte fatty acid status was measured in pre-eclamptic patients and Body Mass Index-matched healthy pregnant controls. Pre-eclamptic patients were found to have a lower percentage of total polyunsaturated fatty acids and total n-6 fatty acids as well as lower amounts of dihomo-γ-linolenic acid, arachidonic acid and docosahexaenoic acid compared to healthy pregnant controls. This change in maternal fatty acid profile would be consistent with a greater synthesis of the potent eicosanoid thromboxane A_2. In summary, these results are overall consistent with a state of enhanced coagulation priming in pre-eclampsia.

List of abbreviations

AA	Arachidonic acid
ABC	Avidin-biotin complex
ADAMTS 13	A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13
APC	Activated protein C
AT	Antithrombin
AU	Absorbance Units
BMI	Body Mass Index
B-OG	N-octyl βD-glucopyranoside
$CaCl_2$	Calcium chloride
CAMs	Cell Adhesion Molecules
COX	Cyclooxygenase
CRH	Corticotrophin-releasing hormone
DAB	Diaminobenzidine
DHA	Docosahexaenoic acid
DHLA	Dihomo-γ-linolenic acid
DNA	Deoxyribonucleic acid
DnsGGACK	1-5 Dansyl-Glu-Gly-Arg-chloromethylketone DiHCL
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPA	Eicosapentaenoic acid
F1+2	Prothrombin fragment 1+2
FDPs	Fibrin Degradation Products
FITC	Fluorescein isothyocyanate
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIIC	Factor VII coagulant activity
FVIII	Factor VIII
FVIIIa	Activated FVIII
FIX	Factor IX
FIXa	Activated factor IX

FX	Factor X
FXa	Activated factor X
FXase	Factor Xase complex
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSA	Human Serum Albumin
HCL	Hydrochloric acid
HELLP	Haemolysis Elevated Liver enzymes and Low Platelets
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMWK	High molecular weight kininogen
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular cell adhesion molecule-1
ICC	Immunocytochemistry
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL-1-β	Interleukin-1-β
IUGR	Intrauterine Growth Restriction
K_2CO_3	Di-potassium carbonate
KCL	Potassium Chloride
LCPUFA	Long Chain Polyunsaturated Fatty Acid
LDL	Low Density Lipoprotein
LOX	Lipoxygenase
LT	Leucotrienes
mRNA	Messenger Ribonucleic Acid
MUFA	Monounsaturated Fatty Acid
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
nM	nanoMolar
OD	Optical Density
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate Buffered Saline

PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGI_2	Prostaglandin I_2 (Prostacyclin)
PPACK	D-Phe-Pro-Arg-chloromethylketone HCL
PS	Phosphatidylserine
PUFA	Polyunsaturated Fatty Acid
RNA	Ribonucleic Acid
SAFA	Saturated Fatty Acid
sICAM-1	Soluble intracellular cell adhesion molecule-1
STBMs	Syncytiotrophoblast membrane fragments
sVCAM-1	Soluble vascular cell adhesion molecule-1
TAT	Thrombin-antithrombin
TBS	Tris- buffered saline
TNF-α	Tumour necrosis factor-α
tPA	Tissue-type plasminogen activator
TTP	Thrombotic thrombocytopenic purpura
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
uPA	Urinary plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
vWF	vonWillebrand Factor

Tables of abbreviations

Fatty acids	
Saturated fatty acids	
12:0	Lauric acid
14:0	Myristic acid
16:0	Palmitic acid
18:0	Stearic acid
20:0	Arachidic acid
22:0	Behenic acid
24:0	Lignoceric acid
Monounsaturated fatty acids	
16:1 n7	Palmitoleic acid
18:1 n9	Oleic acid
20:1 n9	Eicosanoid acid
22:1 n9	Erucic acid
24:1 n9	Nervonic acid
n-6 polyunsaturated fatty acids	
18:2 n6	Linoleic acid
18:3 n6	γ-linolenic acid
20:3 n6	Dihomo-γ-linolenic acid
20:4 n6	Arachidonic acid (AA)
22:4 n6	Docosatetraenoic / Adrenic acid
22:5 n6	n-6 Docosapentaenoic acid (n-6 DPA)
n-3 polyunsaturated fatty acids	
18:3 n3	α-linolenic acid
20:5 n3	Eicosapentaenoic acid (EPA)
22:5 n3	n-3 Docosapentaenoic acid (n-3 DPA)

Placental histology	
Trophoblast layer	
Cytotrophoblast	CyT
Syncytiotrophoblast	SyT
Syncytial sprouts	SySp
Syncytial bridges	SyBr
Syncytial knots	SyKn
Microvilli	MV
Villous structures	
Capillaries	Cap
Artery	Ar
Vein	Ve
Blood vessel	BV
Endothelial cell	EC
Basement membrane	BM
Stem villi	SV
Terminal villi	TV
Intervillous space	IVS
Staining	
Fibrin staining	Fib
Annexin V staining	AnnV
Tissue factor staining	TF
M30 staining	M30

Acknowledgement

I would like to express my gratitude and thanks to the following people for their assistance in my undertaking of this project; Anne Young for preparing the slides for immunocytochemical analyses and assistance in image analyses. Fiona Jordan for preparing BeWo cell cultures. Ann Rumley for routine analyses of markers of coagulation activation. Ann Brown for preparing BeWo cultures, running the microparticle assay of patient groups, performing routine analyses of markers of coagulation activation, endothelial activation, placental function and the measure of fetal CRH mRNA. Barbara Meyer at the University of Wollongong, Australia for Folch extraction and gas chromatography in the identification and quantification of erythrocyte fatty acids.

I would also like to thank my supervisor Dr Dilys Freeman for her valuable advice and guidance throughout this period of research. Lastly, I would like to thank Professor Ian Greer for overseeing this project at its inception.

Authors' declaration

I declare that this thesis is the result of my own work and observations, except as acknowledged.

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Chapter 1 Introduction

1.1 Introduction

1.1.1 Clinical features of pre-eclampsia

Pre-eclampsia is a disease of pregnancy resulting from a maternal physiological response to abnormal placentation. It is a multisystem disorder affecting approximately 2-7% of all pregnancies in the United Kingdom and is a significant cause of maternal and fetal morbidity and mortality(1). According to the International Society for the Study of Hypertension in Pregnancy, pre-eclampsia is defined as having a diastolic blood pressure greater than 110mmg Hg on one occasion, or greater than 90 mmHg on repeated readings, with proteinuria of greater than or equal to 0.3g / 24 hours, or 2+ proteinuria on dipstick testing, in the absence of renal disease or infection. In the seventh report of the Confidential Enquiry into Maternal and Child Health (CEMACH) report 2003-2005, there were 295 maternal deaths in the UK. Of these, 19 maternal deaths were attributable to pre-eclampsia and its associated complications(2). With regards to perinatal mortality, according to the CEMACH report 2007 on perinatal mortality, there were 120 antenatal fetal deaths as a result of pre-eclampsia(3). While there has been a gradual reduction over the years, pre-eclampsia nevertheless remains a significant cause of maternal and fetal morbidity and mortality. Risk factors for developing pre-eclampsia include being primiparous, having a previous history of pre-eclampsia (particularly if onset was before the third trimester), a family history of pre-eclampsia, chronic hypertension, diabetes or kidney disorder, obesity [greater than 30 kg/m^2 body mass index (BMI)], multiple gestation, maternal age under 20 years or over 35 years of age, thrombophilia, renal and connective tissue diseases(4).

Maternal complications of pre-eclampsia include disseminated coagulopathy / HELLP (Haemolysis, Elevated Liver enzymes and Low Platelets) syndrome, cerebral haemorrhage, abruption placentae, pulmonary oedema, renal failure, liver failure or haemorrhage, cerebro-vascular accident and death(1). In severe uncontrolled cases of pre-eclampsia, the patient may go into grand-mal seizures (an eclamptic fit) which can be life threatening and lead to maternal and fetal mortality. Pre-eclampsia can also affect the fetus as the condition is associated with abnormal placentation, placental thrombosis and infarction. This can lead to a reduction in nutrient supply to the fetus and may manifest clinically as reduced fetal movement, reduced liquor volume and a fetal size below that expected for gestational age. Worsening pre-eclampsia, progressive placental thrombosis and subsequent reduced blood supply to the fetus may eventually lead to intrauterine fetal death as a result of hypoxia. Other fetal complications of pre-eclampsia include pre-term delivery (with its associated complications), hypoxic-neurologic injury and perinatal death(1). Normal healthy pregnancy is known to be a pro-thrombotic condition in preparation for the haemostatic demands of delivery and there is an increase in pro-coagulant activity and a decrease in anti-coagulant activity as pregnancy progresses(5;6). In pre-eclampsia, there is a shift in the haemostatic balance towards a pro-thrombotic state, together with changes in endothelial and placental function(1;7). The exact causes of pre-eclampsia are still undefined, however various factors such as an imbalance between pro-and anti-coagulant activity, endothelial dysfunction, endothelial inflammation and syncytiotrophoblast shedding have been proposed.

Pre-eclampsia can sometimes but not always be associated with fetal intrauterine growth restriction (IUGR). IUGR is a failure of the fetus to achieve the growth potential that is expected by its genetic constitution and environmental influences endogenous to the pregnancy. The fetus has subnormal body weight or mass and is growing under the 10^{th} centile for its gestational age. IUGR can manifest as a result of various causes and these causes can be divided into maternal, placental and fetal factors. Maternal factors include cardiac disease, renal disease, cigarette smoking and excessive alcohol consumption. Placental factors include pre-eclampsia, placental abruption, thrombosis, infection and vasculitis. Fetal factors can include intrauterine infection, cardiac disease and chromosomal abnormalities(8). While the causes of pre-eclampsia are likely to be multifactorial, it is accepted that the placenta and the mother’s response to placentally-derived factors and proteins plays a major role in its pathogenesis.

1.2 The Placenta

1.2.1 Development of the normal placenta

The placenta functions as a feto-maternal interface which supplies the growing fetus with essential nutrients obtained from the maternal blood supply. Post-conception, the fertilized zygote develops into a flattened vesicle (the blastocyst) which comprises of an outer wall of cells called trophoblasts, the blastocystic cavity and an inner cell mass. The trophoblasts eventually develop into the placenta while the inner cell mass develops into the embryo, umbilical cord and amnion. Within days after implantation, the trophoblasts proliferate to form a layer which invades into the endometrium. The trophoblast gives rise to three main cell types; the syncytiotrophoblast (which forms the main epithelial covering of the villous tree and is in direct contact with the maternal blood space), the cytotrophoblast (which is the germinative population that proliferates throughout pregnancy and fuses to generate the syncytiotrophoblast) and the extravillous trophoblast cells which are non-proliferative and invade the maternal endometrium. The syncytiotrophoblast layer is a continuous structure, without boundaries between neighbouring syncytiotrophoblast cells. On the villous side of the syncytiotrophoblast are numerous microvilli which serve to increase the cell surface area. As pregnancy progresses, the syncytial layer becomes variable in thickness. It may be thinned to become the vasculosyncytial membrane or the syncytiotrophoblast nuclei may become piled up in areas forming syncytial knots. The syncytiotrophoblast is involved in various activities such as protein and lipid synthesis and breakdown, water and gas diffusion between fetal and maternal circulation, transfer of glucose, amino acids and electrolytes and production of hormones (Human Placental Lactogen and Human Chorionic Gonadotrophin). If there is damage to the trophoblast layer, fibrin deposition occurs and cytotrophoblast cells proliferate to repair the damage(9).

Stem villi form the ‘trunks’ and ‘branches’ of the villous tree. They function to support the structure of the villous trees and but not have a significant role in feto-maternal exchange. The stroma consists of collagen fibre bundles, occasional fibroblasts, macrophages and mast cells. Larger stem villi often have a central artery and vein with smaller vessels and capillaries within its stroma. Approximately half of the stromal volume is taken up by vascular lumen and there are no vessels other than capillaries and sinusoids. Terminal villi

are the final outgrowths of the villous tree. Terminal villi are formed from capillary growth and coiling, not by outgrowths. Terminal villi appear as bulbous structures and may be single or have side branches. Terminal villi have a thin trophoblastic surface in close contact with dilated capillaries within and functions as the main site of feto-maternal exchange. At term, terminal villi will make up 45% or more of placental volume.

The decidua is supplied by uteroplacental arteries which are derived from uterine spiral arterioles. During placental growth and trophoblastic invasion into the decidua, trophoblast cells invade into the walls of uteroplacental arteries. There is proteolysis of the elastic and smooth muscle cells of the spiral artery walls by invasive extravillous trophoblast cells(10). There is 'fibrinoid' material deposition in the walls of these spiral arteries in which cytotrophoblastic cells are embedded. As a result, the spiral arterioles change from flexible channels to large diameter (up to 5 times its original size) rigid channels, now incapable of vasoconstriction. This change to spiral arterioles occurs in the majority of myometrial spiral arteries located in the centre of the placenta and to lesser extent at the peripheral vessels. The net effect of which is the reduction of uteroplacental flow resistance leading to increased blood flow to the placental villi thereby increasing feto-maternal transfer(10). By losing their elastic lamina and smooth muscle, these remodelled spiral arteries also lose their responsiveness to vasoactive agents such as thromboxane(11).

The intervillous space is an area where maternal blood flows freely around the placental villi thereby facilitating feto-maternal exchange. Maternal blood flows into the intervillous space from decidual arteries located near the centres of the villous trees and this blood is drained via outlets to decidual veins located in between the villous trees. Each villous tree has a corresponding perfused portion of the intervillous space and together they make up a feto-maternal circulatory unit (placentone). There are about 40 to 60 placentones in total in a normal placenta.

1.2.2 Placental changes in pre-eclampsia

In pre-eclampsia there is failure of extravillous trophoblast invasion into the myometrium and spiral arteries(10;12) and uterine spiral arteries are not converted into large capacitance

channels as they would in healthy pregnancy. This leads to inadequate maternal blood perfusion into the intervillous spaces to supply the developing fetus and reduced feto-maternal exchange. The failure of extravillous trophoblast spiral artery invasion into the uterine spiral arteries also leads to these spiral arteries maintaining their thick muscular coat which in turn allows them to be influenced by vasoactive agents such as thromboxane A_2.

Within the arterioles of placental bed arteries, there is atherosis of the uterine vascular endothelium (where the vessel wall is replaced by fibrin and the intima is invaded by macrophages which engulf lipid, becoming foam cells) and fibrinoid necrosis. This tends to occur in arterioles which have failed to convert to large capacitance vessels(13). Damage to the endothelium leads to thrombosis and a mural thrombus may develop, further occluding blood flow, leading to reduced intervillous blood flow. If blood flow is significantly impaired, this may lead to placental hypoxia with resultant cell death. This can be seen macroscopically as areas of infarction. In pre-eclampsia placentas, there is a greater percentage of villi with fibrinoid deposits compared to healthy pregnant controls(14).

1.2.3 Placental changes in IUGR

IUGR is associated with a reduced numbers of stem, intermediate and terminal villi as well as a reduction of the intervillous space. There is also an associated reduction in the trophoblast volume, stromal volume and fetal capillary volume. Furthermore there is a reduction in villous surface and capillary surface(15). There is reduced linear growth of villi and fetal capillaries along with reduced capillary volume and surface(16). This results in a reduction in the exchange area between fetal and maternal circulation which contributes to the clinical condition of IUGR.

IUGR has been found to be associated with maternal placental floor infarction. There is gross deposition of perivillous fibrinoid material among the basal villi of the maternal floor of the placenta(17-19) which suggests there is a disorder of coagulation. Increased trophoblastic cell apoptosis in IUGR has been noted using electron microscopy and

confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining(20). A significantly higher index of M30 cytodeath antibody (which recognises a fragment of cytokeratin 18 which is cleaved by caspases in early apoptosis) staining has also been found in the trophoblast of IUGR patients(21), suggesting there is increased trophoblast apoptosis in IUGR.

1.2.4 Coagulation in the placenta

1.2.4.1 Placental Tissue Factor

Tissue factor (TF) is a membrane-bound protein and functions as a potent initiator of coagulation by acting as a cell-surface receptor for activated factor VII(22). TF is expressed on subendothelial cells such as vascular adventitial cells and smooth muscle cells(23). In its subendothelial location, TF forms a boundary around blood vessels. Mechanical or chemical damage of the blood vessel endothelium can lead to blood leakage from the vascular space into the subendothelial space, allowing circulating activated factor VII (FVIIa) to become exposed to subendothelial TF leading to the formation of the FVIIa-TF complex, a key step in coagulation activation. The FVIIa-TF complex increases the enzymatic activity of activated factor VII approximately $2x10^7$-fold towards factors IX and factor X in the extrinsic coagulation cascade [illustrated in figure 1.4.1 and reviewed in (22;24)].

In healthy pregnancy, high concentrations of TF have been detected in the placenta(25). TF expression has been localised to placental macrophages, endothelial cells and fibroblast-like cells in loose connective tissue but was not observed in trophoblast or trophoblastic basement membranes(26) although cultured syncytiotrophoblast cells have been found to express TF(27). Higher levels of TF mRNA have been found in pre-eclamptic placentas compared to healthy pregnant control placentas(28) and Estelles *et al* noted greater amounts of TF antigen and TF mRNA in the placentas of women who had severe pre-eclampsia with associated IUGR compared to healthy pregnant controls(29). Addition of tumour necrosis factor-α (TNF-α) into cytotrophoblasts isolated from healthy and pre-eclamptic placentas *in vitro* has been found to increase the amount of TF expressed by

trophoblasts. The amount of TF expressed was greater in pre-eclamptic samples compared to healthy pregnant samples, suggesting that TF expression by pre-eclamptic placentas may be more sensitive to pro-inflammatory cytokines(30).

1.2.4.2 Placental fibrinoid

In haemostasis, fibrin is the end product in the process of coagulation. In the placenta, placental fibrin-type fibrinoid is extracellularly deposited materials composed mostly of fibrin. Electron microscopy of fibrinoid material reveals a longitudinal filamentous structure similar to that of true fibrin(18). Fibrinoid is a product of maternal blood and is present at all stages of pregnancy. It is present in both healthy and pathological pregnancies [reviewed in(10)]. Perivillous fibrin deposition is a normal finding in healthy placentas. Various functions of perivillous fibrinoid have been proposed, such as having a role in maintaining the mechanical stability of the placenta and regulating blood flow between the villi and villous repair(10). Cultured cellular trophoblasts have been grown on a fibrin matrix and morphological differentiation into trophoblast resembling the trophoblast of term villi occurred, suggesting that perivillous fibrin may be involved in trophoblast repair(31).

As normal pregnancy progresses, there is often loss of areas of syncytiotrophoblast (possibly due to syncytial degeneration or trophoblast turnover) and these areas of trophoblast discontinuity are replaced by perivillous fibrinoid(32;33). Electron microscopy has revealed apoptotic changes of syncytiotrophoblast in these areas of fibrinoid deposits(34). While fibrinoid material may have physiological functions, the deposition of perivillous fibrinoid at the trophoblast surface obstructs feto-maternal exchange and in cases of gross deposition, may lead to placental insufficiency. Excessive perivillous fibrin deposition has been found in conditions of IUGR(17) and pre-eclampsia(14;35), both conditions that are associated with placental insufficiency.

1.2.4.3 Placental annexin V

Annexin V belongs to the family of annexins, which are highly homologous phospholipid binding proteins. Annexin V can be found in various cell types such as platelets and endothelial cells and has also been detected in maternal blood and amniotic fluid(36). While the plasma concentration of annexin V is low (0-5ng/ml), it is high within the placenta at approximately 250mg/kg (37). Within the placenta, annexin V has been found in various cell types such as capillary endothelial cells, fibroblasts, Langhans cells and Hofbauer cells. However annexin V is most abundant within trophoblastic cells(38). Annexin V is expressed constitutively by syncytiotrophoblast cells(39) and has been found to be localised to the microvillous surface of syncytiotrophoblastic cells(40). Annexin V is expressed by the placenta from as early as 7 weeks' gestation and is highly expressed on the syncytiotrophoblast microvillous surface throughout gestation(41). Annexin V has a high affinity to negatively-charged phospholipids such as phosphatidylserine and therefore can act to bind phosphatidylserine-exposing cell membranes [reviewed in(36)]. The presence of annexin V on a cell surface confers anticoagulant properties. Annexin V trimerises on the phospholipid cell membrane and these trimers polymerise to form hexagonal 2-dimensional crystals that form a lattice over the phospholipid membrane(42). This lattice is stabilized by protein-protein interactions(36). The presence of the annexin V lattice reduces lateral mobility of already-bound coagulation factors on cell membranes(39) thus preventing the propagation of these coagulation factors.

A deficiency of annexin V has been implicated in the procoagulant effects of the antiphospholipid syndrome (which is associated with increased arterial and venous thrombosis, placental thrombosis and fetal death) as reduced amounts of annexin V have been found on the syncytiotrophoblast apical membranes of these patients(43). *In vitro* experiments have shown that antiphospholipid immunoglobulins lead to reduced annexin V binding to phospholipid bilayers(43;44) as well as trophoblast cell surfaces(45) with an associated increase in prothrombin binding on these trophoblast cells(45). In pregnant mice, infusions of antibodies towards annexin V were found to lead to placental thrombosis, placental necrosis and fetal loss(46). Injections of phosphatidylserine-containing vesicles (which are prothrombotic) into mice also led to thrombosis of the placental bed and IUGR, while subsequent injection of recombinant annexin V led to a reversal of these effects(47).

Lower amounts of annexin V expression on trophoblasts has been noted in pre-eclamptic patients compared to healthy pregnant controls(39;48). In association with lower annexin V expression on the trophoblasts, higher amounts of fibrin degradation products and thrombin-antithrombin complexes has been noted, suggesting an inverse relationship between the expression of annexin V and coagulation(48). Several studies have suggested that annexin V may afford the syncytial surface protection against clot formation(45-47). Therefore, it is possible there may be a relationship between the distribution of annexin V and fibrin deposition on the syncytial surface of the placenta.

1.2.5 Placental apoptosis

As the placenta develops, its tissues undergo remodelling and central to this process of remodelling is cellular apoptosis. Apoptosis is one of the main types of programmed cell death which involves a series of biochemical events leading to changes in cell morphology and ultimately the cellular death. The characteristic morphology of cells undergoing apoptosis are cell membrane blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Finally, the apoptotic cell breaks into small membrane-wrapped fragments known as apoptotic bodies or microparticles which are eventually engulfed by phagocytic cells. Apoptosis is distinct from necrosis in that apoptosis is an active form of cell death which is controlled, while necrosis is accidental cell death as a result of external factors. The processes associated with apoptosis in the disposal of cellular debris do not damage the organism. In necrosis the whole cell along with its organelles swell as the plasma membrane becomes unable to control the passage of ions and water into and out of the cell, leading to leakage of cellular contents and inflammation of surrounding tissues(49).

Apoptosis occurs during organ development and is part of normal cellular turnover. Apoptosis involves a complex set of signalling molecules such as Fas ligands, Fas receptors and death signalling genes such as Bcl-2. Bcl-2 has been immunolocalised to syncytiotrophoblasts of normal pregnancies throughout pregnancy. Apoptosis can be identified using cellular cytokeratin 18 which has been found to be cleaved in early apoptosis. M30 CytoDEATH (Roche Molecular Biochemicals) is a mouse monoclonal

antibody that detects a specific caspase cleavage site within cytokeratin 18 that is not revealed in normal healthy cells. Thus, M30 is a useful tool for localising apoptotic cells. A previous study looking at M30 localisation within the healthy placenta in the third trimester showed that the majority of M30 staining was localised to extravillous trophoblasts as well as syncytiotrophoblast cells with abundant M30 staining of syncytiotrophoblast cells in areas of greater perivillous fibrinoid deposition(50). As cytokeratin is located within the cell cytoplasm, M30 immunoreactivity is confined to the cytoplasm although non-specific M30 staining of the nuclei of highly proliferating cells has also been recorded(51).

In the developing placenta, cytotrophoblast cells continually proliferate, differentiate and fuse into the syncytiotrophoblast layer. A second differentiation process occurs at the outer syncytiotrophoblast layer where syncytial apoptosis occurs and ageing syncytiotrophoblast nuclei are packed into syncytial knots and extruded into the maternal circulation to be replaced by a younger population (9;49). These apoptotic syncytial knots are cleared away by villous stromal macrophages as well as circulating macrophages (which occurs to a large degree in maternal lungs) [reviewed in(52)]. The ingestion of apoptotic cells (exposing phosphatidylserine) by macrophages has been shown *in vitro* to produce TGFβ-1 which suggests that macrophages may attenuate the maternal inflammatory response in the placenta(53). In normal pregnancy, in the first trimester, there is a greater cytotrophoblast volume in relation to syncytiotrophoblast volume. However this changes towards the end of pregnancy where the syncytiotrophoblast volume becomes greater along with a greater amount of syncytial knots and bridges(54).

Pre-eclamptic and IUGR placentas have a greater degree of syncytiotrophoblast apoptosis as well as a greater number of syncytial knots and bridges in comparison to healthy placentas(20;55;56). In pre-eclampsia, the increased apoptosis is associated with a reduction in syncytiotrophoblast area and almost all terminal villi in late pregnancy show syncytial knots(57). *In vitro,* hypoxia has also been shown to induce apoptosis in trophoblasts(58;59). As the syncytiotrophoblast layer is in contact with the intervillous space which is in turn in contact with the maternal systemic circulation, it is possible that apoptotic syncytiotrophoblasts would release microparticles into the maternal circulation.

1.3 Placental debris and microparticles

1.3.1 Microparticle generation and characteristics

Microparticles range from 0.1 to 2µm in size and are produced during cellular activation and apoptosis. In normal cell conditions, membrane aminophospholipids such as phosphatidylserine and phosphatidylethanolamine are actively transported from the outer leaflet to the inner (cytosolic) leaflet of the cell membrane by cell membrane translocase. During cellular apoptosis, there is release of calcium from the endoplasmic reticulum resulting in an increase in intracellular calcium levels leading to the inhibition of translocase. As a result of inhibition of translocase, phosphatidylserine is no longer actively maintained at the inner leaflet and becomes exposed on the outer surface of the cell membrane[reviewed in (60)]. This increase in calcium also activates cytosolic calpain which cleaves cytoskeletal filaments leading to blebbing of the cell membrane and the eventual release of these cell membrane blebs into the extracellular environment as microparticles[reviewed in(61)]. The released microparticles contain cytoplasmic components with phosphatidylserine exposed on their membrane surface. Cell-specific antigens particular to the cell type that the microparticles originated from are exposed on the microparticle membrane surface(62). For example, microparticles derived from endothelial cells express CD31, CD51, CD54 and CD62E and CD106. Microparticles have been identified from various cell types such as endothelium, T-cells, monocytes and platelets(63-66). Microparticles have been found to activate platelets(66) and they have also been found to increase the adhesion of monocytes to human umbilical vein endothelial cells(HUVEC) via an up-regulation of intracellular cell adhesion molecule-1 (ICAM-1)(64).

Microparticles have been proposed to be procoagulant in nature due to the exposed phosphatidylserine on their surface, as phosphatidylserine provides a surface for the assembly of coagulation factors in the conversion of prothrombin to thrombin via the tissue factor pathway (67;68). Indeed, plasma levels of microparticles have been found to be raised in various prothrombotic conditions such as acute coronary syndrome(69), diabetes(70), heparin induced thrombocytopenia(71), paroxysmal nocturnal

haemoglobinuria(72) and severe hypertension(73). Microparticle levels have also been found to be raised in women with a history of idiopathic pregnancy loss(74). Microparticles may however also possess anti-coagulant properties. Tans *et al* showed increased platelet inactivation of activated factor V after thrombin stimulation and this was associated with microparticle release(75).

1.3.2 Syncytiotrophoblast membrane particles

The syncytiotrophoblast layer of the placenta is in direct contact with the intervillous space and therefore is in contact with maternal blood. In healthy pregnancy, with constant placental remodelling and renewal of the syncytiotrophoblast layer, there is apoptosis of syncytiotrophoblasts (9) and this has been proposed to result in the shedding of syncytiotrophoblast membrane fragments(STBMs) into the maternal side of placental circulation(76). In healthy pregnancy, STBMs have been identified in the maternal uterine vein circulation (77;78). While STBMs have been found in the uterine vein circulation of 60% of healthy pregnant women, only 10 % of these healthy pregnant women had STBMs detected in their peripheral circulation(78). As the mechanism of STBM generation is similar to that of microparticle generation (i.e. a consequence of cellular apoptosis); STBMs may be considered to be microparticles of syncytiotrophoblast origin.

In pre-eclampsia, there is greater trophoblast apoptosis compared to healthy pregnancy(79) and higher levels of STBMs have been found in the maternal blood of pre-eclamptic women compared to healthy pregnant controls(78;80). Goswami *et al*(81) measured STBM levels in pre-eclamptic patients by means of ELISA using an anti-trophoblast antibody (NDOG2) to capture the particles and STBM levels were found to be significantly increased in early onset pre-eclampsia patients (patients who developed pre-eclampsia under 34 weeks' gestation) compared to healthy pregnant controls but they were not statistically different when compared between late onset pre-eclamptics (over 34 weeks' gestation) and healthy pregnant controls. STBMs have been found in the uterine veins of 71% of pre-eclamptic women but only 10% of these women had STBMs detected in their peripheral circulation which suggests that while there is greater trophoblast apoptosis in pre-eclampsia compared to healthy pregnancy, relatively little STBMs reach the maternal peripheral circulation(78). There was also an increased amount of STBMs found in the

peripheral circulation of early-onset pre-eclamptic women compared to normotensive IUGR pregnancies (81). In *in vitro* studies, STBMs from pre-eclamptic patients have been shown to interfere with endothelial cell proliferation (82) as well as impair small artery relaxation(83;84) although another study of myometrial arteries perfused with STBMs in levels up to 100 times that reported in pre-eclampsia failed to show any effect on bradykinin-mediated arteriolar dilatation(85). Therefore, there is evidence that STBMs may affect endothelial function and blood flow in pre-eclampsia.

1.3.3 Microparticles in healthy pregnancy

Microparticle levels in peripheral blood have been measured in healthy pregnant and non-pregnant controls using flow cytometry. In a study of 15 healthy pregnant women and 19 non-pregnant controls, Bretelle *et al* showed that platelet and endothelial-derived microparticles were higher in healthy pregnant compared to non-pregnant controls(86). In contrast, in another study of 10 healthy pregnant individuals and 10 non-pregnant controls, Van Wijk *et al* did not find any significant difference in platelet, monocyte, granulocyte or endothelial-derived microparticle levels between pregnant and non-pregnant controls(87). With regards to coagulation, using a prothrombinase assay, Bretelle *et al*(86) found that total microparticle pro-coagulant activity was significantly increased in healthy pregnant individuals compared to their non-pregnant controls. However, VanWijk *et al* measured microparticle thrombin generating ability *in vitro* and did not find any significant difference between healthy pregnant and non-pregnant controls(88).

1.3.4 Microparticles in pre-eclampsia

When microparticle levels were compared between pre-eclamptic women and healthy pregnant controls, increased numbers of microparticles derived from endothelial, T-helper, T-suppressor, monocyte, granulocyte cells were found in the plasma of pre-eclamptic patients compared to healthy pregnant controls(80;87;89;90) although some studies did not detect any significant difference in endothelial-derived microparticles between pre-eclamptic and healthy pregnant controls(86;87). The highest proportion of microparticles in both pre-eclamptic and healthy pregnant women has been found to be platelet-derived

microparticles(87) although in contrast, Bretelle *et al* found a lower amount of platelet-derived microparticles in pre-eclamptic pregnancies compared to healthy pregnant controls(86). Despite differences in microparticle levels in these cell subpopulations, no differences have been found in the total number of microparticles in maternal plasma between pre-eclamptic and healthy control groups(86;87). With regard to microparticle pro-coagulant activity, in a study of 10 pre-eclamptic and 10 healthy controls, VanWijk *et al* measured microparticle thrombin generating ability *in vitro* and did not find any significant difference between pre-eclamptic and healthy pregnant controls (88). Bretelle *et al*(86) using a prothrombinase assay did not find any difference in microparticle pro-coagulant activity between pre-eclamptic and healthy pregnant control groups.

1.3.5 Fetal Corticotrophin-releasing hormone mRNA

For purposes of measuring placental cell debris in maternal circulation, a measure of fetal cellular products in maternal circulation may be undertaken. Several studies have shown that fetal DNA was detectable in maternal plasma from as early as 6 weeks' gestation onwards with a gradual increase of fetal DNA levels as pregnancy progresses(91-93). However, the use of fetal DNA as a measure has limitations as in order to differentiate placental cellular debris from maternal cellular debris, only male offspring DNA could be used and this precluded the study of pregnancies carrying female fetuses(94). Fetal mRNA was then discovered to be detectable in maternal plasma by using a 2-step reverse transcription-PCR assay(95) and fetal mRNA appeared to be protected from serum RNAses as they were circulating within apoptotic bodies(96;97). Fetal mRNA in maternal plasma would be presumed to originate from the placenta as the placenta is genetically identical to the fetus and is in direct contact with the maternal circulation. The measure of fetal mRNA in maternal blood would therefore be a reflection of the amount of placental cell debris in maternal systemic circulation.

In human physiology, Corticotrophin-releasing hormone (CRH) is a neurotransmitter synthesised and released from the hypothalamus into the hypothalamo-hypophyseal portal system where it acts on the anterior pituitary gland. Due to the blood-brain barrier, maternal CRH mRNA is not detectable in the maternal systemic circulation. The placenta and fetal membranes produce CRH (98) and immunohistochemical studies have shown

intense CRH staining at the syncytiotrophoblast layer of the chorionic villi, fetal membranes and the invasive trophoblast cells in the decidua(99;100). Therefore, any measurable CRH mRNA in maternal circulation should come from the placenta. The measurement of fetal corticotrophin releasing hormone mRNA (CRH mRNA) can therefore be used as a measure of placental-derived cell debris in maternal systemic circulation.

Fetal CRH mRNA levels have been measured in maternal plasma in healthy pregnancy and have been found to increase as pregnancy progresses(101) and in labour(102). The presence of fetal CRH mRNA was found to clear from maternal circulation within 2 hours after caesarean delivery(103). In pre-eclampsia, higher levels of placental-derived CRH have been found in the maternal serum as well as in the placental circulation compared to healthy pregnant controls(104;105) and the fetal CRH mRNA concentration in maternal plasma has also found to be higher in pre-eclampsia compared to healthy pregnant controls(106;107). Fetal CRH mRNA levels have been found to be higher in early-onset pre-eclamptics compared to late-onset cases, possibly reflecting increased placental damage in early onset pre-eclamptic patients(107).

1.4 Blood coagulation (haemostasis)

In order to place the coagulation changes in pregnancy and pre-eclampsia in context, it is useful to review the function of various coagulation factors in normal haemostasis and then examine the changes in pregnancy and pre-eclampsia. Normal haemostasis is an important physiological reaction to prevent loss of blood from damaged blood vessels into the extravascular space. This is achieved by the formation of a localised insoluble blood clot (a fibrin mesh) to plug the damaged area of the blood vessel. As the damage is repaired, the clot is gradually removed by fibrinolysis.

Haemostasis is initiated and terminated in a controlled fashion. This is necessary to ensure formation of a blood clot in the correct location and to prevent excessive propagation of the clot. Various components of blood and the blood vessel wall are involved in haemostasis, such as the haemostatic factors, platelets, tissue factor and the endothelium. A

fine balance of activation and inactivation of various coagulation factors is necessary to control clotting and this occurs by various feedback loops in the haemostatic network. Dissolution of the fibrin mesh is controlled by the plasminogen activation system. The normal haemostatic network will now be reviewed.

1.4.1 The haemostatic network

With reference to figure 1.4.1, there are two main pathways (the intrinsic pathway and the extrinsic pathway) that leads to the formation of activated factor X (FXa) which is involved in the formation of the prothrombinase complex. The intrinsic pathway is initiated when the proteins prekallikrein, kallikrein, factor XI (FXI) and factor XII (FXII) are exposed to a negatively-charged surface such as collagen on the blood vessel wall. This is the contact phase. Prekallikrein is converted to kallikrein which then activates FXII. Activated factor XII (FXIIa) hydrolyses more prekallikrein to kallikrein thus amplifying this cascade. FXIIa also activates factor XI (FXI). Activated factor XI (FXIa) in the presence of calcium ions then activates factor IX (FIX). The main function of activated factor IX (FIXa) is to combine with activated factor VIII (FVIIIa) to form the factor Xase (FXase) complex. The FXase complex then activates factor X (FX).

The extrinsic pathway (see figure 1.4.1) is initiated when circulating activated factor VII (FVIIa) comes into contact with tissue factor (TF). Tissue factor is normally located at the subendothelial matrix of blood vessels. Damage to the vasculature leads to circulating FVIIa coming into contact with TF at the subendothelial matrix leading to formation of the FVIIa-TF complex which then activates factor X (FX). Activated factor X (FXa) then activates factor V by proteolysis. FXa assembles with prothrombin, activated factor V (FVa) and calcium ions on the phospholipid surface provided by cell membranes *in vivo* to form the prothrombinase complex. FXa can also directly generate small amounts of thrombin from prothrombin (the ‘initiation’ stage) and these small amounts of thrombin back-activate FV and factor VIII (FVIII) initiating the ‘amplification’ phase of coagulation.

Prothrombin is a protein. It is converted to thrombin by cleavage of 2 peptide bonds by the prothrombinase complex. Fully cleaved thrombin is termed α-thrombin and is active in free solution. Thrombin cleaves the fibrinogen molecule at it's α- and β- chains thus exposing N-terminal sequences that allow attachment of another cleaved fibrinogen molecule. This allows progressive polymerization in either direction of cleaved fibrinogen molecules thus forming elongating fibrils of fibrin, the blood clot. Thrombin has several positive feedback mechanisms to enhance coagulation (see figure 1.4.1). Thrombin can activate factor V. Thrombin can also activate circulating factor XIII to form activated factor XIII (FXIIIa) which functions to crosslink fibrin and other proteins in the clot thereby stabilising the whole clot structure. Factor VIII (FVIII) circulates in blood bound to VonWillebrand Factor (vWF) and is proteolysed by thrombin to form activated factor VIII (FVIIIa) which functions as an essential co-factor in the FXase activation of Factor X. Thrombin can also affect the intrinsic coagulation cascade by cleaving FXI into FXIa.

There are several anticoagulant pathways in the haemostatic network (see figure 1.4.1). Protein C, a potent anticoagulant is synthesized by the liver and is activated by cleavage to form activated protein C (APC). APC acts to inactivate FVa as well as FVIIIa, thus interfering with the formation of the prothrombinase complex as well as the FXase complex. The cleavage of protein C to APC can be accelerated by thrombin, thus acting as a negative feedback loop toward the generation of further thrombin. Antithrombin (AT) is synthesized by the liver and functions to inactivate thrombin by cleavage resulting in an inactive complex, the thrombin-antithrombin complex (TAT). Antithrombin can also inactivate FIXa, FXIa and FXIIIa, but to a much lesser degree than thrombin itself. Tissue factor pathway inhibitor (TFPI) is synthesized by platelets, endothelial cells, smooth muscle cells, monocytes and fibroblasts. When there is endothelial damage, platelets aggregate and when activated by thrombin, release TFPI into the site of coagulation. TFPI forms a complex with TF-FVIIa-FXa, inactivating these co-factors and directly affecting the formation of the prothrombinase complex.

1.4.2 The plasminogen-activation system of fibrinolysis

Fibrinolysis is important to ensure that fibrin deposition does not exceed beyond that which is required to prevent loss of blood from the vasculature. Following repair to the

vasculature, the process of fibrinolysis ensures that the fibrin mesh is removed as part of the process of tissue repair and remodelling. Fibrinolysis mainly occurs on the surface of cells in close proximity with the cross-linked fibrin itself. These steps are illustrated with reference to figure 1.4.2.

Factor XII (FXII) is known as Hageman factor and is synthesised in the liver. FXII is cleaved by kallikrein and plasmin into activated FXII (FXIIa). FXIIa activates prekallikrein on the surface of endothelial cells to become kallikrein. Kallikrein then breaks down high molecular weight kininogen (HMWK) to bradykinin. Bradykinin then stimulates the release of endothelial cell tissue-type plasminogen activator (tPA) from endothelial cells(108). tPA is also secreted by endothelial cells when stimulated by other factors such as venous occlusion, adrenaline and thrombin. Circulating tPA is inactive until it becomes bound to fibrin, forming tPA-fibrin which converts fibrin-bound plasminogen to plasmin. Urinary plasminogen activator (uPA) is synthesised by kidney tubules and collecting ducts. uPA is secreted in an inactive form (pro-urokinase) which is cleaved by kallikrein and plasmin to become active uPA.

Plasmin plays a major role in the plasminogen-activation pathway of fibrinolysis (see figure 1.4.2). Plasmin hydrolyses the fibrinogen and fibrin components of the blood clot into small soluble peptides [known collectively as fibrin degradation products (FDPs)] leading to clot dissolution. Fibrin-bound plasminogen is converted to plasmin by tPA and uPA. It can be seen that fibrin combines with tPA to activate plasmin. Thus, fibrin plays a major role in its own lysis. Plasmin can enhance its anticoagulant effect indirectly by activating FXII (thus leading to greater conversion of prekallikrein to kallikrein) as well as by activating pro-urokinase to active uPA. The action of these plasminogen activators are regulated by plasminogen activator inhibitors (PAI) (see figure 1.4.2). Plasminogen activator inhibitor 1 (PAI-1) is secreted by endothelial cells and platelets and functions to inactivate tPA, uPA and plasmin. Plasminogen activator inhibitor 2 (PAI-2) is mainly produced by the placenta where it is synthesised by monocytes and trophoblast cells. It acts to bind both tPA and uPA but its potency is at least 10-fold less than PAI-1.

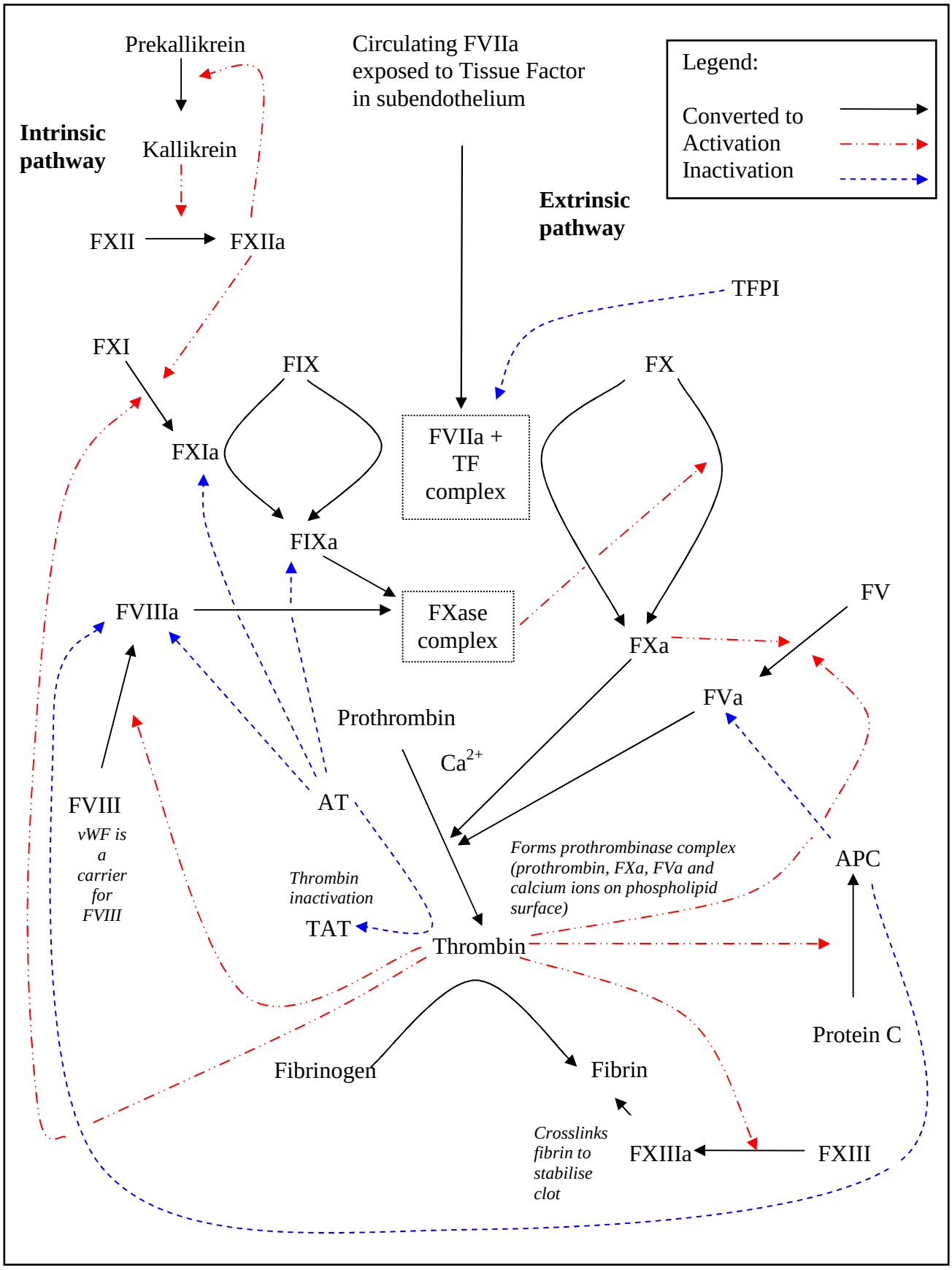


Figure 1.4.1: The haemostasis network (adapted from Postgraduate Haematology 5th Edition, Blackwell Publishing 2005. Hoffbrand AV, Catovsky D, Tuddenham EDG).(24)

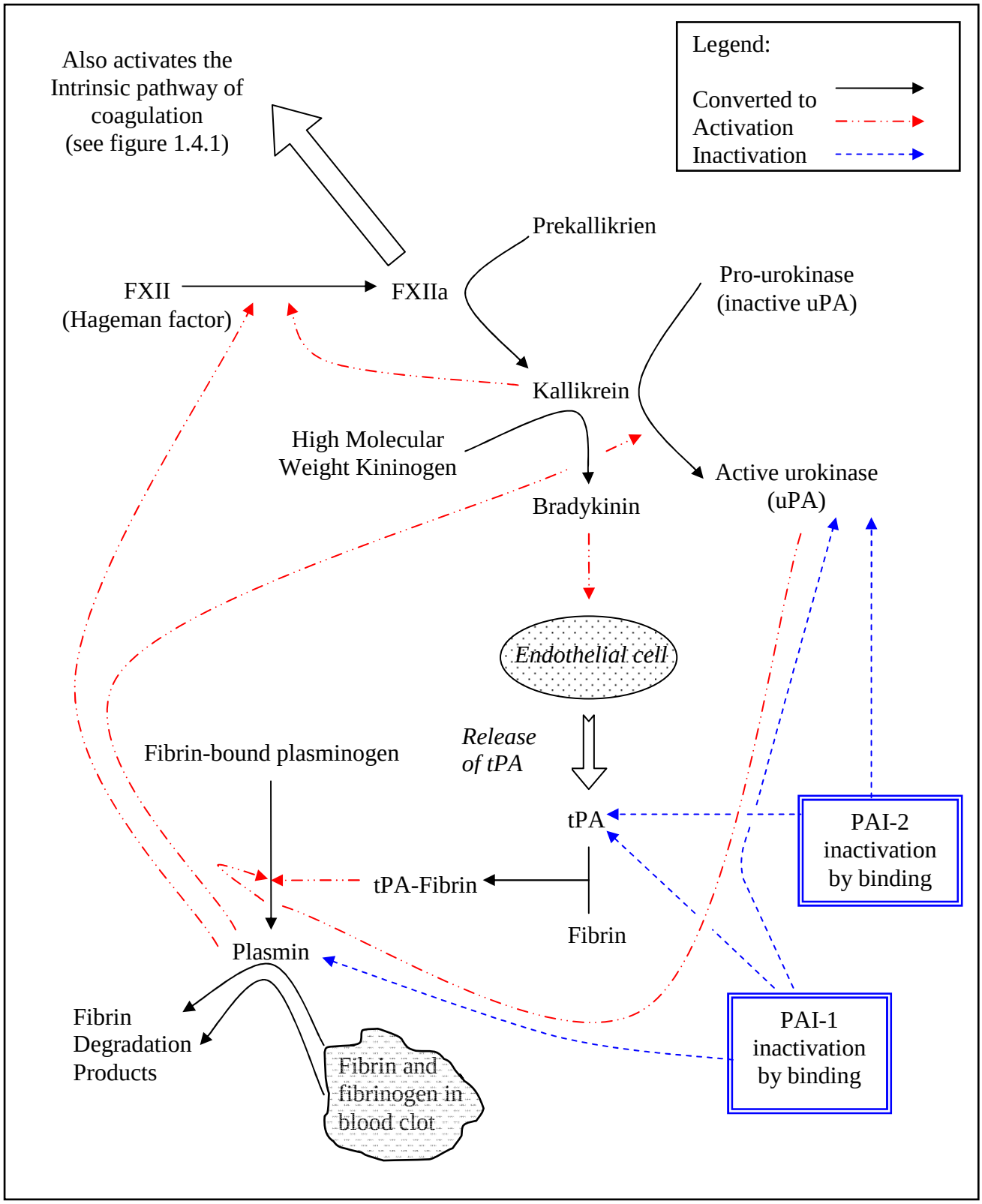


Figure 1.4.2: The plasminogen-activation pathway of fibrinolysis (Adapted from Postgraduate Haematology 5th Edition, Blackwell Publishing 2005. Hoffbrand AV, Catovsky D, Tuddenham EDG)(24)

1.4.3 Markers of coagulation in pregnancy and pre-eclampsia

1.4.3.1 Soluble Tissue Factor

Tissue factor is not expressed by the endothelium or circulating monocytes under normal physiological conditions and therefore, there is little appreciable contact of TF with circulating blood. However, when stimulated by various cytokines or mediators, TF can be expressed by the endothelium, monocytes and macrophages [reviewed in(22;23)]. The endothelium has been shown to express TF when stimulated by cytokines such as tumour necrosis factor-α (TNF-α) and interleukin 1-β (IL-β)(109) or when stimulated by mediators such as thrombin, oxidized LDL(110) or vascular endothelial growth factor. Monocytes can express tissue factor when stimulated by endotoxins, C-reactive protein(CRP) and oxidised LDL (111)[reviewed in(23)].

Soluble TF has been found to be present in blood(112) as well as expressed by endothelial cells after stimulation by proinflammatory cytokines such as TNFα and interleukin-6(113). Giesen *et al* (112) has identified neutrophils and monocytes containing tissue factor in the circulation and this tissue factor was found to be actively procoagulant. Higher plasma levels of soluble TF have been associated with sepsis, atherosclerosis and diabetes [reviewed in (114)], diseases which are associated with a procoagulant state. Therefore, increased levels of tissue factor reflect a state of increased coagulation potential.

TF expression by monocytes was found to be significantly lower in healthy pregnancy compared to non-pregnant controls(115). In pre-eclampsia, plasma levels of TF has been found to be significantly elevated compared to healthy pregnant controls(28;116;117). However in a prospective study involving 2190 pregnant women, Djurovic *et al* did not find any difference in plasma TF levels at 18 weeks' gestation between women who subsequently developed pre-eclampsia and their matched controls(118).

1.4.3.2 Tissue Factor Pathway Inhibitor (TFPI)

Tissue factor pathway inhibitor (TFPI) antagonises the effects of TF by inactivating the FVIIa/TF complex, thereby preventing the production of FIXa and FXa(119). Higher plasma levels of TFPI have been found in various pro-thrombotic conditions such as acute myocardial infarction, diabetes mellitus and disseminated intravascular coagulation [reviewed in(119;120)]. Plasma levels of TFPI have also been found to be raised in hypercholesterolemic patients and TFPI was strongly correlated with total cholesterol and low density lipoprotein levels(121). It was hypothesised that this increase may be a compensatory mechanism to prevent activation of the extrinsic coagulation system by tissue factor and factor VII in hypercholesterolemic patients(121).

TFPI can be synthesized by the liver, monocytes, macrophages, megakaryocytes and cultured human umbilical vein endothelial cells. TFPI is produced constitutively by microvascular endothelial cells [reviewed in (119)]. Approximately 50 to 80% of TFPI is located in the endothelium with 10 to 50% circulating in plasma and a small amount in platelets [reviewed in (122)]. Plasma levels of TFPI has been shown to be positively associated with markers of endothelial cell activation such as t-PA, thrombomodulin and von Willebrand factor (vWF)(123). The plasma form of TFPI may therefore be a marker of endothelial cell activation(120). In the placenta, TFPI has been found to be expressed by cytotrophoblasts, syncytiotrophoblasts and the vascular endothelium(124). Cultured syncytiotrophoblasts express higher TF levels but lower TFPI levels in comparison to cultured human umbilical vein endothelial cells. This suggests there is a need for an increased pro-coagulant activity in the placental intervillous space, perhaps in preparation to prevent excessive haemorrhage [reviewed in (122)]. Higher levels of TFPI in plasma have been found in pre-eclamptic patients compared to healthy pregnant controls(117;125;126).

1.4.3.3 Thrombin-antithrombin complex

Once thrombin is formed, it circulates in the systemic circulation bound to endogenous serine protease inhibitors such as antithrombin. This results in an inactive complex, the

thrombin-antithrombin complex (TAT)(24). A measurement of TAT levels can therefore be used to reflect the amount of thrombin in circulation(127). Serum TAT levels have been found to gradually increase as pregnancy progresses(88;128-131). In one study, the median TAT levels went up between 4.1 to 7.8 times from the start of labour to the end stages of labour(132) which may reflect increased levels of circulating thrombin in preparation for haemostasis at delivery. TAT levels have been found to reduce and normalise approximately 24 hours postpartum(128).

Higher TAT levels have been found in the maternal plasma of pregnancies complicated by pre-eclampsia when compared against healthy pregnant controls(125;128;129;133) and TAT levels have been found to significantly increase in tandem with both systolic and diastolic blood pressures(88). In pre-eclampsia, higher TAT levels have been found in uterine veins compared to the maternal systemic circulation, which may reflect increased pro-coagulant activity of the placental circulation in pre-eclampsia(134). Lower levels of anti-thrombin have been found in patients with severe pre-eclampsia(125;129;133) possibly reflecting antithrombin utilisation by increased levels of thrombin in this pro-coagulant state. In an attempt to reduce the harmful effects of increased coagulation in pre-eclampsia, a double-blinded randomised controlled trial was undertaken to administer antithrombin to patients with pre-eclampsia. Antithrombin was administered over a 7 day period which resulted in an improvement in maternal symptoms, fetal biophysical profile and fetal weight gain(135). This suggested a beneficial effect of antithrombin, possibly by reducing available circulating thrombin, reducing coagulation activation, reducing placental thrombosis and leading to a consequent improvement in the clinical condition.

1.4.3.4 Prothrombin fragment 1+2

During the conversion of prothrombin to thrombin, prothrombin fragment 1+2 (F1+2) is released. F1+2 may therefore be used as a surrogate marker to quantify the amount of thrombin generated(127;136). Increased levels of F1+2 have been found in various pro-thrombotic conditions such as end-stage renal disease(137), disseminated intravascular coagulation(138), septic shock(139), haematological malignancies(140), unstable angina and acute myocardial infarction(140-142).

In healthy pregnancy, it was found that there was a progressive rise in F1+2 levels as pregnancy progressed(131;140;142-144). This rise was found to be associated with increasing levels of fibrinogen(143), TAT(88;131;140) and APC resistance(88). In pre-eclampsia, levels of F1+2 were found to be higher when compared to healthy pregnant patients(88). Patients who had a history of severe pre-eclampsia were found to have increased levels of F1+2 and thrombin-antithrombin(TAT) six to fifteen months after delivery(145).

1.4.3.5 Factor VIIc

Factor VII (FVII) is activated by cleavage, yielding a 2-chain disulphide-linked FVIIa molecule. With damage to the blood vessel and disruption of the endothelial cell layer, circulating factor VIIa comes into contact with tissue factor at the subendothelial matrix. This leads to the formation of the TF-FVIIa complex that initiates coagulation by directly activating FX and FIX(24). Recombinant FVIIa has been successfully used as a procoagulant to control excessive postpartum haemorrhage(146) and also in bleeding disorders such as haemophilia(147). The factor VII coagulant activity (FVIIc) can be measured in a bioassay that is sensitive to FVIIa, which would be a more accurate assessment of the activity of FVIIa in comparison to measuring levels of FVIIa antigen in blood. FVIIc levels have been found to be raised in healthy pregnancy(148). There is little in the literature regarding FVII levels in pre-eclampsia although one study did not find any significant difference in FVII levels between women who had hypertension and superimposed pre-eclampsia and healthy control groups(149). Postnatally, FVII levels have been found to be significantly raised in pre-eclamptic pregnancies 6 to 15 months after delivery compared to healthy pregnant controls(145).

1.4.3.6 Factor XIIa

The precise role of FXIIa is not well defined as FXIIa may have both pro- and anti-coagulant effects. It has a pro-coagulant effect in that it activates factor XI to become factor XIa which participates in the intrinsic coagulation pathway. It also has anti-coagulant effects in that it leads to production of plasmin which is involved in blood clot

dissolution. FXII deficiency has been found in recurrent venous thrombosis(150), but found to be raised in diabetes mellitus(151) and septic shock(139;152). Several studies have shown an association of FXII deficiency with recurrent miscarriage(153-155) and as early pregnancy loss is associated with placental thrombosis, FXII deficiency may predispose to thrombosis.

In normal pregnancy, FXII has been shown to be raised up to 150% in the third trimester compared to non-pregnant controls(156). Higher levels of FXIIa were also found in the third trimester compared to the first trimester(152). A longitudinal study of FXIIa in normal pregnancy showed a progressive increase of FXIIa in pregnancy but which decreased postpartum. This increase was most significant at 3 points between 12 to 35 weeks' gestation(157). In a study of 12 pre-eclamptic patients, FXII levels were significantly higher compared to healthy pregnant controls(158). Therefore, FXII levels appear to be raised in pregnancy and further raised in pre-eclampsia.

1.4.3.7 Activated protein C (APC)

FVIIIa and FVa are potent coagulation factors in the formation of the FXase and prothrombinase complexes respectively. FVIIIa and FVa activity is inhibited by the 'protein C pathway'. Protein C is a vitamin K-dependent serine protease that is synthesised in the liver. Protein C is activated by cleavage of its heavy chain by thrombin to form activated protein C (APC). This reaction is accelerated on the surface of endothelial cells by receptors for both thrombin and protein C. APC functions to bind to Protein S in the presence of thrombin and thrombomodulin leading to the inactivation of FVa and FVIIIa in the extrinsic coagulation cascade. Therefore, APC is anticoagulant in nature [reviewed in(24)].

The activity of protein C can be measured as the APC ratio. A lower ratio corresponds to APC resistance and reduced anticoagulant properties. The APC ratio has been found to progressively reduce in healthy pregnancy and this is most significant after 20 weeks' gestation(142;159;160). APC resistance has been found to be higher in patients who suffered first and second trimester losses(161). Administration of APC has been found to

reverse the effects of IUGR induced by procoagulant phospholipid vesicles in mice(162). A point mutation in the factor V gene (FV:Q506) causes a condition known as factor V Leiden which leads to APC resistance. A prospective study of 2480 women in early pregnancy showed less intrapartum haemorrhage in patients who had factor V Leiden(163). Factor V Leiden is also associated with an increased risk of developing pre-eclampsia (88;164-166).

1.4.3.8 Plasminogen activator inhibitor type 1 (PAI-1)

Plasminogen activator inhibitor type 1 (PAI-1) is produced by endothelial cells, platelets, kidney tubule cells, adipose tissue, liver, the placenta and myometrium(167). Active PAI-1 may be freely circulating in plasma or bound to and stabilised in its active form by vitronectin which is found in abundance in the subendothelial matrix. With damage to the endothelium and activation of the pro-coagulant system, PAI-1 bound to vitronectin in the subendothelial matrix is ideally placed to participate in and influence the process of clot dissolution and vascular repair(168). Platelets store large amounts of PAI-1 within its α-granules and is released during platelet aggregation(168). PAI-1 indirectly inhibits the process of fibrinolysis by binding and inactivating the 3 main proteases involved in fibrinolysis (tPA, uPA and plasmin). These complexes are then removed by the liver. PAI-1 is the primary inhibitor of tPA. Raised PAI-1 levels have been found in obesity, ischemic cardiovascular disease(169) and type II diabetes(24).

In normal pregnancy, there is an increase in maternal plasma levels of PAI-1(6;170) but this has been found to fall quickly after delivery(170-172). Maternal plasma levels of PAI-1 in pre-eclampsia have been found to be significantly raised when compared to healthy pregnant controls(125;173-175). Patients with pre-eclampsia with extensive placental infarction have been found to have increased plasma levels of PAI-1 when compared to pre-eclamptics without extensive placental infarction(172). However in cases of pre-eclampsia complicated with IUGR, lower levels of PAI-1 in maternal serum have been found compared to patients with just pre-eclampsia alone(125). Greater levels of PAI-1 have been found in the syncytiotrophoblasts of pre-eclamptic patients compared to normal healthy controls, with greater PAI-1 expression at areas of placental infarction(176).

1.4.4 Markers of vascular inflammation and endothelial function

1.4.4.1 Intercellular adhesion molecule1 (ICAM-1)

Intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) belong to the immunoglobulin superfamily of Cell Adhesion Molecules (CAMs). ICAM-1 and VCAM-1 are involved in the leukocyte-endothelial cell adhesion cascade, where circulating leukocytes are attracted to areas of inflammation. In the leukocyte-endothelial cell adhesion cascade, circulating leukocytes are attracted by chemotaxis to the site of inflammation and margination towards the vascular endothelium occurs mediated by E- and P-selectin. Integrins on the surface of the leukocytes interact with ICAM-1 on the surface of the endothelium leading to leukocyte adherence. This then leads to leukocyte flattening and eventual transmigration through junctions between endothelial cells into the basement membrane. After a brief pause, they migrate into the interstitial tissue [reviewed in(177)]. In inflammation, pro-inflammatory cytokines such as interleukin-1, tumour necrosis factor-α and interferon-γ activate endothelial cells and leukocytes leading to shedding of CAMs from their surface into the systemic circulation. This leads to the presence of soluble ICAM-1(sICAM-1) and soluble VCAM-1(sVCAM-1) in plasma. Thus, measurement of these soluble forms may serve as markers of endothelial activation and vascular inflammation [reviewed in(178)]. ICAM-1 is produced by macrophages and endothelial cells and is present on these cell surfaces.

ICAM-1 may have a role in coagulation as a relationship has been discovered between ICAM-1 expression and acquired APC resistance(179). Monocytes have thrombin receptors and thrombin has been shown to upregulate monocyte ICAM-1 expression *in vitro*(179). Soluble ICAM-1 levels have been measured in the maternal plasma of healthy pregnant women and no significant difference in levels have been found between pregnant and non-pregnant women(180;181) although Clark *et al* noted higher levels of monocyte-derived ICAM-1 in healthy pregnant compared to non-pregnant women(179). Heimrath *et al* noted higher levels of sICAM-1 in patients with pregnancy-induced hypertension (PIH), compared to healthy pregnant controls(182). In patients with pregnancy-induced hypertension, higher levels of ICAM-1 have been found on the surface of lymphocytes in the peripheral circulation as well as decidual lymphocytes, with decidual lymphocytes

expressing greater amounts of ICAM-1 than peripheral lymphocytes(183). In IUGR patients without pre-eclampsia, sICAM-1 levels in maternal plasma have been found to be higher when compared to healthy pregnant controls(184).

In pre-eclampsia, the literature is conflicting with several studies showing maternal plasma sICAM-1 levels significantly higher in pre-eclamptic patients compared to healthy pregnant controls(181;184;185) but in a study of 55 pre-eclamptic patients, Chaiworaposanga *et al* did not find any significant difference in sICAM-1 levels between pre-eclamptic and healthy pregnant controls(180). Kim *et al* also did not find any significant difference in sICAM-1 levels between healthy pregnant and mild pre-eclamptic women but sICAM-1 levels were significantly higher in severely pre-eclamptic women compared to the healthy pregnant patients(186). Using immunocytochemistry, Lyall *et al* did not find any difference in ICAM-1 expression on the endothelium between pre-eclamptic and healthy controls(187;188). In the placenta, ICAM-1 was found to be expressed by the endothelium of spiral arteries and the trophoblastic cells. There was no difference in the amount of ICAM-1 expression between pre-eclamptic pregnancies and healthy pregnant controls(189).

1.4.4.2 Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 is produced mainly by endothelial cells but can also be expressed by macrophages, myoblasts and dendritic cells(190). Maternal serum soluble VCAM-1 (sVCAM-1) levels have been measured in normal healthy pregnancy and no significant differences were found between healthy pregnant and non-pregnant controls(180;181). However maternal plasma sVCAM-1 levels have been found to be higher in IUGR pregnancies compared to healthy pregnant controls(184). In pre-eclampsia, several studies have shown that maternal plasma levels of sVCAM-1 were significantly raised in pre-eclamptic pregnancies compared to healthy pregnant controls (180;181;184;186;191). sVCAM-1 levels have also been shown to be significantly higher in severe pre-eclampsia compared to mild pre-eclampsia(186;191). In pre-eclamptic pregnancies complicated with IUGR, greater levels of sVCAM-1 have been found in pre-eclamptic patients with IUGR compared with patients who had pre-eclampsia without IUGR(192). A prospective longitudinal study of 1543 pregnancies showed that taken together, raised levels of

prenatal sICAM-1 and sVCAM-1 had an overall predictive value of 64% towards developing pre-eclampsia(193).

Lyall *et al* detected significantly higher levels of sVCAM-1 in pre-eclamptic patients, compared to healthy pregnant controls(187;194). Heyl *et al* detected increased amounts of VCAM-1 and ICAM-1 on the surface of cultured human umbilical vein endothelial cells after stimulation with sera from pre-eclamptic pregnancies suggesting that there was endothelial cell activation as a result of exposure to sera(195). However, using immunocytochemistry, no difference was found in the amount of VCAM-1 expression in the endothelium of spiral arteries and the trophoblastic cells of pre-eclamptic pregnancies compared to healthy pregnant controls suggesting that the increased levels of sVCAM-1 in pre-eclampsia may not be of placental origin(189;196).

1.4.4.3 Von Willebrand factor

Von Willebrand factor (vWF) is synthesised predominantly within vascular endothelial cells(197). Von Willebrand factor may be released at a constant rate into plasma (constitutive pathway) or may be stored within Weibel-Palade bodies in endothelial cells to be released when stimulated (regulated pathway). Megakaryocytes also synthesise vWF which are stored in the α-granules of platelets(198). However, very little platelet-synthesized vWF is released into plasma. The release of vWF is stimulated by thrombin. There are two functions of vWF in haemostasis; it serves as a carrier for factor VIII by forming a complex with circulating factor VIII to protect it from degradation and vWF also functions to mediate platelet adhesion to the vascular subendothelium. Damage to the vasculature exposes circulating blood to collagen which is present at the subendothelial matrix. Circulating vWF attaches to collagen leading to platelet adhesion, aggregation and platelet activation with the release of α-granules at the site of injury. Locally-activated endothelial cells can also release stored vWF. Thrombin formation at the site of injury further stimulates vWF release. This leads to an increased local concentration of vWF at the site of injury encouraging further platelet plugging at the site of endothelial damage. The physiological release of vWF by the endothelium allows it to be a useful marker of endothelial activation. Increased levels of vWF have been found in various prothrombotic

conditions such as deep venous thrombosis(199;200), active inflammatory bowel disease(201) and acute stroke(202).

Von Willebrand factor levels rise as pregnancy progresses and levels resolve to normal after delivery(203;204). Von Willebrand factor is inactivated by cleavage of a plasma metalloproteinase; a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS 13) and ADAMTS 13 activity in healthy pregnant women have been found to reduce from around 12 weeks' gestation until early puerperium, then found to increase again(205). In patients with pregnancy-induced hypertension, vWF levels have been found to be higher compared to healthy pregnant controls(206) and plasma levels of vWF were found to be linearly correlated to the severity of pregnancy-induced hypertension(207). Higher levels of vWF have been found in women with pre-eclampsia compared to healthy pregnant controls(208-210). Elevated levels of vWF were also found in patients with HELLP syndrome compared to healthy pregnant controls. However levels of vWF returned to normal when measured 6 months postpartum(211). Patients with HELLP syndrome also showed reduced levels of ADAMTS 13 when compared to healthy pregnant controls. However level of ADAMTS 13 returned to normal when measured 6 months postpartum(211).

1.4.5 Measure of placental function

1.4.5.1 Plasminogen activator inhibitor type 2 (PAI-2)

PAI-2 is a serpin and is produced by placental villous syncytiotrophoblasts as well as monocytes and macrophages. PAI-2 is mainly located intracellularly but has been found to be released during cell activation or apoptosis [reviewed in (212)]. PAI-2 functions to inhibit fibrinolysis by inhibiting uPA and tPA in the plasminogen activation system of fibrinolysis by forming stable complexes. Inactivation of uPA and tPA leads to reduced conversion of plasminogen to plasmin and consequently reduced degradation of fibrin by plasmin. PAI-2 levels in plasma are normally undetectable but have been found to be raised in healthy pregnancy(170;172) and there is a progressive increase with increasing gestation(172). A positive correlation has been found between maternal serum PAI-2 levels

and birth weight in healthy pregnancy(172). After delivery, maternal PAI-2 levels may remain elevated for up to 11 days, suggesting that it may still be secreted from placental remnants still present in the uterus(172).

In pre-eclampsia, maternal serum PAI-2 levels have been found to be significantly lower compared to their healthy pregnant controls(125;170;172;213). Maternal plasma PAI-2 levels have also been found to be low in severe pre-eclampsia, with an associated increase in tPA levels (214). PAI-2 levels in pre-eclamptic patients with associated IUGR have been found to be lower compared to patients with pre-eclampsia alone(125). Immunohistochemical staining of PAI-2 and uPA antigens in pre-eclamptic placental tissue was not found to be as intense compared to healthy pregnant control placental tissue suggesting there is less PAI-2 expression in pre-eclampsia(170). The PAI-1/PAI-2 ratio has been found to be significantly raised in pre-eclamptic pregnancies compared to healthy pregnant controls(213). As plasma levels of PAI-1 have been found to be raised in pre-eclampsia and the plasma levels of PAI-2 decreased in pre-eclampsia, the PAI-1/PAI-2 ratio can be a useful marker for pre-eclampsia. Indeed, 2 prospective studies found elevated PAI-1/PAI-2 ratios in pregnant women who subsequently went on to develop pre-eclampsia (215;216).

1.5 Eicosanoids in coagulation

1.5.1 Eicosanoids in coagulation and vasoactive properties

Platelets are small anucleate cells in the systemic circulation that play a critical role in thrombosis. Activated platelets provide a negatively-charged surface for the assembly of the prothrombinase complex by externalising phosphatidylserine onto the surface of its cell membrane. Activated platelets form a bridge with surrounding polymerised fibrin which is generated in the coagulation cascade. Activated platelets also provide additional coagulation factors such as factor V, factor VIII and fibrinogen. Platelet activation results in the exposure of phosphatidylserine on its plasma membrane surface as well as forms membrane blebs with the release of microparticles [reviewed in (217)].

Thromboxane is an eicosanoid and is involved in the process of coagulation, as activated platelets release thromboxane at the site of platelet adhesion. Released thromboxane in turn activates other platelets via G-protein coupled thromboxane receptors. This leads to a positive feedback loop of activation and recruitment of more platelets into the growing thrombus [reviewed in (217)]. Thromboxane is synthesised by platelets as well as by placental trophoblasts(218). Thromboxanes are synthesised *in vivo* in platelets from polyunsaturated fatty acids (PUFAs) liberated from cell membrane phospholipids by the action of phospholipase A_2.

Prostacyclin (PGI_2) is also an eicosanoid. Prostacyclin is synthesised mainly by the vascular endothelium and also by the vascular cells of the placenta, the membranes, the myometrium and the endothelium of umbilical, placental and uterine vessels(218) Prostacyclin is a major endothelium-derived inhibitor of platelet activation, acting through a G_s-coupled receptor [reviewed in (217)]. Prostacyclin has been shown to inhibit platelet adhesion and thrombus formation on vascular wall subendothelium(219). Prostacyclin, as well as being an inhibitor of platelet aggregation is also a potent vasodilator (220). Thromboxane and prostacyclin therefore have antagonistic effects.

1.5.2 Thromboxane and prostacyclin in pregnancy and pre-eclampsia

In healthy pregnancy, thromboxane and prostacyclin as produced in equal amounts(218). In pre-eclampsia, both the trophoblast and the placental villous core secrete higher levels of both thromboxane and prostacyclin compared to healthy placentas(221). Pre-eclamptic trophoblasts have been found to produce over three times as much thromboxane but less than 50% as much prostacyclin compared to healthy placentas(218;222). Greater amounts of thromboxane B_2 (the metabolite of thromboxane A_2) has been found compared to 6-keto-prostaglandin F1α (the metabolite of prostacyclin) in the maternal plasma of pre-eclamptic pregnancies compared to healthy pregnant controls(223). Higher levels of thromboxane B_2 and lower levels of 6-keto-prostaglandin F1α have also been found in the urine of pregnant women who have gone on to develop pre-eclampsia(224). A higher ratio of maternal urinary thromboxane A_3 to 6-keto-prostaglandin F1α has also been found in IUGR pregnancies compared to healthy controls(225).

Women with pre-eclampsia have also been found to have higher amounts of thromboxane-induced platelet aggregation compared to healthy controls in both early and late pregnancy(226). Plasma levels of prostacyclin are reduced in both mild and severe pre-eclampsia. In contrast, thromboxane levels were unchanged in mild pre-eclampsia but significantly higher in severe pre-eclampsia, which is consistent with platelet activation in pre-eclampsia(218). Cultured pre-eclamptic trophoblasts have been found to produce more thromboxane B_2 (a metabolite of thromboxane A_2) and phospholipase A_2 when compared to cultured trophoblasts from healthy pregnancies and the production of thromboxane B_2 and phospholipase A_2 was further increased when cultured under hypoxic conditions (227).

Thromboxane is a vasoactive substance and stimulates vasoconstriction. In contrast, prostacyclin is a vasodilator. In pre-eclampsia, there is failure of uterine spiral arteriole conversion by extravillous trophoblasts into large capacitance vessels. As a result of failure of conversion, these arterioles maintain their muscular coat and they would be influenced by the vasoconstrictive properties of thromboxane. Therefore, the increased thromboxane: prostacyclin ratio can lead to the features of pre-eclampsia such as vascular hypertension, increased platelet aggregation and reduced uteroplacental blood flow (218).

1.5.3 Thromboxane and prostacyclin synthesis from fatty acids

The eicosanoids thromboxane and prostacyclin are synthesised from their fatty acids precursors arachidonic acid and eicosapentaenoic acid (EPA) [illustrated in figure 1.5.3]. Arachidonic acid is the parent compound for the synthesis of the 2-series thromboxane and prostacyclin while EPA is the parent compound for the synthesis of the 3-series thromboxane and prostacyclin. The 2-series thromboxanes and prostacyclines have a greater potency of action than 3-series thromboxanes and prostacyclines. As the 2-series thromboxanes and prostacyclins are synthesised from n-6 fatty acids while 3-series thromboxanes and prostacyclins are synthesised from n-3 fatty acids, the balance of n-6 to n-3 fatty acids in the body can, by virtue of having greater substrate, determine the quantity of which of these types of eicosanoids will be produced. For example, a greater proportion of n-6 fatty acids to n-3 fatty acids in membrane phospholipids may shift the balance of

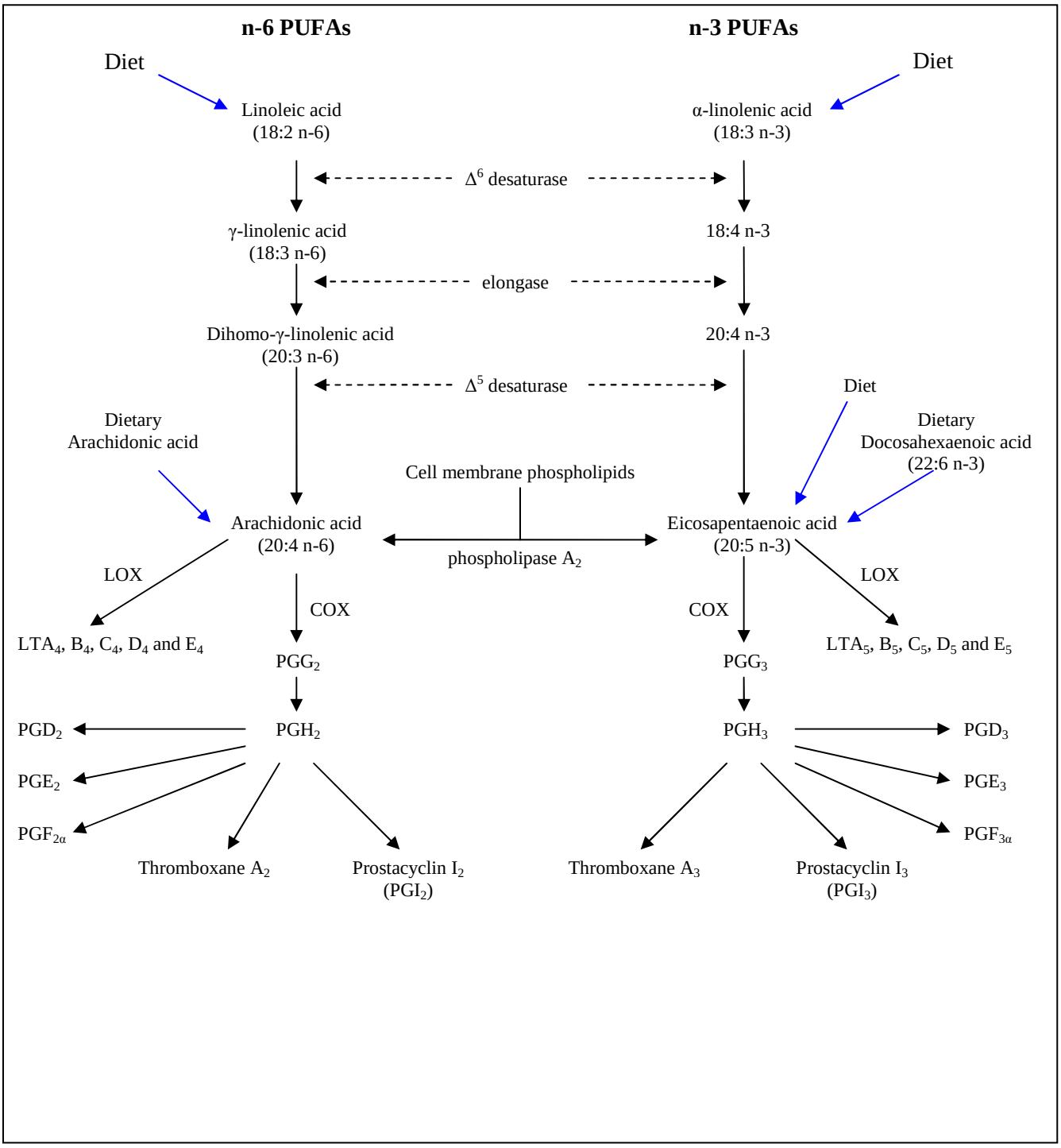


Figure 1.5.3: Eicosanoid synthesis from the essential fatty acid precursor's linolenic acid (n-6 series) and α-linolenic acid (n-3 series). Adapted from: Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. Larsson et al. *Am J Clin Nutr 2004.* LT, leucotrienes; PG, prostaglandins; LOX, lipoxygenase; COX, cyclooxygenase.

eicosanoid synthesis to favour the production of thromboxane A_2. As thromboxane A_2 has a greater potency of action compared to thromboxane A_3 [reviewed in (228)], greater thromboxane A_2 synthesis in platelets will lead to greater platelet activity and thus an greater procoagulant state.

1.5.4 Fatty acids

Fatty acids are important constituents of the phospholipid layer of cell membranes as well as precursors of eicosanoids. In polyunsaturated fatty acid (PUFA) metabolism in humans, the precursor of the n-6 series PUFAs is linoleic acid and the precursor of the n-3 series PUFAs is α-linolenic acid. Linoleic acid and α-linolenic acid are essential fatty acids. These precursors are metabolised into their respective products by the sequential actions of desaturases and elongases. These desaturases and elongases are shared between the different groups of fatty acids (such as the n-3, n-6, n-7 and n-9 series). There is competitive inhibition by these fatty acid groups for desaturases and elongases and the major determinant of which fatty acid group is preferentially metabolised into its products is the amount of substrate present. PUFAs are important precursors of eicosanoids such as thromboxanes, prostacyclins and leucotrienes (refer figure 1.5.4).

In Western diets, there is a tendency towards greater n-6 fatty acids intake compared to n-3 fatty acids and this leads to arachidonic acid being the predominant substrate for eicosanoid synthesis. This leads to the majority of eicosanoids produced in the body being of the 2-series prostaglandins (including thromboxane A_2) which have greater potency of action compared to the 3-series prostaglandins. Low levels of the n-3 fatty acids EPA and DHA have been found to be associated with increased risk of death from cardiovascular disease(229) and high intakes of n-3 fatty acids in diet are associated with a significant reduction in cardiovascular mortality(230). The balance of n-6 and n-3 fatty acids has been shown to affect platelet function as platelet cultures with the n-3 fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) showed a decreased platelet aggregation response to collagen(231) and in culture, DPA was found to suppress thromboxane A_2 formation by platelets which were exposed to collagen(232).

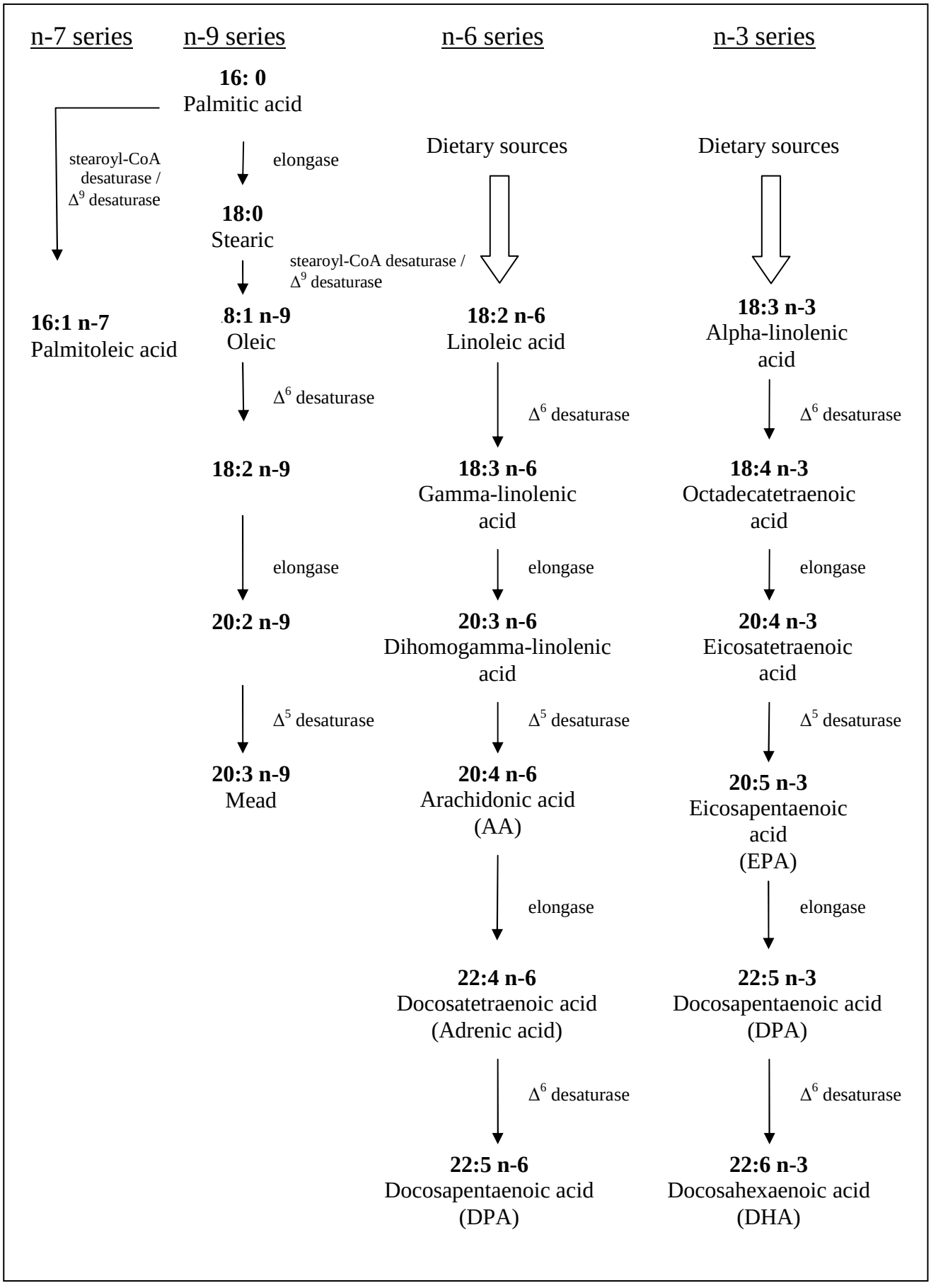


Figure 1.5.4: Pathways of the n-3, n-6, n-7 and n-9 groups of fatty acid metabolism. Adapted from: *Unsaturated fatty acids- Nutritional & physiological significance. The report of the British Nutrition Foundation Task Force (1992).*

1.5.4.1 Fatty acid synthesis

Fatty acids may be saturated, mono- or polyunsaturated. Saturated fatty acids do not have a double bond in its hydrocarbon chain. Monounsaturated fatty acids have a single double bond while polyunsaturated fatty acids have more than one double bond in its hydrocarbon chain. A double bond leads to a 'kink' in the hydrocarbon chain and a greater number of double bonds will lead to increased 'kinking'. Cell membranes are composed of phospholipid molecules arranged in a bilayer and phospholipid molecules have two fatty acid tails. Increased 'kinking' of fatty acids in adjacent phospholipids leads to the phospholipid molecules being less densely packed together leading to an increase in membrane fluidity. Therefore, greater amounts of polyunsaturated fatty acids in cell membranes lead to greater membrane fluidity(233).

Fatty acids may be obtained from the diet or synthesised by the human body (refer figure 1.5.4). Fatty acids that can be synthesised by the human body are termed non-essential fatty acids while fatty acids that cannot be synthesised in the human body are termed essential fatty acids. In mammals, fatty acids consisting of up to 16 carbons in length are synthesised by cytoplasmic fatty acid synthase (234). Further lengthening of the carbon chain is performed by elongase enzymes which are membrane-bound enzymes located in the endoplasmic reticulum. Elongases function to add 2 carbon units to the hydrocarbon chain of the fatty acid molecule thereby lengthening the hydrocarbon chain. There are different elongase systems specific for fatty acids of different chain lengths and different degrees of unsaturation [reviewed in(235)]. At present, the regulation of elongase enzymes are not fully understood [reviewed in(234)]. Desaturase enzymes introduce a double bond into the carbon chain of the fatty acid molecule. For example, Δ^5 desaturase introduces a double bond between carbon atoms 5 and 6 in dihomo-γ-linolenic acid to form arachidonic acid (20:4 n-6). Fatty acids of various series utilise the same elongase and desaturase enzymes and in fatty acid synthesis, there is competition of each of the fatty acid classes for the same elongation and desaturation enzymes [reviewed in(236)]. For example, both n-6 and n-3 class fatty acids use the same Δ^6 desaturase enzymes to introduce double bonds between carbon atoms 5 and 6 into their respective metabolites.

Stearoyl-Co-A desaturase (Δ^9 desaturase) is a highly regulated enzyme that catalyses a crucial step in the biosynthesis of monounsaturated fatty acids from saturated fatty acids leading to the synthesis of membrane phospholipids, triglycerides, cholesterol esters and alkyl-1,2-diacylglycerol. A deficiency in Δ^9 desaturase can lead to reduced fatty acid oxidation and thus, reduced lipid synthesis and storage. Δ^9 desaturase can also lead to increased insulin sensitivity and increased metabolic rate(237;238). It is known that there is dysfunction of lipid metabolism in pre-eclampsia and pre-eclampsia is associated with hypertriglyceridemia(239), raised low density lipoproteins and raised free fatty acids(240).

1.5.5 n-6 and n-3 series polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are fatty acids with 18 carbons or more in the hydrocarbon chain with two or more double bonds. PUFAs are important components of cell membranes and they confer fluidity and selective permeability to the cell membrane. Two important groups are the n-6 and n-3 PUFAs(235). The pathways of n-6 and n-3 fatty acid metabolism are illustrated in figure 1.5.4. The precursors of n-6 and n-3 fatty acid metabolism (linoleic acid and α-linolenic acid respectively) are classed as essential fatty acids as humans lack the 12-desaturase enzyme that converts oleic acid into linoleic acid. The human body also lacks the 15-desaturase enzyme and so cannot convert linoleic acid to α-linolenic acid. These desaturase enzymes are found in plants and so linoleic and α-linolenic acid must be obtained from the diet. Corn, sunflower and soybean oils are high in linoleic acid. Linseed oil is high in α-linolenic acid. In a typical diet of the UK population the main polyunsaturated fatty acid (PUFA) is linoleic acid (14g/day) with α-linolenic acid comprising approximately 2g/day(241).

Linoleic acid and α-linolenic acid are metabolised down their respective pathways into long-chain polyunsaturated fatty acids (LCPUFAs). The Δ^6 desaturase enzyme is the rate limiting enzyme in n-3 and n-6 metabolism to LCPUFAs and it has the highest affinity first for α-linolenic acid (n-3 PUFA), then linoleic acid (n-6 PUFA) and the lowest for oleic acid (n-9 PUFA)(242). In the n-6 series, linoleic acid is metabolised into dihomo-γ-linolenic acid (20:3 n-6) and arachidonic acid (20:4 n-6). In the n-3 series, α-linolenic acid is metabolised into eicosapentaenoic acid (20:5 n-3) (EPA), docosapentaenoic acid (22:5 n-3) (DPA) and docosahexaenoic acid (22:6 n-3) (DHA).

Arachidonic acid (20:4 n-6) is the most abundant PUFA in cell membranes and the typical Western diet contains approximately 20% of total long chain PUFAs as arachidonic acid, with the n-6 fatty acid dihomo-γ-linolenic acid making up 2%. In contrast, the n-3 fatty acid eicosapentaenoic acid makes up less than 1% of total fatty acids consumed [reviewed in(228)]. LCPUFAs may also be obtained directly from fish. Fish is high in n-3 fatty acids. In particular, oily fish such as herring, mackerel and sardines contain high amounts of the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are also high in the liver of certain fish, for example cod. Both EPA and DHA are present in 10 to 100-fold greater amounts in fish compared to land animals (243). Fish oil preparations contain approximately 20-30% EPA and DHA.

It has been suggested that humans originally evolved on a diet where n-6 to n-3 fatty acid intake was in the ratio of 1:1. In contrast, the current western diet n-6: n-3 ratio ranges from 10:1 to 25:1(244). As both the n-6 and n-3 fatty acids share desaturase and elongase enzymes, an increase in one class of fatty acid results in substrate competition for the shared desaturase and elongase enzymes leading to a reduced amount of metabolites of the other class. For example, an increase of the n-6 fatty acid linoleic acid in the diet will lead to an increased amount of its n-6 metabolites by the Δ^6 desaturase enzyme but will result in a reduced amount of n-3 fatty acid metabolites(242).

1.5.6 Fatty acids in healthy pregnancy and pre-eclampsia

1.5.6.1 Maternal fatty acid changes in healthy pregnancy

Plasma maternal fatty acids have been measured in many studies but were measured from plasma lipoproteins and thus affected by maternal fasting status. The results show a general trend towards an increase in absolute amounts of total fatty acids, n-6, n-3 fatty acids as well as percentages of some fatty acids(245-247) and this is in keeping with the increased state of lipolysis in pregnancy. A method of circumventing the problem of maternal fasting status would be to analyse the fatty acid composition of maternal erythrocyte cell membranes. The half-life of an erythrocyte if 120 days and so a measure of its membrane fatty acid composition would be representative of the mother's fatty acid status over the

preceding 3 months, although, Skeaff *et al* had noted that dietary-induced changes in the fatty acid composition of plasma fatty acids were reflected in erythrocyte cell membrane fatty acids after just 2 weeks(248). Nevertheless, a measure of fatty acids from erythrocyte membrane would not be confounded by the subject's immediate fasting status.

Stewart *et al* performed a longitudinal assessment of erythrocyte cell membrane fatty acid composition in 47 healthy pregnant women measured in each trimester of pregnancy(249). There were significant increases in the percentages and absolute amounts of DPA (22:5 n-6) α-linolenic acid (18:3 n-3) and DHA (22:6 n-3). There were also significant increases in the absolute amounts of palmitoleic acid (16:1 n-7) and nervonic acid (24:1 n-9) but not as a percentage of total fatty acids. These changes occurred between the first and second trimester(249).

Other groups have also measured erythrocyte fatty acid composition in pregnancy(250-254) and have found that in healthy pregnancy, with regards to n-6 fatty acids, linoleic acid, dihomo-γ-linolenic acid, arachidonic acid, and n-6 docosapentaenoic acid (n-6 DPA) were found to be higher either as a percentage of total fatty acids or in absolute amounts or both. With regards to n-3 fatty acids, α-linolenic acid and DHA were found to be higher both as a percentage of total fatty acids and in absolute amounts. With regards to other fatty acid classes, nervonic acid (24:1 n-9) and palmitoleic acid (16:1 n-7) were found to be higher in healthy pregnancy compared to healthy control. This general increase of fatty acids may be due to increased maternal mobilisation of fatty acids in healthy pregnancy. In the third trimester, the levels of fatty acids such as arachidonic acid, EPA, n-6 DPA and DHA may become reduced compared to the second trimester levels. Therefore, in healthy pregnancy, most fatty acids are increased as pregnancy progresses and this is probably as a result of increased maternal mobilisation from her stores(247). DHA has been found to be higher in women than in men and is further increased in women taking the combined contraceptive pill (which contains estrogen)(255). It is possible that estrogen promotes the synthesis of DHA which might explain why it can be raised in pregnancy. In the third trimester, some fatty acids such as arachidonic acid, EPA, n-6 DPA and DHA may be reduced. Arachidonic acid and DHA are important structural components of fetal central nervous system and so a reduction may be due to greater fetal accretion in the third trimester. In healthy pregnancy, supplementation of fish oil high in n-3 fatty acids was shown to be associated with a reduction of urinary excretion of thromboxane A_2

metabolites, suggesting that high dose n-3 supplementation in pregnancy may reduce maternal thromboxane A_2 synthesis(256).

1.5.6.2 Maternal fatty acid changes in pre-eclampsia

There is some evidence that n-3 fatty acids may be protective against the development of pre-eclampsia. A prospective cohort study of 1718 women found that individuals who had an increased intake of DHA and EPA (both n-3 PUFAs) were associated with a lower risk of developing pre-eclampsia(257). A case-control study of maternal erythrocyte n-3 and n-6 fatty acids of 99 pre-eclamptic and 100 healthy pregnant women was carried out and it was shown that low maternal levels of total n-3 fatty acids as well as low levels of EPA and DHA was associated with an increased risk of pre-eclampsia(258). It was also found that pregnant women with low levels of total n-3 fatty acids and pregnant women with the lowest ratio of n-3 to n-6 fatty acids had a higher risk of developing pre-eclampsia (258;259). However there was no reduced risk of developing pre-eclampsia with reduced n-6 fatty acid intake(257) and there was no association between arachidonic acid levels with risk of developing pre-eclampsia(258).

Various changes in fatty acid levels in maternal plasma have been noted in pre-eclampsia compared to healthy pregnant women. Serum fatty acids have been measured in the maternal plasma of pre-eclamptic pregnancies and there was a trend towards lower levels of total PUFAs (260;261) in pre-eclampsia. There were higher absolute levels of palmitic (16:0), palmitoleic (16:1 n-7), oleic (18:1 n-9) and dihomogamma-linoleic acid (20:3 n-6) in the plasma of pre-eclamptic women in late pregnancy compared to healthy pregnant controls(260;262). The relative percentage of 18:1n-9 to total fatty acids was found to increase and the relative percentage of linoleic acid (18:2 n-6) was found to decrease as pregnancy progressed in pre-eclamptic patients compared to healthy controls (260). Higher levels of arachidonic acid both as absolute levels (262)and as a percentage of total fatty acids have been found in pre-eclampsia(263). With regard to n-3 fatty acids, lower absolute amounts of total n-3 fatty acids, EPA and DHA have been found in pre-eclamptic patients compared to healthy pregnant controls(261;264). A greater n-6: n-3 ratio has also been found in pre-eclamptic patients compared to healthy controls(264). While these fatty

acid changes were noted in maternal plasma, there did not appear to be any studies measuring maternal erythrocyte cell membrane fatty acid changes in pre-eclampsia.

1.5.6.3 Fatty acid utilisation by the fetus

In the third trimester, the fetus begins to deposit fat subcutaneously and a healthy fetus at term would have deposited 500g of fat subcutaneously. Fetal adipose tissue is rich in saturated fatty acids, especially palmitic acid and oleic acid but is low in linoleic acid(265). In normal pregnancy, the developing fetus is dependent on the mother for its supply of essential fatty acids. Al *et al* showed supplementation of linoleic acid (n-6) rich foods from 20 weeks gestation resulted in a significant increase in the total amount of n-6 PUFAs and a significant reduction in n-3 PUFAs(266), while fish oil supplements (rich in n-3 fatty acids) given from 30 weeks gestation onwards led to a significant increase in the total amount of n-3 PUFAs (including DHA) with a significant reduction in n-6 PUFAs in cord blood (267). In the last trimester of pregnancy, the fetal requirements of arachidonic acid and DHA as especially high as there is rapid development of fetal retinal as well as brain tissue(247;268). DHA is selectively accumulated in the membrane phospholipids of the developing fetal retina and the brain grey matter [reviewed in (268)] where it is involved in the structure and function of the retina [reviewed in (269;270)]. A reduction in DHA supply to the fetus has been found to be associated with visual and learning difficulties (268). Placental transfer of arachidonic acid and DHA from the maternal circulation into the placenta occurs by active transport by fatty acid binding proteins and arachidonic acid and DHA are preferentially taken up by the placenta compared to their parent fatty acids linoleic acid and α-linolenic acid(269;271;272).

1.6 Aims and objectives

Pre-eclampsia is a state of enhanced coagulation as evidenced by an increased amount of clotting factors in maternal circulation and increased fibrin deposition in the placenta. Microparticles are released from apoptotic cells and may have procoagulant properties. Microparticle levels have been found to be raised in various prothrombotic diseases. My

primary hypothesis is that there may be a relationship between microparticle procoagulant activity and coagulation in the placenta as well as maternal circulation.

1.6.1 Coagulation in the placenta

There is greater trophoblast apoptosis in pre-eclampsia and IUGR and microparticles (which may be procoagulant in nature) are released from apoptotic cells and this may lead to fibrin deposition. Annexin V is located on the maternal surface of syncytiotrophoblasts and has anticoagulant properties. Tissue factor is expressed by the placenta and is a potent initiator of coagulation. I hypothesise that there may be associations between fibrin deposition, annexin V and tissue factor expression in pre-eclamptic, healthy and IUGR placentas. Immunocytochemistry was chosen as the method to localise fibrin, annexin V and tissue factor staining within the placenta. Back-to-back staining was used as a method to identify any relationships between these stainings.

1.6.2 Coagulation in maternal systemic circulation

The levels of circulating microparticles in plasma have been found to be raised in various prothrombotic diseases. As pre-eclampsia is a pro-thrombotic state with associated endothelial activation, platelet activation and trophoblast apoptosis, microparticles may play a significant part in this procoagulant state. Microparticle procoagulant activity has previously been measured using a plate-based microparticle capture assay(65). I wished to develop and validate this assay to measure microparticle procoagulant activity in pre-eclamptic patients and matched healthy pregnant control patients to identify if there is any difference in microparticle procoagulant activity between these two groups. With greater trophoblast apoptosis in pre-eclampsia, the placenta may be a significant source of microparticles in maternal circulation. The measure of fetal CRH mRNA in maternal blood would allow the identification and measure of placental cellular debris in maternal circulation.

In pre-eclampsia, there is coagulation activation, endothelial activation and reduced placental function. I hypothesised that there may be a relationship between microparticle procoagulant activity as well as placental cellular debris with coagulation activation, endothelial activation and placental function in pre-eclamptic and healthy pregnant patients.

1.6.3 Fatty acid changes in pre-eclampsia

Fatty acids of the n-6 and n-3 groups are precursors towards the synthesis of thromboxane and prostacyclin. The n-6 fatty acids are the precursors of the potent 2-series thromboxanes while n-3 fatty acids are precursors of the less potent 3-series thromboxanes. Thromboxane is a potent platelet aggregatory factor as well as vasoconstrictor. Pre-eclampsia is a state of enhanced coagulation along with maternal hypertension, placental ischemia and insufficiency. In pre-eclampsia, there is greater thromboxane and lesser prostacyclin synthesis by the placenta. There is also greater thromboxane-induced platelet aggregation in pre-eclampsia. I hypothesised that maternal fatty acid patterns may be different between pre-eclamptic and healthy pregnant women, which may lead to a shift of balance towards the synthesis of greater amounts of the potent thromboxane A_2. There may also be a relationship between the maternal fatty acid composition with maternal coagulation activation, endothelial function and placental function in pre-eclamptic and healthy pregnancies. Fatty acid composition was measured by gas chromatography.

Chapter 2 Methodology

2.1 Ethics approval

This project was approved by the Ethics Committee of North Glasgow NHS Trust at Glasgow Royal Infirmary and all recruits gave written informed consent.

2.2 Pre-eclamptic, healthy and type II diabetic subjects

2.2.1 Pre-eclamptic cases and healthy pregnant controls

Pre-eclamptic and healthy pregnant matched controls were recruited as the primary test subjects of this project. Pre-eclamptic patients and matched healthy pregnant controls were recruited in their third trimester of pregnancy (28 to 40 weeks' gestation) from Glasgow Royal Infirmary Maternity Unit. There were 32 pre-eclamptic patients and 32 healthy pregnant controls recruited into this study. They were matched by age (+/- 2 years), body mass index (BMI +/- 2 kg/m^2) and parity (0, 1, greater than 1). Pre-eclamptic patients were defined using the International Society for the Study of Hypertension in Pregnancy criteria (diastolic blood pressure greater than 110mmg Hg on one occasion, or greater than 90 mmHg on repeated readings, with proteinuria of greater than or equal to 0.3g / 24 hours or 2+ proteinuria on dipstick testing in the absence of renal disease or infection). Patients were excluded from this study if they had multiple gestation, any medical conditions which led to a procoagulant state or if they were on any anticoagulant medication such as aspirin or heparin. Patients with any suspected fetal anomalies which were likely to lead to intrauterine growth restriction were also excluded.

All pre-eclamptic and healthy pregnant controls were not in labour at the time of sampling. For each patient, blood pressure, smoking status as well as any treatment for hypertension was noted. Eleven pre-eclamptic patients were on labetalol at the time of sampling. These

patients were on treatment for less than 3 days prior to blood sampling. Pregnancy outcome data such as the mode of delivery, fetal weight, and sex of the fetus was obtained from the patients' notes after delivery. Placental weight was not always obtainable as it was seldom recorded. The fetal weight values were converted into birth weight centiles calculated using the Gestation Network Centile calculator version 5.4 from:

http://www.gestation.net/birthweight_centiles/centile_online.htm

2.2.2 Healthy volunteers

Healthy subjects were recruited as a pilot study to assess the range of microparticle procoagulant activity in healthy population. Healthy lab staff volunteers who did not have any medical problems and who were not on any medication were recruited. A total of 13 volunteers were recruited.

2.2.3 Complicated and uncomplicated type II diabetic patients

Type II diabetes is a chronic disease marked by high blood glucose levels which occurs as a result of insulin resistance. Diabetes may lead to retinopathy, neuropathy, nephropathy and cardiovascular disease and patients who develop these conditions are defined as 'complicated diabetics'. Diabetics without symptoms or signs of these complications are defined as 'uncomplicated diabetics'. Higher microparticle levels have been found in complicated type II diabetic patients compared to uncomplicated type II diabetics (273). This may translate to greater microparticle pro-coagulant activity in complicated type II diabetics compared to uncomplicated controls. Type II diabetic patients with and without complications were recruited as a pilot study to assess if differences in microparticle procoagulant activity could be found between complicated and uncomplicated type II diabetic patients. This data would also inform the power calculation for the main study.

Type II diabetic patients who attended for their annual review at the diabetic outpatients department at Glasgow Royal Infirmary were recruited. Six patients with complications of diabetes (3 with ischaemic heart disease, 2 with neuropathy and 1 with retinopathy) and 10 uncomplicated diabetics were recruited into the study. Patients were excluded from this study if they had any other medical conditions which predisposed them to a procoagulant state. Patients who were taking anticoagulant medication such as aspirin, heparin or warfarin were also excluded.

2.3 Blood and placental tissue collection and storage

2.3.1 Blood collection and storage

Approximately 50 mls blood was taken by venepuncture from pregnant women into various Vacutainer blood tubes (Becton Dickinson Pty Ltd): 15% EDTA (10mls volume), 0.105 M citrate (4.5mls volume), 145 i.u lithium heparin (6mls volume), plain silicone (6mls volume) and fluoride/oxalate (2mls volume). With regards to complicated and uncomplicated diabetic and healthy volunteer groups, 10mls of blood was collected from each individual into 0.105M citrate Vacutainer tubes. All samples were immediately put on ice and transferred to the lab. The samples were then spun for 20 minutes at 2000g at a temperature of 4ºC to separate plasma from blood cells. The plasma from all samples was immediately aliquoted and stored in a -80ºC freezer. Packed cells from EDTA tubes were removed and stored in a -80°C freezer. The collection of platelet-poor plasma was achieved by spinning the plasma collected in 0.105M citrate tubes at 13000g for 4 minutes to pellet the platelets to the bottom of the tube. The supernatant (now platelet-free) was removed and stored in a -80ºC freezer for later analysis.

2.3.2 Placental tissue collection and storage

The placental samples analysed were archival samples held at the department of reproductive medicine, Glasgow Royal Infirmary. The samples were previously obtained by other researchers and collected with informed consent. Samples were analysed from 6

IUGR pregnancies, 6 pre-eclamptic pregnancies, 6 healthy first trimester and 6 healthy third trimester pregnant controls. The procedure for placental sample collection was thus; the placenta was immediately brought to the lab once delivered. Samples of approximately 2 cubic centimeters in size were taken randomly from 4 quadrants of the placenta, then immediately and copiously washed in Phosphate Buffered Saline (PBS). These samples were then fixed in 10% buffered formalin (BDH, UK) for 24 hours, followed by 50% ethanol for 8 hours and finally 70% ethanol for 24 hours. These placental samples were then brought to the Pathology Department of Glasgow Royal Infirmary to be embedded into paraffin blocks.

2.4 Immunocytochemistry (ICC)

Immunocytochemistry is the application of immunological methods to cells or tissue sections to enable protein visualization by the demonstration of a marker conjugated to the final reactant. The method employed was the avidin-biotin complex (ABC) method, diagrammatically shown in figure 2.4.1 where (a) is the tissue antigen (b) the primary antibody (c) the biotinylated secondary antibody and (d) the avidin-biotin peroxidase complex.

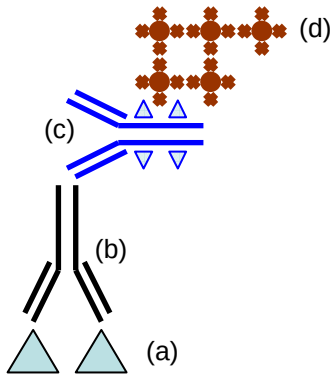


Figure 2.4.1: The Avidin-Biotin Complex [adapted from the Handbook of Immunochemical Staining Methods, 3rd Edition, *Boenisch et al 2001* (274)].

Annexin V, fibrin, tissue factor and M30 cytodeath immunostaining within the placental tissues were studied. Tissue sections were prepared from paraffin embedded tissue using a

microtome (Leica model RM2135), cutting sections of 5µm thickness. In back-to-back section preparations, consecutive sections were taken and stained for comparison. Slides were placed in plastic racks, heated in an oven at 56°C for 35 minutes and then deparafinised using xylene 2 x 10 minutes. They were then rehydrated through graded alcohols: 100% ethanol for 2 x 5 minutes, 95% ethanol for 2 x 5 minutes, 70% ethanol for 1 x 5 minutes and finally Phosphate Buffered Saline (PBS) (1.2g NaH_2PO_4, 9.0g NaCl, 1L distilled water, pH 7.6) for 5 minutes. Endogenous tissue peroxidase activity was inactivated by immersion in freshly prepared 0.5% hydrogen peroxide (prepared using 5 ml of 30% hydrogen peroxide in 300 ml methanol) for 30 minutes. Samples were then washed twice for 10 minutes in PBS.

Antigen retrieval was performed by microwaving for 5 minutes under pressure. One litre of 0.01M citrate buffer (pH 6.0) was prepared and poured into a sealed container. This solution was brought to boil within the microwave oven, the slides added, the container covered, locked and then further boiled at full power for 8 minutes (the container was at pressure after 3 minutes, leaving the slides to be microwaved for 5 minutes under pressure). The slides were then allowed to cool within the sealed container for 20 minutes and then washed in water for 5 minutes followed by PBS for 2x5 minutes. Antigen retrieval was not performed in the preparation of annexin V slides. Wax rings were carefully drawn around the sections and the samples were blocked with the appropriate serum (see table 2.4.1) in PBS for 30 minutes at room temperature in a humidified box. Excess serum was tapped off. The primary antibody was diluted in serum, added to each slide and incubated overnight for 16 hours at 4°C in a humidified box (see table 2.4.1 for details). The samples were then washed with PBS for 2 x 5 minutes.

The secondary antibody was prepared by diluting the appropriate serum with 5% human serum added. Details of the secondary antibody and diluting serum are shown in table 2.4.2. This prepared secondary antibody was added to the slides and incubated for 30 minutes at room temperature in a humidified box. The samples were then washed in PBS for 2 x 5 minutes. Positive controls are listed in table 2.4.3. For negative controls, the tissue of interest was incubated with the primary antibody substituted with a monoclonal IgG mouse antibody (DAKO code no. X 09312) directed towards Aspergillus niger glucose oxidase which is an enzyme neither present nor inducible in mammalian tissues.

Antigen	Pre-treatment	Blocking serum	Primary antibody	Type	Diluting serum	Dilution factor	Source
Annexin V	none	20% rabbit / 20% human serum	Goat anti-human annexin V	Poly IgG	2% rabbit serum	1:500	Santa Cruz (SC-1929)
Fibrin	microwave citrate buffer, pH 6.0	20% horse / 20% human serum	Mouse anti-human fibrin	Mono IgG	2% horse serum	1:200	Immunotech (Cat. No 0541)
Tissue Factor	microwave	20% horse / 20% human serum	Mouse anti-human tissue factor	Mono IgG	2% horse serum	1:50	Calbiochem (Cat.No 612161)
M30 Cytodeath	microwave	20% horse / 20% human serum	Mouse anti-human M30 cytodeath	Mono	2% horse serum	1:10	Roche Molecular Biochemicals Cat. No 2 140 322

Table 2.4.1: Primary antibodies and blocking serum

Antigen	Secondary antibody	Diluting serum	Dilution factor
Annexin V	Biotinylated anti-goat IgG	2% rabbit serum with 5% human serum added	1:200
Fibrin	Biotinylated anti-mouse IgG	2% horse serum with 5% human serum added	1:200
Tissue Factor	Biotinylated anti-mouse IgG	2% horse serum with 5% human serum added	1:200
M30 Cytodeath	Biotinylated anti-mouse IgG	2% horse serum with 5% human serum added	1:200

Table 2.4.2: Secondary antibodies and diluting serum

Antigen	Positive control tissue
Annexin V	Placenta
Fibrin	Placenta and decidua
Tissue Factor	Breast tissue
M30 Cytodeath	Tonsil

Table 2.4.3: Positive controls

Preparation of the avidin/biotin solution was as described by the manufacturer Vectastain ABC (Vector Laboratories) working solutions kit instructions. The avidin/biotin solution was added to the slides and incubated for 30 minutes at room temperature in a humidified box. The slides were then washed in PBS for 2 x 5 minutes. Diaminobenzidine (DAB) solution (1 DAB tablet [DAB/Sigma UK] in 15mls of 50mM Tris with 12µl 30% hydrogen peroxide, pH 7.6) was prepared, added to cover each slide and left for 10 minutes at room temperature. DAB was oxidised by the peroxidase attached to the secondary antibody forming an insoluble brown precipitate. The DAB solution was tapped off into sodium hypochlorite to be inactivated and the slide was washed in water for 5 minutes. The slides were then counterstained with Harris haematoxylin (Sigma) for 15-20 seconds and then washed in running tap water. The samples were dehydrated through alcohols (70%, 90%, 95%, and 100%) to xylene. Finally they were mounted in DPX.

An Olympus BX50 microscope equipped with x4, x10, x20 and x40 lenses, connected to a 3-CCD colour camera (JVC) was used for digital image capture of the slides. Computer visualisation of the images was achieved with the image analysis program Image-Pro Plus version 4.5.1 (Media Cybernetics Inc). For back-to-back sections, the corresponding photo was taken and turned mirror-image by the Image-Pro Plus program to facilitate an easier comparison of the staining pattern between the two back-to-back slides. Adobe Photoshop Elements version 2.0 was used to correct any background discoloration (as a result of the microscope light source) as well as to insert text into the pictures.

2.5 Preparation of synthetic phosphatidylcholine: phosphatidylserine vesicles

Synthetic phosphatidylcholine (PC): phosphatidylserine (PS) vesicles were prepared as per the method of Pigault *et al*(275) to be used as a standard in the prothrombinase assay to measure the procoagulant activity of microparticles in blood samples.

2.5.1 Materials and reagents

NaCl and chloroform were purchased from BDH Lab Supp. NaN_3, Hepes, Lα-phosphatidylcholine (PC) and Lα-phosphatidylserine (PS) was purchased from Sigma Chemical Co. N-octyl βD-glucopyranoside (β-OG) was purchased from Roche Chemical Co. Spectra/Por No.1 dialysis tubing was purchased from Spectrum Laboratories Inc.

The following reagents were prepared:

Buffer A: This solution was made with HEPES 10mM, NaCl 150mM, NaN3 0.02%, distilled water and made to a pH of 7.4.

40mg/ml Lα-phosphatidylcholine: 10mg Lα-phosphatidylcholine was dissolved in 250µl chloroform.

5mg/ml Lα-phosphatidylserine: 5mg Lα-phosphatidylserine was dissolved in 1000µl chloroform.

20% N-octyl βD-glucopyranoside (β-OG): 0.5g β-OG was dissolved in 2.5mls chloroform.

2.5.2 Preparation of synthetic PC:PS vesicles

Positive displacement pipettes were used. Lα-phosphatidylcholine (85µl of 40mg/ml) and Lα-phosphatidylserine (320µl of 5mg/ml) were added into a glass tube, covered with metal foil and shaken gently. To this, 2 mls of 20% N-octyl βD-glucopyranoside solution was added, the tubes covered with metal foil and shaken gently again. The tube was put into a rotary evaporator (Jouan RC10-22) and dried for 2 hours at 55°C +/- 5° resulting in a pearly white residue at the bottom of the tube. The tube was then removed and allowed to cool. Two mls of buffer A solution was then added, vortexed gently until the pearly white residue at the bottom of the tube had completely dissolved. The solution was then allowed to stand for 1 hour. The solution was then poured into a Spectra/Por No.1 Dialysis tube and was dialysed against 200mls buffer A with gentle stirring of the buffer. Three bath changes of 200mls buffer A were performed with 24 hours between each bath change. The solution was then removed and stored in glass screw cap tubes. This solution of PC: PS vesicles had a concentration equivalent to 1mM phosphatidylserine.

2.6 Preparation of microparticles from BeWo cell culture

BeWo cells are a commercially available choriocarcinoma cell line. Induced apoptosis of BeWo cells in culture by tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) would lead to the release of 'natural' microparticles into the culture medium. These microparticles could then be isolated and utilised as a standard in the prothrombinase assay. These 'natural' microparticles would have externalised phosphatidylserine on its surface as well as contain various cell membrane lipids and proteins that would stabilise these microparticles in storage.

2.6.1 BeWo cell culture

BeWo cells were obtained from ATCC (catalogue number: CCL98) and grown as per manufacturers instructions. BeWo cells were cultured in Hams medium (ICN 12-467-S4),

10% Foetal Calf Serum (Sigma F9665), 1% Penicillin/Streptomycin (Gibco BRL 15140-122), 1% 200mM Glutamine (ICN 1680149) and 1% Fungazone (ICN 16-723-48). BeWo cells were cultured in flasks with 15 mls of culture medium in each flask. These flasks were stored in the incubator at 37°C (5% CO_2), vented and the medium was changed every other day. BeWo cells were subcultured once a week and were grown to confluence of 60 to 70%.

2.6.2 Preparation of tumour necrosis factor α (TNFα) and interferon γ (IFNγ) solutions

Tumour necrosis factor alpha (TNFα) [R+D systems 210-TA] was made up to a concentration of 1μg/ml in PBS. Interferon gamma (IFNγ) [R+D systems 285-IF] was made up to a concentration of 2μg/ml in PBS. These solutions were stored at -20°C until use.

2.6.3 Stimulation of microparticle release from BeWo cell culture

Apoptosis was induced by adding 150μl of 1μg/ml TNFα and 75μl of 2μg/ml IFNγ into each flask to give a final concentration of 10ng/ml TNFα and 10ng/ml IFN in each flask. The culture was then incubated for another 24 hours at 37°C. The culture medium was then removed and centrifuged for 20 minutes at 2000g to remove any non-adherent cells, then the supernatant further ultracentrifuged at 13000g for 4 minutes. The supernatant was removed and 5 mls Biostab biomolecule stabilising storage solution (Fluka 92889, Sigma-Aldrich) added. This supernatant was then measured for microparticle activity in the prothrombinase assay. In cases where further concentration was required to increase microparticle pro-coagulant activity, this was performed by dialysis through a semi-permeable membrane on sucrose. Storage for analysis was in a 4°C fridge.

2.7 Prothrombinase assay

The prothrombinase assay was chosen as a method to measure the procoagulant activity of microparticles in the blood sample. The method of Aupiex *et al* (65) was used. This assay is based on the principle that phosphatidylserine is externalised on the surface of microparticles and phosphatidylserine catalyses the conversion of prothrombin to thrombin. The prothrombinase assay captures microparticles in the sample and measures the coagulation potential of microparticles in the sample by quantifying the amount of thrombin generated.

2.7.1 Materials

NaCl, KCl, $CaCl_2$, EDTA, NaOH pellets and HCl were purchased from BDH Lab Supp. Human Serum Albumin (HSA) and Tris were purchased from Sigma Chemical Co. Annexin-V-Biotin, Streptavidin coated 96 well microtitration plates, Factor Xa and Chromozym TH were purchased from Roche Chemical Co. D-Phe-Pro-Arg-chloromethylketone HCL (PPACK) and 1-5 Dansyl-Glu-Gly-Arg-chloromethylketone DiHCL (DnsGGACK) were purchased from Calbiochem Chemicals. Factor Va was purchased from American Diagnostica Inc and prothrombin was purchased from Hyphen Biomed.

2.7.2 Assay reagents

TBS-Calcium solution: Tris 50mM, NaCl 120mM, KCl 2.7mM, $CaCl_2$ 1mM, pH of 7.5.

TBS-Calcium HSA 0.3% (TBS-Ca-HSA): Human serum albumin (HSA) was added to TBS-Calcium solution and made up to a concentration of 3g/L.

Annexin-V-Biotin 350ng/ml: Annexin-V-Biotin (0.1 μg/μl concentration) was diluted into TBS-Ca-HSA (175 μl into 49825 μl) to make a 50 ml solution. This was stored as 2ml aliquots at -20°C.

$CaCl_2$ 13mM solution: $CaCl_2$ (14.4mg) was dissolved into 10 mls TBS-Ca-HSA.

Factor Va 3.6nM: Factor Va (5µl of 15.5µM) was added to 210 µl TBS-Ca-HSA to make a final concentration of 360nM. This was stored as 10µL aliquots at -20°C. Prior to use, 990µl TBS-Ca-HSA was added to make a 3.6nM solution.

Factor Xa 500pM: Factor Xa (10µl of 350µM) was added to 7mls TBS-Ca-HSA to make a 500nM solution. This was stored at -20°C. Prior to use, 5µl was added to 4995µl TBS-Ca-HSA to make 500pM solution.

Prothrombin 3.9M: TBS-Ca-HSA (3.6mls) was added to 13.9nmols prothrombin to make a 3.9µM solution for immediate use.

Chromozym TH 1.52mM: Distilled water (19.87mls) was added to 30.2µmols Chromozym to make a final concentration of 1.52mM. This was stored at 4°C.

EDTA 25mM: This was prepared by adding 2.326 g EDTA to 250mls distilled water. NaOH pellets were gradually added until EDTA dissolved. The pH was adjusted to 8.0 and the solution was then stored at room temperature until ready for use.

PPACK 1mM: PPACK (5mg) was dissolved in 9.54 mls of 10mM HCl solution to make a 1mM concentration of PPACK.

1-5 Dansyl-GGACK 1mM: 1-5 Dansyl-GGACK (5mg) was dissolved in 7.15mls of 10nM HCl solution to make a 1mM concentration of DnsGGACK.

HCl 10mM: This was made by diluting 87µl of 11.5M HCl with 100ml distilled water.

$CaCl_2$ 1M: This was made by dissolving 11.099g $CaCl_2$ in 100mls distilled water.

'Inhibitor mix': This was prepared by adding 5.5mls of 1mM PPACK, 5.5mls of 1mM GGACK and 14.85 mls of 1M $CaCl_2$ to 24.15mls TBS-Ca solution and the pH was adjusted to 7.4. This was stored in aliquots of 1ml at -20°C.

'Master mix': The amounts and reagents required for each well was 15µl of 13mM $CaCl_2$, 15µl of 3.6nM FVa, 15µl of 500pM FXa and 55µl of TBS-Ca-HSA. To calculate the amount of 'master mix' required for the assay, the total number of wells to be used was determined. The required amounts of each reagent were then mixed to make a single solution of 'master mix' and 100µl of this 'master mix' was added into each well.

2.7.3 Methodology of prothrombinase assay

Into each well of a streptavidin coated 96 well microtitration plate, 100µl of 350ng/ml Annexin-V-Biotin (Roche) in TBS-Ca-HSA was added. This was incubated at 37ºC for 30 minutes with gentle shaking. The plate was washed 3 times with 250µl TBS-Ca-HSA per well. 'Inhibitor mix' (10µl) was added into each well followed by 100µl of the sample to be measured. For negative control wells, 100µl TBS-Ca-HSA was used. This was incubated at 37ºC for 30 minutes with gentle shaking. Each well was then washed out 3 times with 250µl TBS-Ca-HSA. 'Master mix' (100µl) was added into each well followed by 50µl of 3.9µM prothrombin solution. The plate was incubated at 37ºC for 30 minutes with gentle shaking. EDTA (50µl of 25nM) was then added into each well. The plate was immediately brought to the plate reader. Chromozym TH (50µl of 1.52nM) was added into each well, the plate was gently shaken and immediately put into the microtitration plate reader equipped with kinetics software. Linear absorbance changes were recorded at 405nm wavelength over 20 minutes.

2.8 Fetal corticotrophin-releasing hormone (CRH) mRNA measurement

While maternal CRH mRNA is undetectable in the maternal systemic circulation, fetal CRH mRNA has been detected in maternal plasma in healthy pregnancy and levels have found to increase as pregnancy progresses. As CRH has been immuno-localised to the placental syncytiotrophoblast layer, the measurement of fetal CRH mRNA can therefore be used as a measure of placental-derived cell debris in maternal systemic circulation. The method of Ng *et al* was used(106)

Maternal plasma was collected in EDTA and stored in a -80°C freezer as described in section 2.3.1. When required, the plasma sample was thawed; 1.6mls removed and centrifuged at 16000g for 10 minutes at 4°C. The supernatant was removed and 2mls of Trizol LS (Invitrogen) added to the pellet. The RNA was then isolated as per the manufacturer's instructions. The RNA in ethanol was concentrated on an RNeasy mini column (Qiagen) and eluted in 30mls RNase-free water according to the manufacturer's instructions. Dithiothreitol (0.3µl, 1nM) and 40 Units RNaseOUT (Invitrogen) were then added into each RNA sample before storing at -80°C. This RNA (4µl) was then reverse transcribed to cDNA in a final reaction volume of 20µl using a High Capacity cDNA Archive kit (from Applied Biosystems, 4322171). The CRH and GAPDH mRNA levels were then quantified (in triplicate) in 2µl cDNA using commercially available primer probes sets (Hs00174941_ml and 4310884E respectively from Applied Biosystems) on a ABI/7900 sequence detection system (Applied Biosystems) according to the manufactures instructions. TaqMan Universal Master Mix, No Amperase UNG was used in a final assay volume of 50µl and the number of PCR cycles was increased to 60. Expression of CRH was expressed relative to the expression of GAPDH(106) (as an index of total plasma RNA). The ratio of CRH to GAPDH was calculated by subtracting the Ct values (threshold cycle - logarithmic) values (delta Ct), taking the antilog and multiplying by 100. Ct values for the control gene GAPDH did not differ between pre-eclampsia and control groups (median Ct 33.0 vs. 33.8, p=0.18).

2.9 Markers of coagulation activation, endothelial activation and placental function

The blood samples to be analysed were collected and stored as detailed in section 2.3.1. Coagulation markers, markers of endothelial activation and markers of placental function were analysed using commercially available kits as detailed in table 2.9.1.

2.10 Erythrocyte cell membrane fatty acid composition

2.10.1 Extraction of fatty acids from erythrocyte cell membranes

The method of Stewart et al was used (249). Packed erythrocyte samples were collected and stored as detailed in section 2.3.1. Total fatty acid extraction from erythrocyte cell membranes was performed by a modified Folch extraction. Packed erythrocytes (400µl) were suspended in 10nM Tris buffer at pH 7.0 and incubated at room temperature for 30 minutes. They were then centrifuged in a Beckman L8-60M Ultracentrifuge Type 50.4 rotor (Fullerton, CA, USA) at 49000rpm at 4°C for 30 minutes. The erythrocyte membrane pellet was then resuspended in 200µl of distilled water and 150µl was transferred to a glass screw-top tube. Methanol:toluene at 4:1 ratio (2mls) containing heneicosanoic acid internal standard (0.2mg $C_{21}H_{42}O_2$/ml toluene) was then added followed by 200µl of 100% acetyl chloride while mixing. The tubes were capped, sealed with teflon tape, and then heated at 100°C for 1 hour. When cooled, 10% K_2CO_3 (3mls) was slowly added to each tube followed by 100µl toluene. The tube was then centrifuged for 8 minutes at 3000 rpm, 5°C and the upper toluene phase was then transferred to gas chromatography vials and stored at -20°C.

2.10.2 Identification and quantification of fatty acids

Fatty acid analysis was carried out using gas chromatography. Methyl fatty acids were separated (1µL injection volume), identified and quantitated on a Shimadzu GC 17A gas chromatograph (Kyoto, Japan) using flame ionisation detection and Class VP software. A DB-23 fused silica capillary column (J&W Scientific, Folson, CA, USA), 30m x 0.25mm internal diameter with a film thickness of 0.25µm was used in conjunction with a Hewlett-Packard 7673B on-column auto-injector (Palo Alto, CA, USA). Ultra high purity hydrogen and air was used as carrier gases at a flow rate of 2mL/min. A temperature gradient programme was used with an initial temperature of 150°C, increasing at 20°C/min up to 190°C, then at 5°C/min up to 210°C, then at 2°C/min up to 230°C and then at 4°C/min up to 240°C (final time 18.5 minutes), and with an equilibration time of 1 minute. The total programme time was 22 minutes. The identification of fatty acid methyl esters was made

by comparison with the retention times of authentic standard mixtures (fatty acid methyl ester mixture no. 189-19, product no. L9405, Sigma, Stockholm, Sweden). Absolute values of fatty acids were calculated by comparison against the heneicosanoic acid internal standard. Percentage values of fatty acids were calculated as a percentage of total fatty acids in the sample.

Measure	Method	Manufacturer of assay kit
Coagulation markers		
TF	ELISA	Imubind, American Diagnostica, Axis-Shield
TFPI	ELISA	Imubind, American Diagnostica, Axis-Shield
TAT	ELISA	Enzygnost, Dade Behring
F1+2	ELISA	Enzygnost, Dade Behring
FVIIc	assayed in MDA 180 coagulometer	reagents from Biomerieux Ltd, Basingstoke, UK
FXIIa	ELISA	Shield Diagnostics, Axis-Shield
APC	assayed in MDA 180 coagulometer	Chromogenix APC resistance kit, Quadratech, UK
PAI-1	ELISA	Tint-Elize, Alpha Laboratories
Markers of endothelial activation		
sICAM-1	ELISA	R&D Systems
sVCAM-1	ELISA	R&D Systems
vWF	ELISA	Imubind, American Diagnostica, Axis-Shield
Marker of placental function		
PAI-2	ELISA	Imubind, American Diagnostica, Axis-Shield

Table 2.9.1: List of commercially available kits used in the measurement of coagulation markers, markers of endothelial activation and marker of placental function.

2.11 Statistical analysis

An *a priori* sample size calculation was based on data from a study of acute coronary syndrome patients which observed a 2-fold increase in microparticles above control(69). A sample size of 8 predicted 95% power at the 5% significance level to detect a difference of 20% in microparticle levels measured with a 10% standard deviation. All statistical analysis was performed using Minitab version 13.32.

2.11.1 Prothrombinase activity, measures of coagulation activation, endothelial activation, placental function and fetal CRH mRNA measurement

The Ryan-Joiner test was used to assess the distribution of values for normality. It was found that to achieve a normal distribution, values of F1+2, PAI-1, PAI-1/PAI-2 ratio and the microparticle prothrombinase activity required to be transformed to log values. The values of sVCAM-1 and TAT required to be transformed to square root values. Analysis for significant differences was carried out using two-sample T-tests for continuous variables, on transformed data where necessary and using the chi-square test for categorical variables. To analyse for correlations, Pearson's correlation coefficients were measured, using transformed data where necessary. As the CRH/GAPDH mRNA ratio of some samples included some zero values, the Wilcoxon rank sum test was used to test the difference between groups and Spearman rank correlation analysis was used. Multivariate analysis was used to assess the independence from smoking by using a General Linear Model. For the purposes of multivariate analysis, the CRH/GAPDH mRNA ratio was divided into 3 categories: equal to 0, range from 0 to 1.0 and range greater than 1.0. Statistical significance was set at $p<0.05$.

2.11.2 Fatty acids

The Ryan-Joiner method was used to assess the distribution of values for normality. It was found that to achieve a normal distribution, the percentage values of 24:0, 22:6 n-3 and the absolute value of 22:6 n3 required to be transformed to log values. Analysis for significant

differences was carried out using two-sample T-tests for continuous variables, on transformed data where necessary. To analyse for correlations, Pearson's correlation coefficients were measured, using transformed data where necessary. Statistical significance was set at $p < 0.005$ to account for repeated analysis.

Chapter 3 Immunocytochemical study of the relationship between annexin V, fibrin and tissue factor at the trophoblast

3.1 Introduction

3.1.1 Coagulation at the placenta

Fibrin is the end product in the process of coagulation. While fibrin has a useful function by forming a plug to prevent blood loss from the vascular space, fibrin deposition on the surface of the syncytial layer of the placenta can physically obstruct and reduce the surface area available for feto-maternal exchange. Large clots may reduce the flow of blood within the intervillous space, thus impeding fetal growth and development. Perivillous fibrin deposition is a normal finding in healthy placentas but excessive perivillous fibrin deposition has been found in conditions of IUGR and pre-eclampsia, both conditions that are associated with placental insufficiency(14;17;18).

Annexin V is expressed constitutively by syncytiotrophoblast cells and annexin V has been localised to the microvillous surface of syncytiotrophoblast cells. Several studies have suggested that annexin V may afford the syncytial surface protection against clot formation(45-47). Therefore, it is possible there may be a relationship between the distribution of annexin V and fibrin deposition on the syncytial surface of the placenta. Annexin V staining intensity in trophoblasts have been found to be reduced in pre-eclamptic patients compared to healthy pregnant controls(39;48).

Tissue factor (TF) is a powerful initiator of coagulation. In the coagulation cascade, TF combines with activated factor VII forming the activated factor VII-tissue factor (FVIIa-TF) complex, a key step in coagulation activation. Cultured syncytiotrophoblast cells have been found to express TF(27). In healthy pregnancy, TF has been detected in the placenta and myometrium(25) and TF expression has also been localised to placental macrophages, endothelial cells and fibroblast-like cells in loose connective (26). Estelles *et al* noted

greater amounts of TF antigen and TF mRNA in the placentas of women suffering severe pre-eclampsia with IUGR compared to healthy pregnant controls.

3.1.2 Placental development

In pregnancy, there is progressive remodelling and development within the placenta with constant turnover of the trophoblast layer of placental villi. The trophoblast layer consists of cytotrophoblasts and syncytiotrophoblasts. Cytotrophoblasts are the stem cells of and are located basal to the syncytiotrophoblasts (which is the trophoblast layer in contact with the intervillous space). As part of the renewal process of syncytiotrophoblasts, cytotrophoblast cells proliferate, differentiate and eventually become incorporated into the syncytiotrophoblast layer via syncytial fusion. As syncytiotrophoblasts age, their nuclei accumulate and become packed into membrane-sealed fragments within the syncytium forming syncytial knots. These syncytial knots are eventually extruded into the intervillous space where they enter the maternal circulation and are eventually taken up by maternal macrophages (which occurs to a large degree in maternal lungs). In the first trimester, there is a greater cytotrophoblast volume in relation to syncytiotrophoblast volume. Towards the end of pregnancy, the syncytiotrophoblast volume becomes greater than the cytotrophoblast volume and there is also a greater amount of syncytial knots and bridges(54). Cytotrophoblast cells can be visualised as a distinct layer in the first trimester but this layer becomes thinner and less distinct as pregnancy progresses. In comparison to placentas in healthy pregnancy, IUGR and pre-eclamptic placentas have a greater number of placental knots and bridges along with a greater degree of trophoblast apoptosis(20;56).

Apoptosis is the process of programmed and controlled cell death which is initiated through the cleavage of native intracellular proteins by caspases (Cysteinyl-aspartic acid proteases). Apoptosis can be identified using cellular cytokeratin 18 which is cleaved in early apoptosis. M30 CytoDEATH (Roche Molecular Biochemicals) is a mouse monoclonal antibody that detects a specific caspase cleavage site within cytokeratin 18. Thus, M30 is a useful tool for identifying apoptotic cells. A previous study looking at M30 localisation within the healthy placenta in the third trimester showed that the majority of M30 staining was localised to extravillous trophoblasts as well as syncytiotrophoblast cells, with abundant M30 staining of syncytiotrophoblast cells in areas of greater

perivillous fibrinoid deposition(50). As cytokeratin is located within the cell cytoplasm, M30 immunoreactivity is confined to the cytoplasm. However non-specific M30 staining of the nuclei of highly proliferating cells has also been recorded(51).

3.1.3 Histological features of placental villi

A brief review of healthy placental villi would be useful to interpret the immunocytochemical findings in this chapter. In first trimester placentas both the cytotrophoblast and syncytiotrophoblast layers are clearly seen to be separate. The syncytiotrophoblast layer has flattened nuclei, is the most superficial layer of the placental villi and is in direct contact with the intervillous space. The cytotrophoblast layer is directly under the syncytiotrophoblast layer. Cytotrophoblast cells appear cuboidal or ovoid in shape and have well demarcated cell borders with lightly staining large nuclei. Syncytial sprouts are outgrowths and proliferation of chorionic villi. Syncytial sprouts appear as a prolongation of the villous tip often with multiple syncytial nuclei present at the end. The stromal core of each placental villous is comprised of collagen fibres with fibroblasts, tissue macrophages (Hofbauer cells), mast cells, plasma cells and capillaries. The space between the placental villi is the intervillous space within which flows maternal blood.

As the placenta develops, the cytotrophoblast layer becomes thinned and the cytotrophoblast cells become incorporated into the syncytial layer by membrane fusion. In comparison to first trimester placentas, third trimester placentas have a very much thinner cytotrophoblast layer as most cytotrophoblast cells have differentiated and become incorporated into the syncytial layer at this stage. The syncytial layer is variable in thickness. It may be thinned to become the vasculosyncytial membrane or the nuclei may be piled up in areas forming syncytial knots. Syncytial bridges can also be seen and they are a result of fusion of adjacent villi. On the intervillous space side of the syncytiotrophoblast cell membrane, there are numerous microvilli present and on higher magnification, can be identified as a fuzzy appearance on the cell surface. Stem villi are present which are large villi involved in the physical support of the villous tree. Larger stem villi may have a central artery and vein within the stroma with branching vessels and capillaries leading to terminal villi. Villous arteries and veins have an endothelial lining.

Terminal villi appear as bulbous structures and may be single or have side branches. They have a thin trophoblastic surface in close contact with the dilated capillaries within.

3.1.4 Hypothesis

There is greater trophoblast apoptosis in pre-eclampsia and IUGR in comparison to healthy pregnancy and this may lead to greater microparticle generation. Microparticles may be procoagulant and this may lead to fibrin deposition. Annexin V is located on the maternal surface of syncytiotrophoblasts and has anticoagulant properties. Tissue factor is expressed by the placenta and is a potent initiator of coagulation. I hypothesise that there may be associations between fibrin deposition, annexin V expression and tissue factor expression in pre-eclamptic, healthy and IUGR placentas.

Objectives:

1) Identify areas of fibrin staining in healthy pregnant, IUGR and pre-eclamptic placentas.

2) Identify areas of annexin V staining in healthy pregnant, IUGR and pre-eclamptic placentas.

3) To investigate a possible relationship between annexin V expression and fibrin deposition in healthy pregnant, IUGR and pre-eclamptic placentas using back-to-back samples.

4) To investigate a possible relationship between annexin V expression and TF expression in healthy pregnant, IUGR and pre-eclamptic placentas using back-to-back samples.

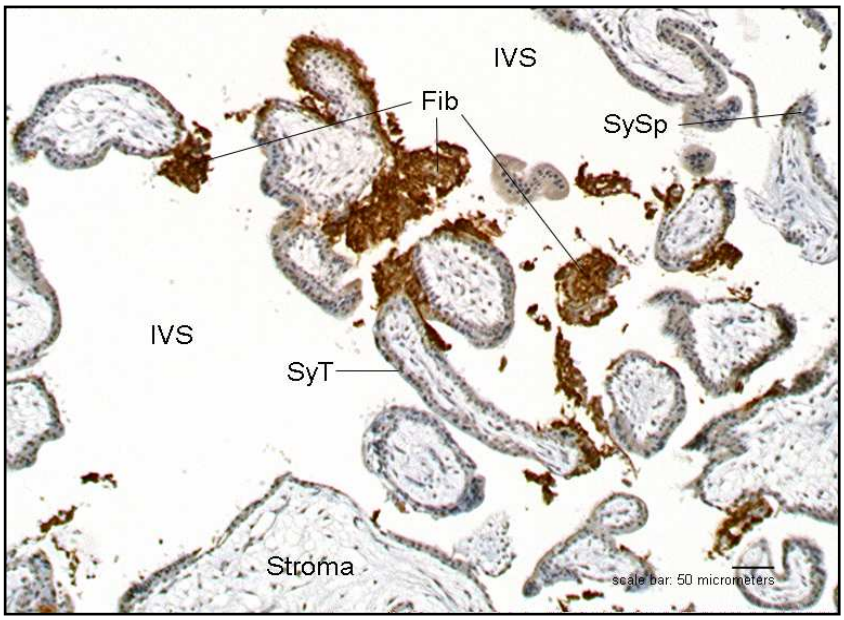
5) Identify areas of apoptosis in healthy pregnant, IUGR and pre-eclamptic placentas using the M30 antibody as a marker for apoptotic cells.

3.2 Results

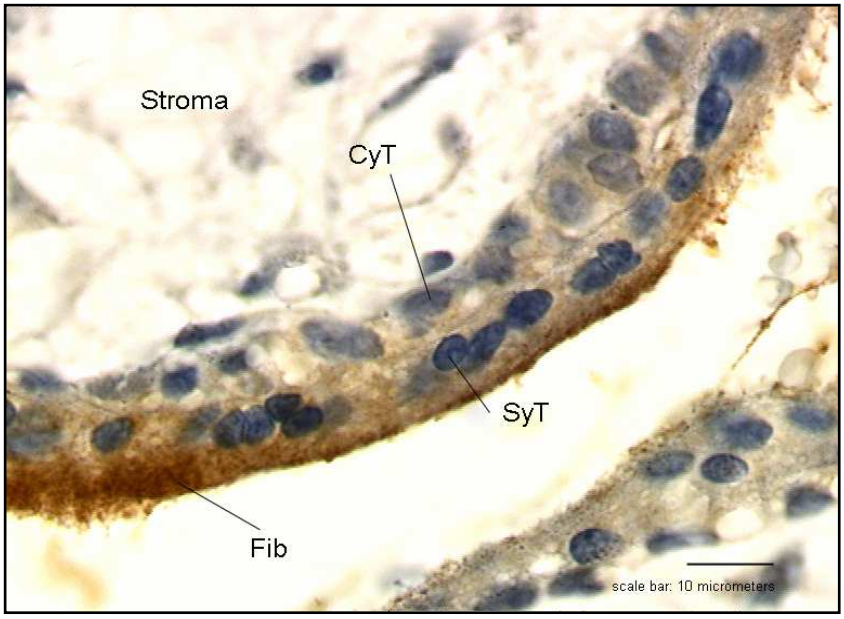
Six first trimester placentas, six third trimester placentas, six IUGR placentas and six pre-eclamptic placentas were stained for annexin V, fibrin and tissue factor. For M30 staining, placental samples from eight first trimester aborted pregnancies and two healthy third trimester pregnancies that were delivered normally were analysed. All samples were archival samples.

3.2.1 Fibrin localisation in first and third trimester healthy placentas, IUGR and pre-eclamptic placentas

In healthy first trimester placentas, there were a few sporadic areas of fibrin **(Fib)** deposition seen (as a brown precipitate) at x 10 magnification. There was perivillous fibrin staining seen at lower magnification (figure 3.2.1.1 A). At higher magnification, fibrin was identified on the maternal surface of syncytiotrophoblast cells (figure 3.2.1.1 B). The large areas of perivillous fibrin deposition appeared to have a foundation on the surfaces of syncytiotrophoblasts. Some typical characteristics of first trimester placenta are seen in figures 3.2.1.1 A and 3.2.1.1 B: for example the syncytial sprouts **(SySp)** and the clearly defined and abundant cytotrophoblast **(CyT)** layer (with large pale staining cuboidal-shaped nuclei) separate from the syncytiotrophoblast layer **(SyT)**. In healthy third trimester placentas, perivillous fibrin **(Fib)** deposition appeared to be more widespread in comparison to first trimester samples (figure 3.2.1.2 A, B). Once again, the foundation for the deposition of fibrin appeared to be the syncytiotrophoblast layer **(SyT)** (figure 3.2.1.2B). Various characteristics of the third trimester placenta were present, such as the thin cytotrophoblast layer, with just the syncytial layer visible in some parts of the villi surface. There was also greater development of the placental villi, with branching of intermediate villi into terminal villi and syncytial knots **(SyKn)**. There was fibrin deposition within the villous capillaries **(Cap)** (figure 3.2.1.2 B).

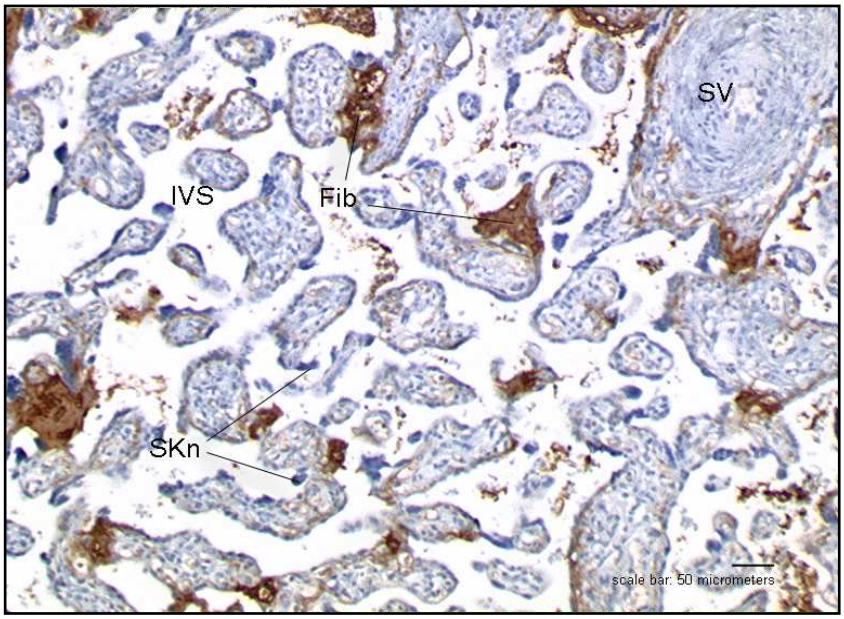


A

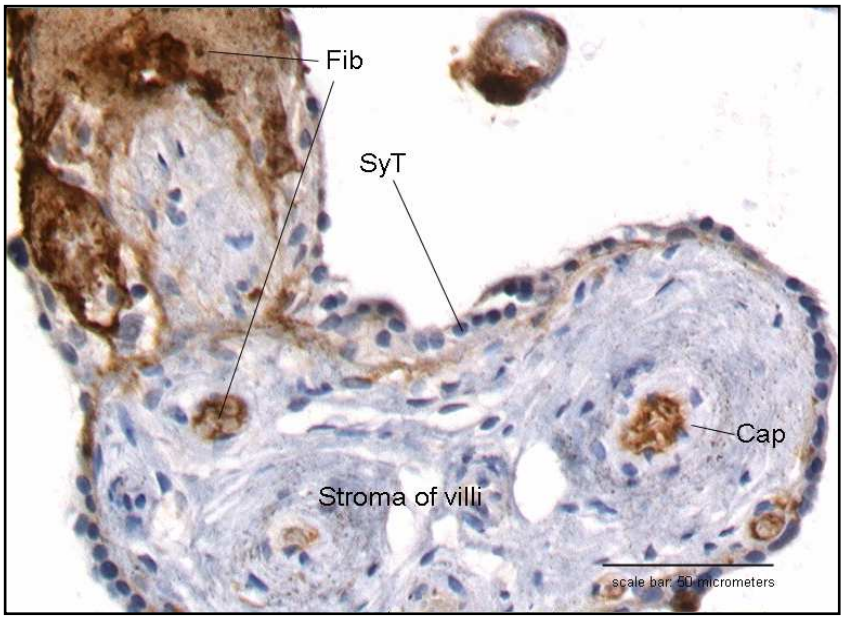


B

Figure 3.2.1.1: Fibrin staining in healthy first trimester placenta at x 10 magnification (**A**) and x 100 magnification (**B**).



A



B

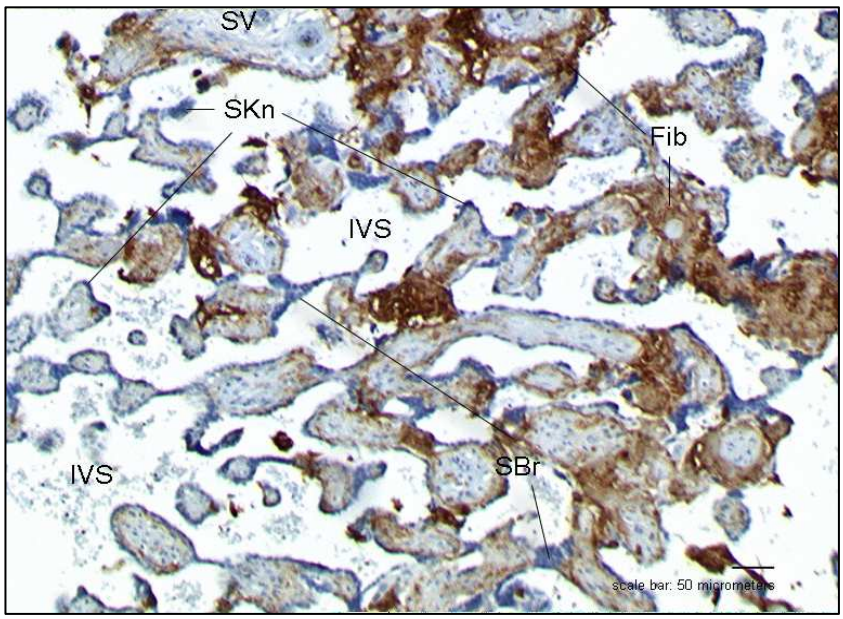
Figure 3.2.1.2: Fibrin staining in healthy third trimester placenta at x 10 magnification (**A**) and x 40 magnification (**B**).

In IUGR placentas, perivillous fibrin **(Fib)** deposition was widespread. There appeared to be a greater amount of syncytial knots and bridges (figure 3.2.1.3 A, B) and the terminal villi appeared smaller and shorter compared to samples from healthy pregnancies. There was also a 'congested' appearance to the distribution of villi. In pre-eclamptic placentas, large areas of fibrin staining were seen at the perivillous areas (figure 3.2.1.4 A). There appeared to be a greater amount of syncytial knots and bridges compared to healthy pregnant samples. On higher magnification (figure 3.2.1.4 B), there appeared to be fibrin staining of some syncytial knots. The terminal villi appeared short and branched.

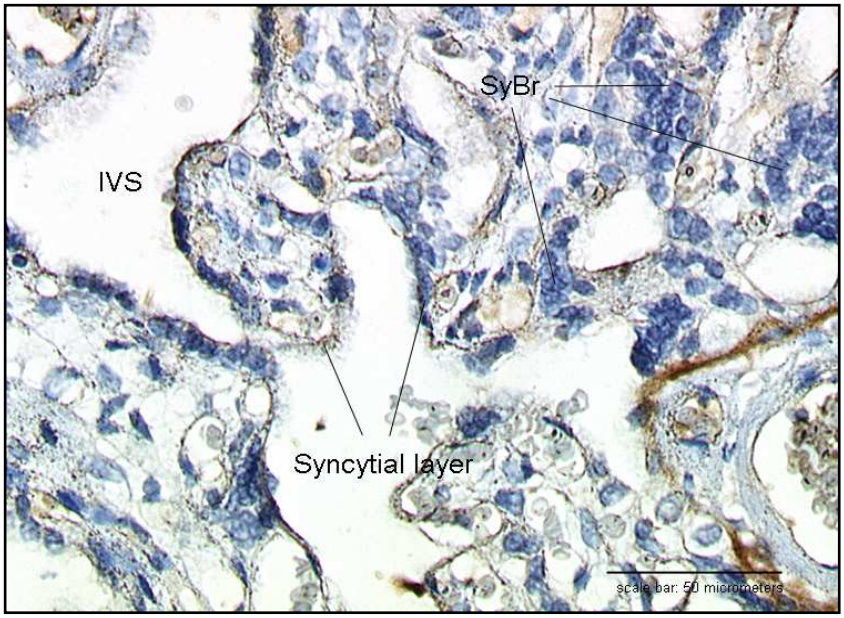
3.2.2 Annexin V localisation in third trimester healthy placentas, IUGR and pre-eclamptic placentas

In healthy third trimester samples, localisation of annexin V **(AnnV)** was clearly identified. The distribution was extensive and was mainly localised to the syncytiotrophoblast layer **(SyT).** Annexin V was seen in the trophoblast layer of all villi types, from stem villi **(SV)** to terminal villi **(TV)** (figure 3.2.2.1A). However at higher magnification, there were areas of absent annexin V staining at the syncytiotrophoblast and in these areas, there appeared to be less numbers of syncytial nuclei seen (figure 3.2.2.1 B).

In IUGR placentas, there appeared to be larger areas of absent annexin V staining compared to healthy third trimester samples (figure 3.2.2.2 A, B) and in some areas of absent annexin V staining, there appeared to be an associated loss of underlying trophoblast and stromal architecture (figure 3.2.2.2 B). In these areas of abnormal architecture, the trophoblast cell surface and trophoblast nuclei appeared indistinct. The underlying stromal tissue appeared disorganised and there appeared to be less cellular nuclei seen. There were no clear features of fetal capillaries in these disorganised areas. In pre-eclamptic samples, there were also large areas of absent annexin V staining and this appeared greater in comparison to healthy third trimester samples. In these areas of absent annexin V staining, there were large areas of villi with underlying loss of stromal architecture similar to that seen in IUGR samples (figures 3.2.2.3 A, B).

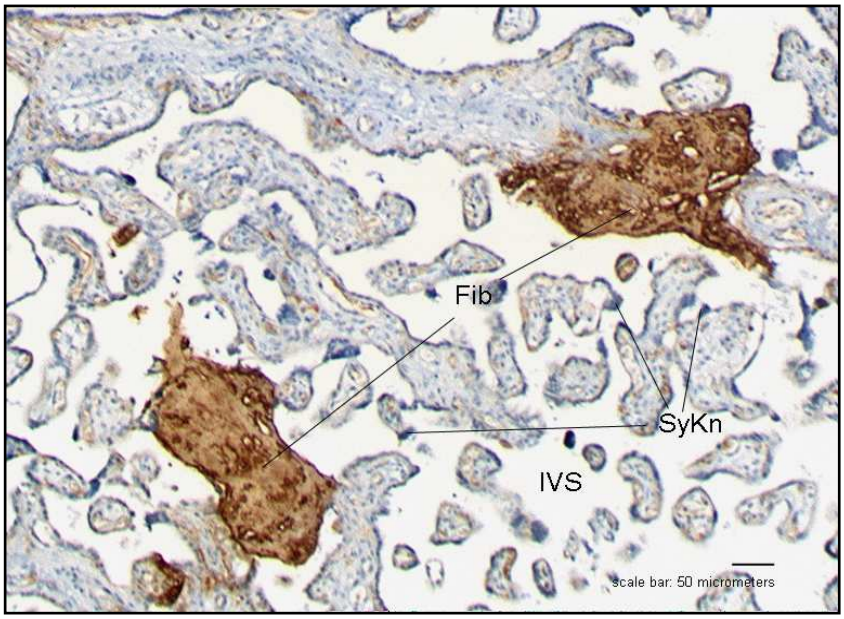


A

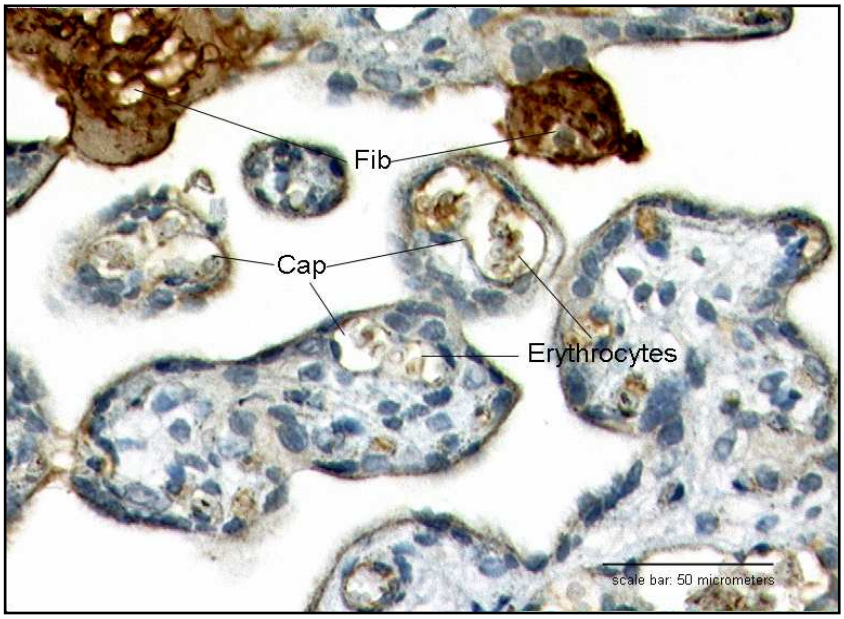


B

Figure 3.2.1.3: Fibrin staining in IUGR placenta at x 10 magnification (**A**) and x 40 magnification (**B**).

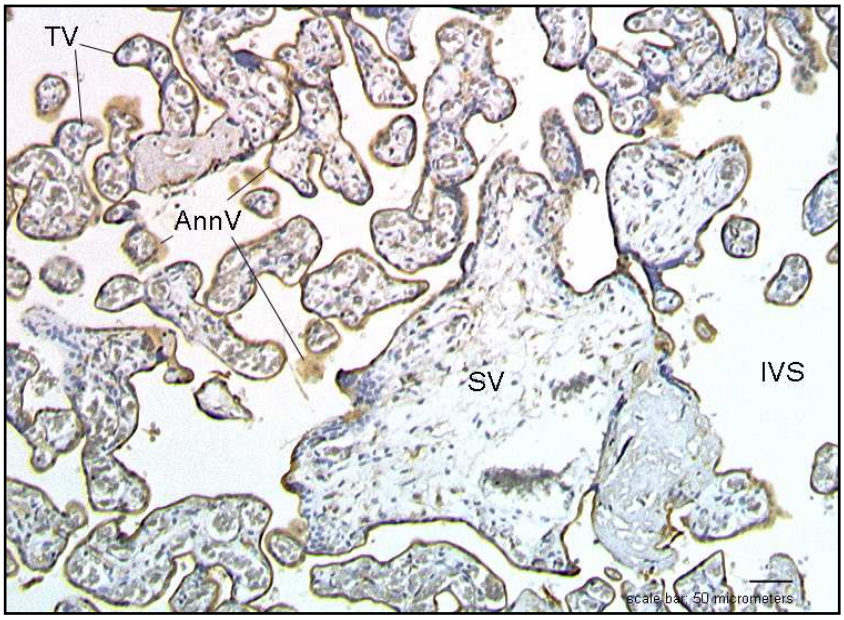


A

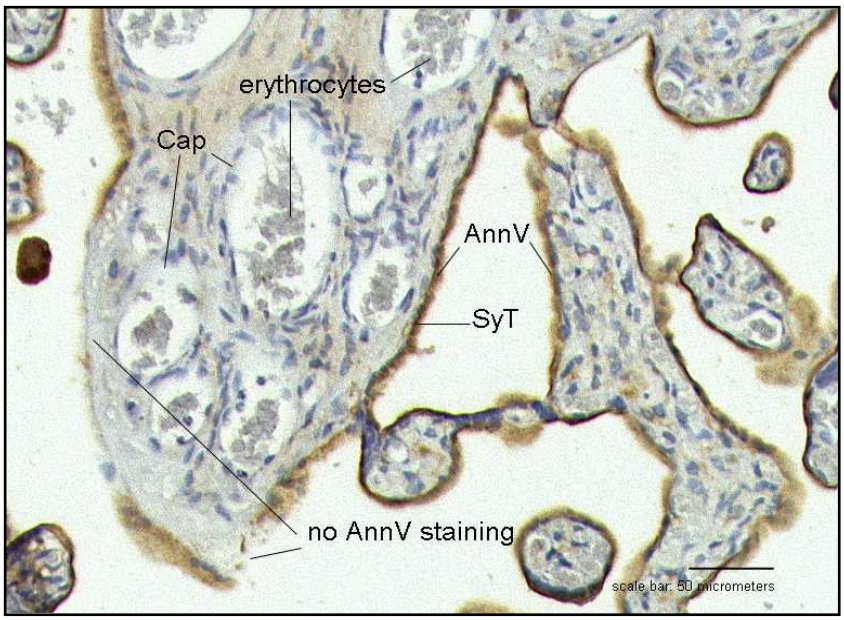


B

Figure 3.2.1.4: Fibrin staining in pre-eclamptic placenta at x 10 magnification (**A**) and x 40 magnification (**B**).

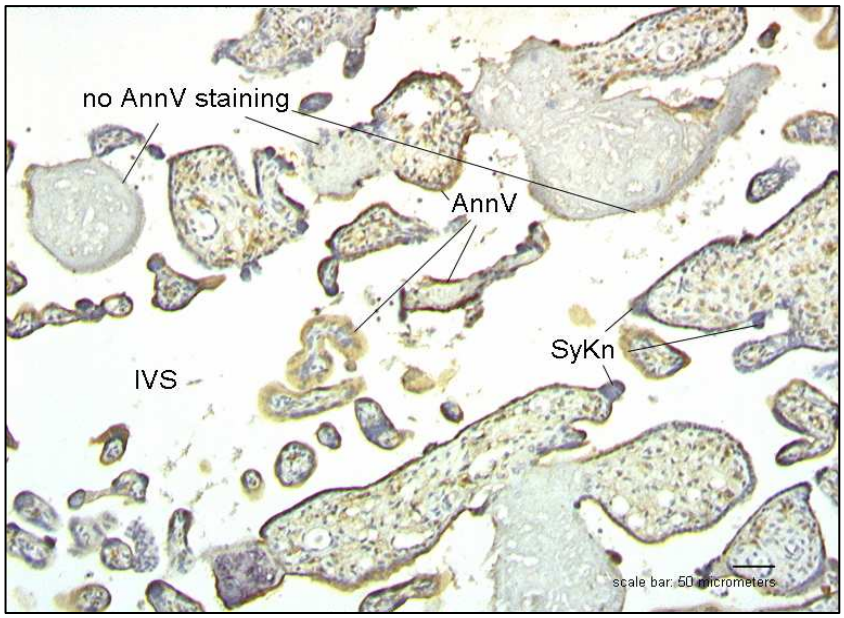


A

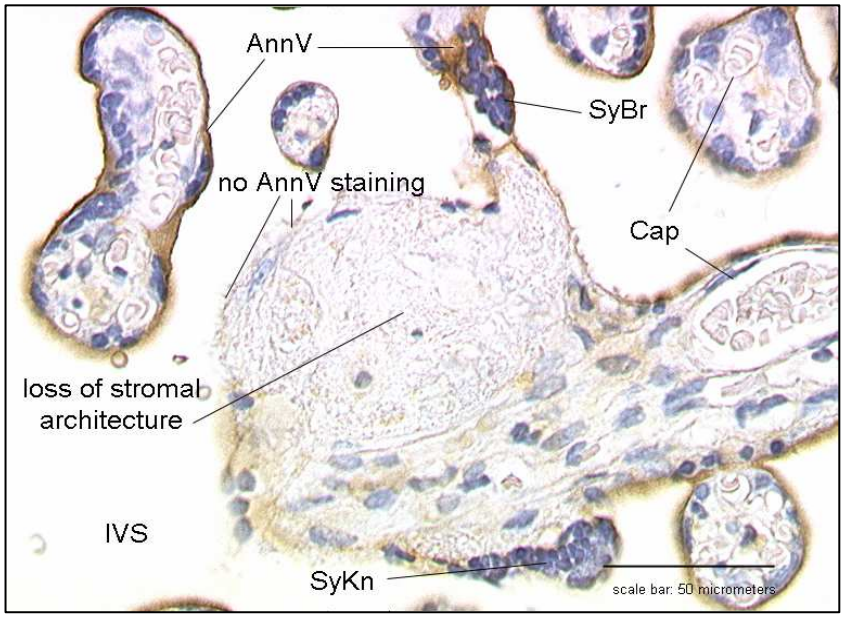


B

Figure 3.2.2.1: Annexin V staining in healthy third trimester placenta at x 10 magnification (**A**) and x 20 magnification (**B**)

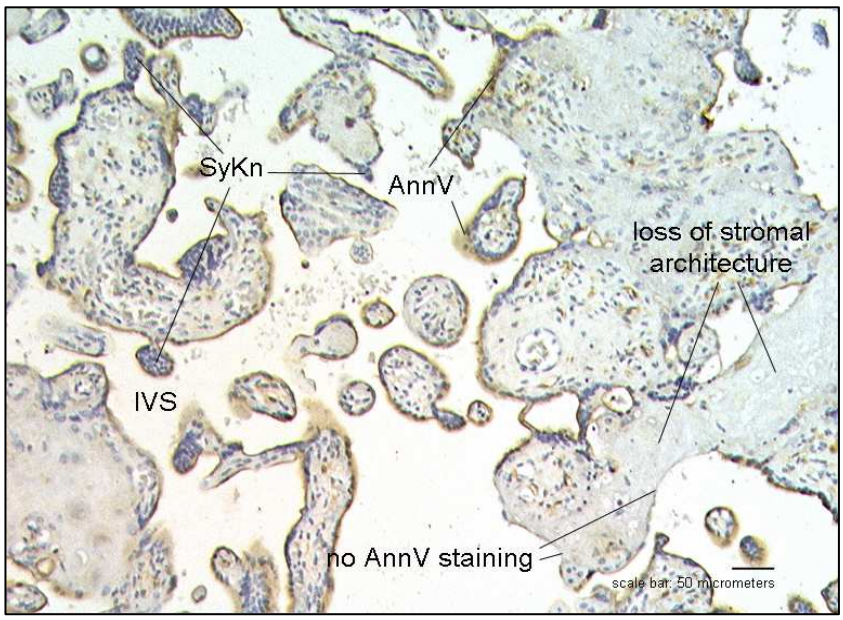


A

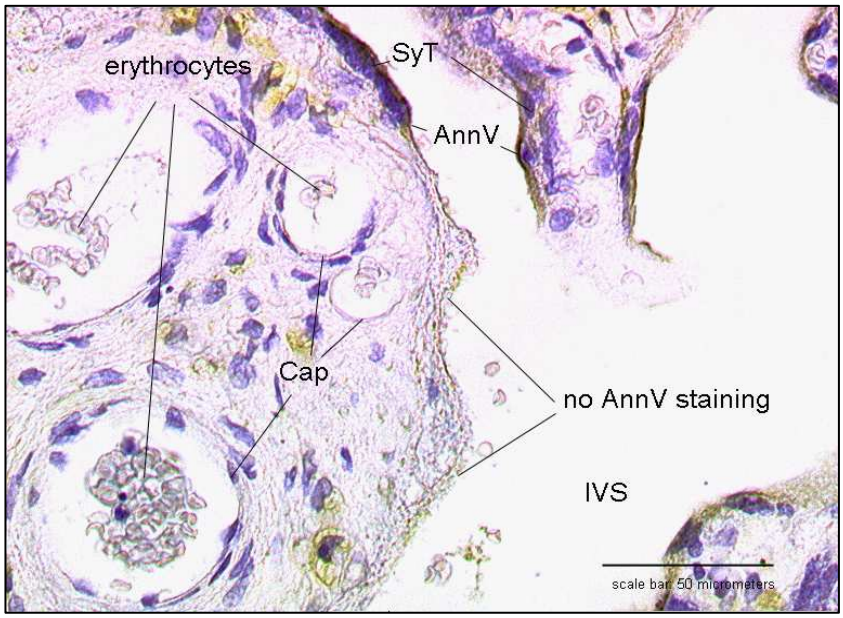


B

Figure 3.2.2.2: Annexin V staining in IUGR placenta x 10 magnification (**A**) and x 40 magnification (**B**).



A



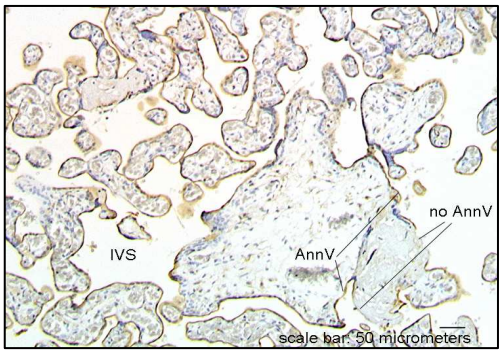
B

Figure 3.2.2.3: Annexin V staining in pre-eclamptic placenta at x 10 magnification (**A**) and x 40 magnification (**B**).

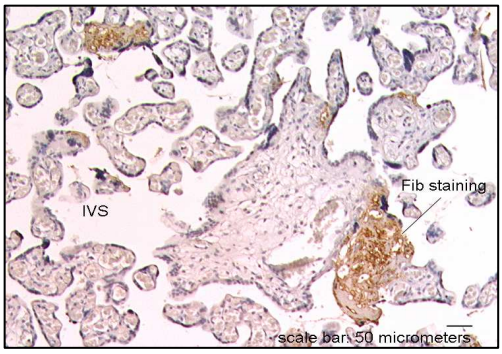
3.2.3 Comparison of annexin V and fibrin localisation in back-to-back samples in healthy third trimester, IUGR and pre-eclamptic placentas

In healthy third trimester samples, there appeared to be an inverse relationship between annexin V and fibrin staining. Where there was annexin V staining, there was little fibrin staining in the same area of the corresponding slide that was cut back-to-back. Conversely, in areas of minimal annexin V staining, there was strong fibrin staining in the same place on the corresponding slide (figures 3.2.3.1A to F). Essentially there appeared to be an inverse localisation between annexin V **(AnnV)** and fibrin **(Fib)** between corresponding slides that were cut back-to-back.

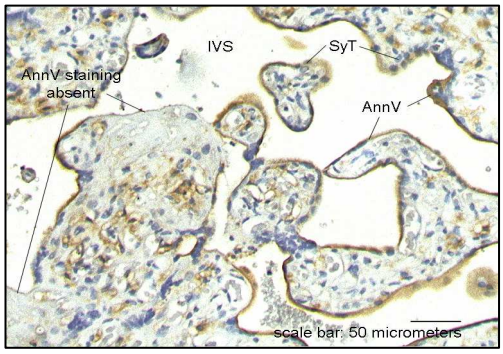
In IUGR samples, there was also inverse localisation between the staining of annexin V and fibrin between corresponding back-to-back slides (as shown in figures 3.2.3.2 A to F). In figure 3.2.3.2A, numerous syncytial knots were present and these knots were stained with annexin V. In the back-to-back slide shown in figure 3.2.3.2B, there was little fibrin deposition on these knots. There was prominent fibrin staining in areas of disorganised villi architecture (figure 3.2.3.2D and figure 3.2.3.2F) both on the syncytial surface and within the underlying area of villous disorganisation. In pre-eclamptic samples, there was also inverse localisation between the staining of annexin V and fibrin in the corresponding back-to-back slides (figure 3.2.3.3 A to F). This pattern was consistent for all of the slides examined. Again, there was prominent fibrin staining in areas of disorganised villi architecture (figure 3.2.3.3 D). There was also the impression of more and larger areas of fibrin deposition in pre-eclamptic samples compared to samples from healthy pregnancies.



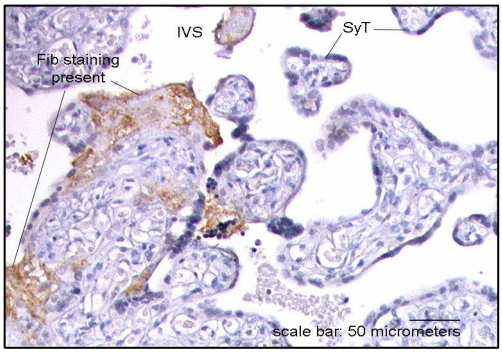
A



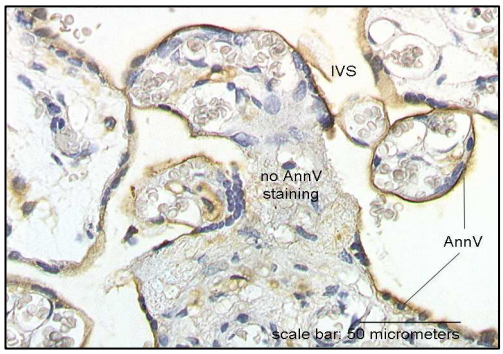
B



C



D



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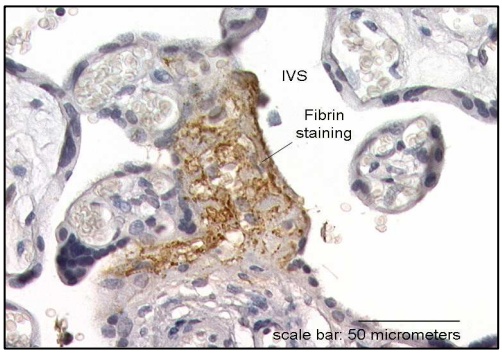
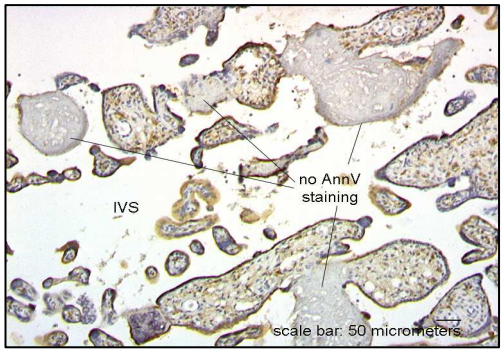
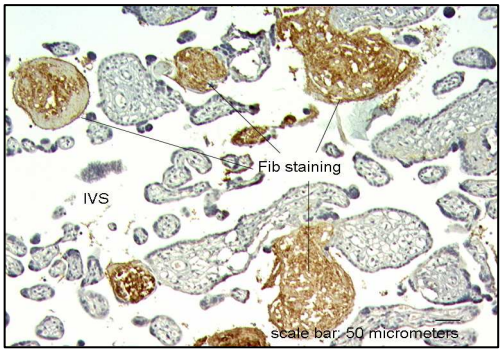


F

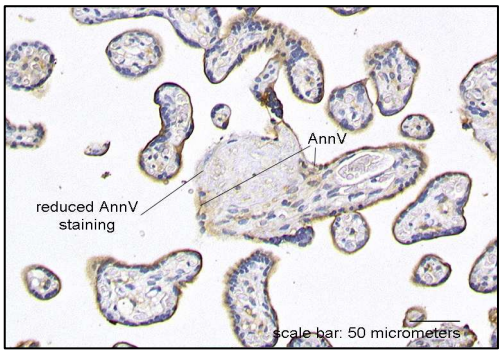
Figure 3.2.3.1: Inverse localisation between annexin V and fibrin immunocytochemical staining in healthy third trimester placentas at x 10 magnification [annexin V (**A**), fibrin (**B**)], x 20 magnification [annexin V (**C**), fibrin (**D**)] and x 40 magnification [annexin V (**E**), fibrin (**F**)].



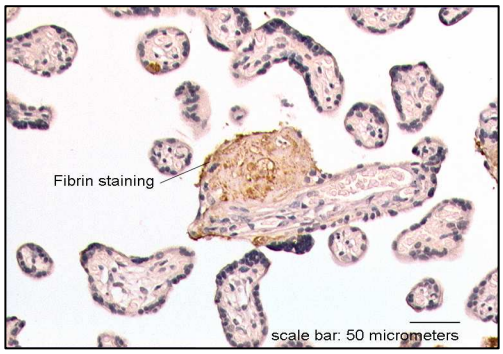
A



B



C



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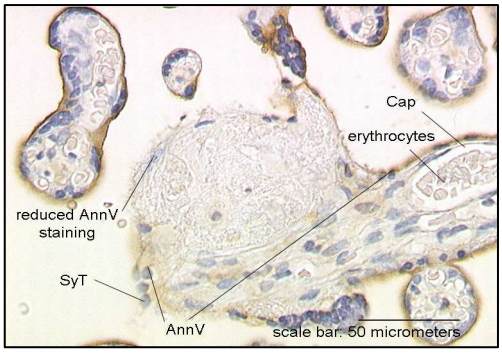
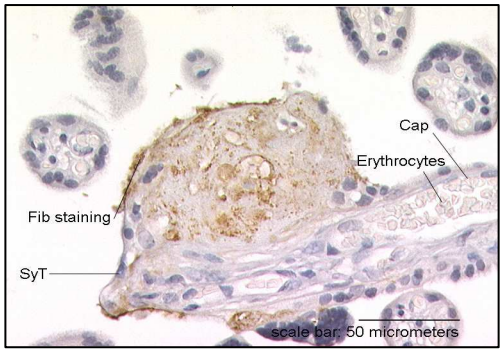
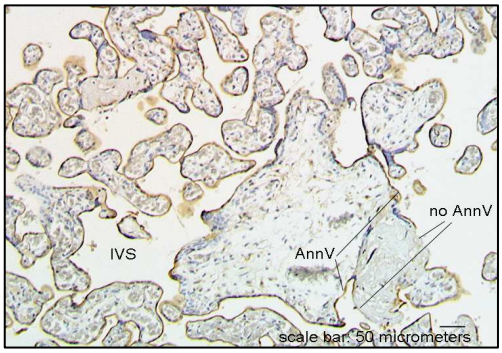


E

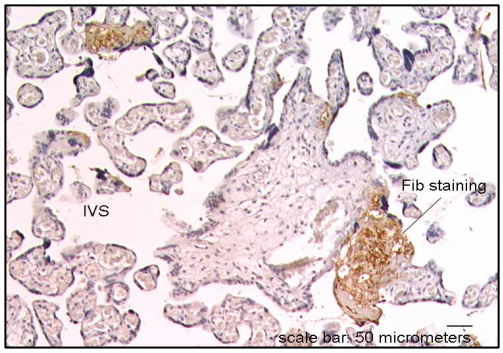


F

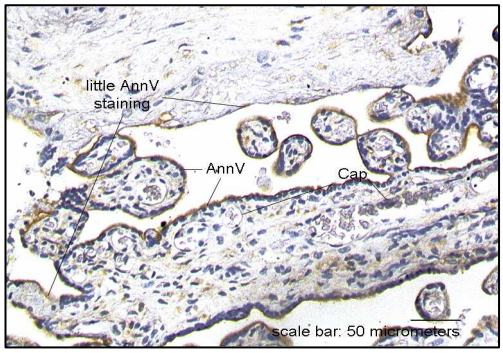
Figure 3.2.3.2: Inverse localisation between annexin V and fibrin immunocytochemical staining in IUGR placentas at x 10 magnification [annexin V (**A**), fibrin (**B**)], x 20 magnification [annexin V (**C**), fibrin (**D**)] and x 40 magnification [annexin V (**E**), fibrin (**F**)].



A



B



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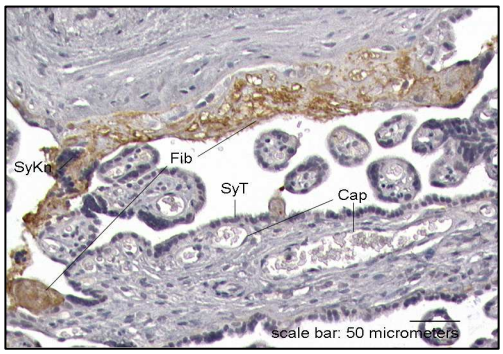

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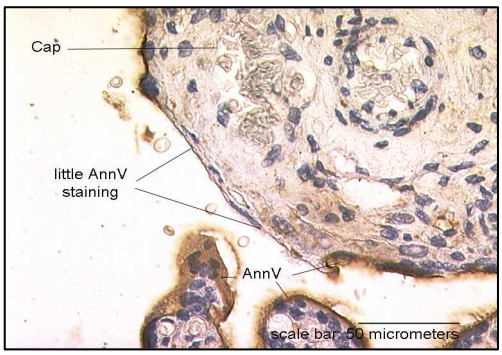
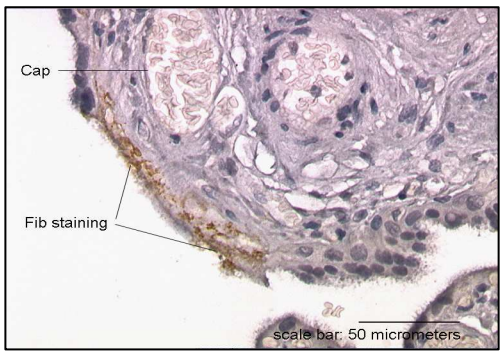


E



F

Figure 3.2.3.3: Inverse localisation between annexin V and fibrin immunocytochemical staining in pre-eclamptic placentas at x 10 magnification [annexin V (**A**), fibrin (**B**)], x 20 magnification [annexin V (**C**), fibrin (**D**)] and x 40 magnification [annexin V (**E**), fibrin (**F**)].

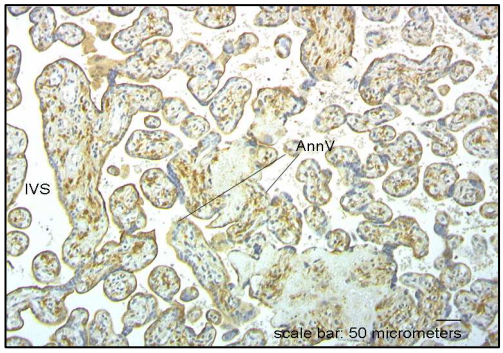
3.2.4 Comparison of annexin V and TF staining in back-to-back samples in normal, IUGR and pre-eclamptic placentas

In healthy third trimester samples, very little tissue factor **(TF)** staining was seen with only approximately only 4 to 5 small areas of distinct TF seen throughout the whole slide. For example, only a single area of TF staining was seen as a brown precipitate in the middle of figure 3.2.4.1B at x 10 magnification. TF staining was mainly at the trophoblast layer (figures 3.2.4.1 D and 3.2.4.12 F). There was very little TF staining within the fetal vascular endothelium in these healthy pregnancies. In comparison, there were large areas of annexin V **(AnnV)** staining on the surface of the syncytiotrophoblasts with some smaller areas of non-staining (figure 3.2.4.1 A). There was no clear relationship in staining pattern between annexin V and TF in these healthy pregnant samples (see figures 3.2.4.1 A to F).

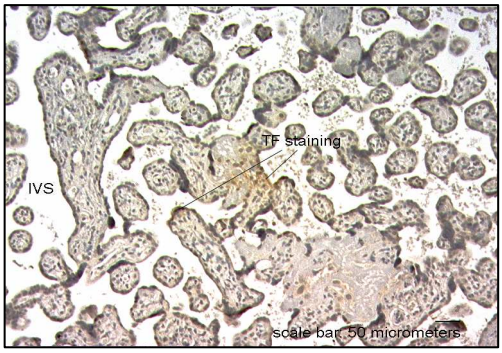
In IUGR samples, there were very few scattered small areas of TF staining throughout the whole slide (figure 3.2.4.2B). In back-to-back slides comparing TF with annexin V, there was no consistent relationship between the TF and annexin V staining. For example, comparing between figures 3.2.4.2C with 3.2.4.2D and between figures 3.2.4.2E and 3.2.4.2 F, there was TF staining in areas of annexin V staining and also TF staining in areas with no annexin V staining. In pre-eclamptic samples, there were very few scattered small areas of TF staining throughout the whole slide (shown in figure 3.2.4.3 B) but staining appeared to be at the trophoblast layer. In back-to-back slides comparing TF with annexin V, there was no consistent relationship between TF and annexin V staining patterns, (figures 3.2.4.3 C and D and figures 3.2.4.3 E and F).

3.2.5 M30 localisation in first and third trimester healthy placentas

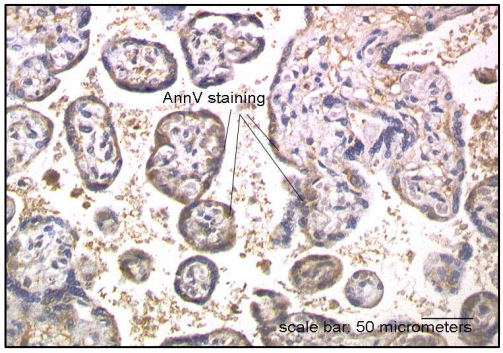
In first trimester healthy placentas, there was distinct M30 staining at the trophoblast layer (figure 3.2.5.1A). There was greater M30 staining at the cytotrophoblast **(CyT)** compared to the syncytiotrophoblast **(SyT)** layer and this is quite evident when seen at greater magnification at 40 x (figure 3.2.5.1B) and 100 x magnification (figure 3.2.5.1C). This



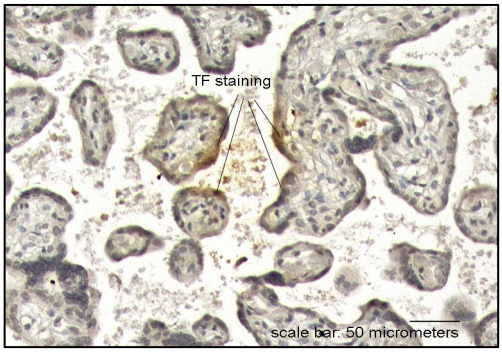
A



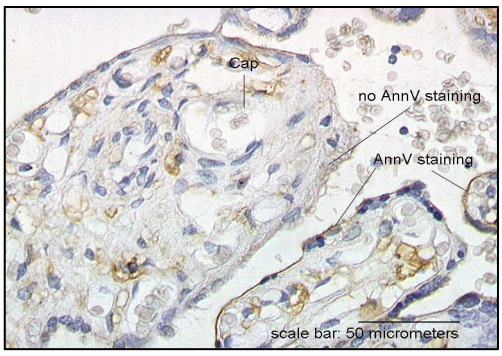
B



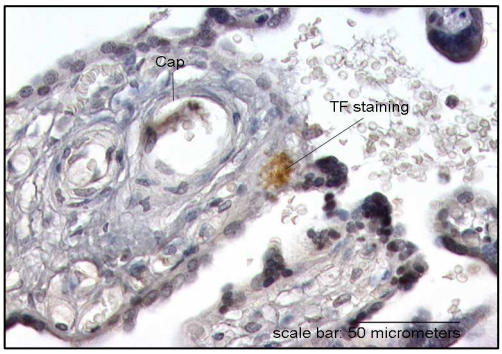
C



D

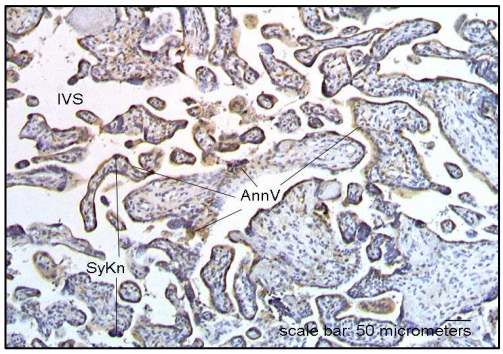


E

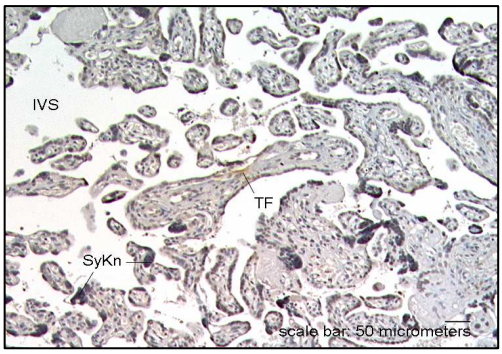


F

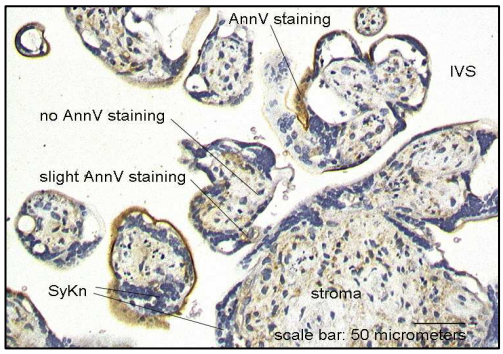
Figure 3.2.4.1: Annexin V and TF immunocytochemical staining in healthy third trimester placentas at x 10 magnification [annexin V (**A**), TF (**B**)], x 20 magnification [annexin V (**C**), TF (**D**)] and x 40 magnification [annexin V (**E**), TF (**F**)].



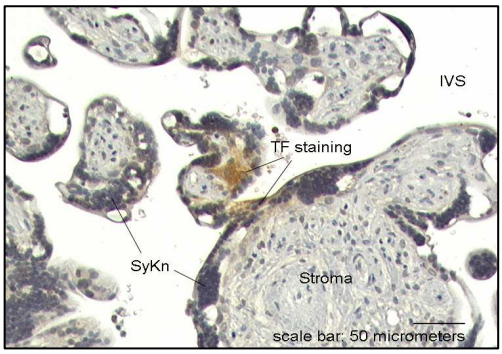
A



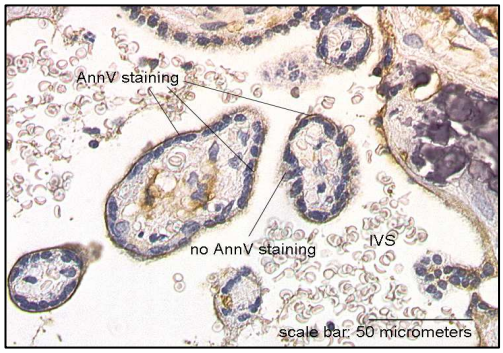
B



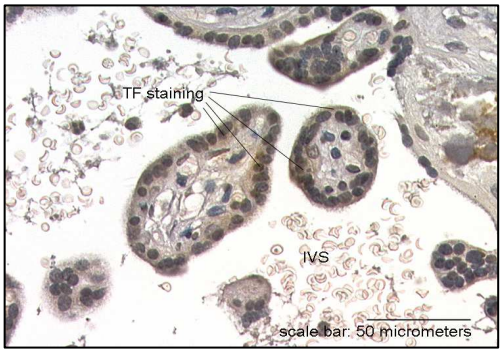
C



D

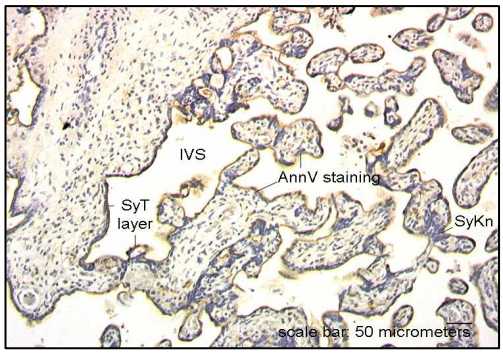


E

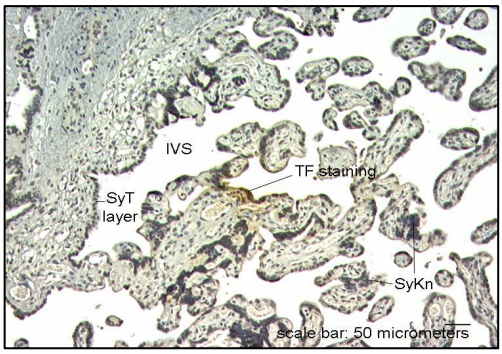


F

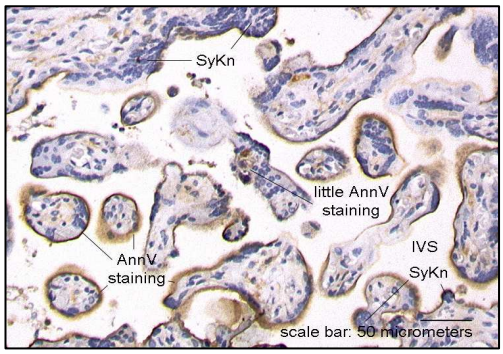
Figure 3.2.4.2: Annexin V and TF immunocytochemical staining in IUGR placentas at x 10 magnification [annexin V (**A**), TF (**B**)], x 20 magnification [annexin V (**C**), TF (**D**)] and x 40 magnification [annexin V (**E**), TF (**F**)].



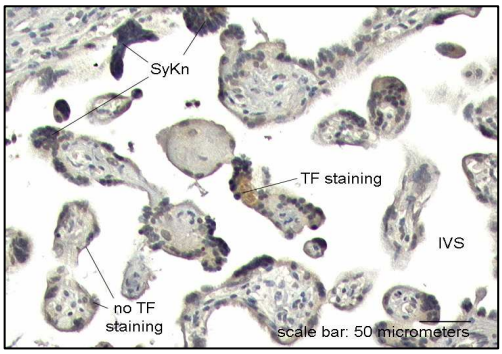
A



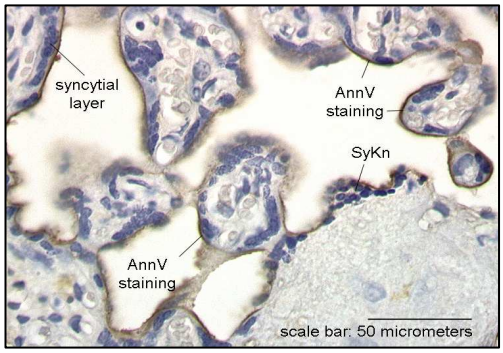
B



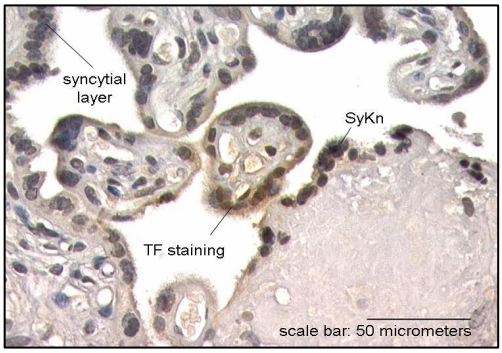
C



D

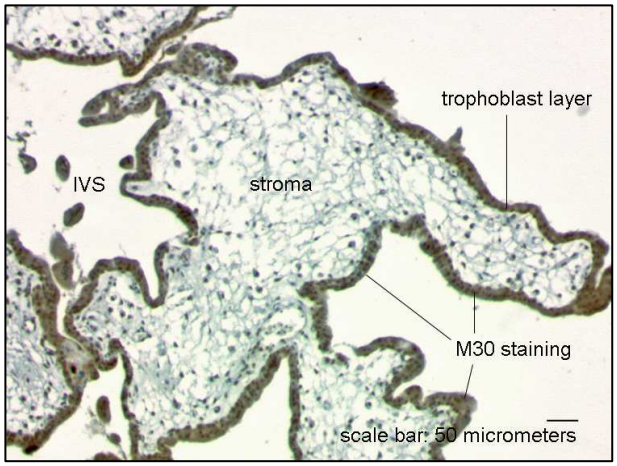


E

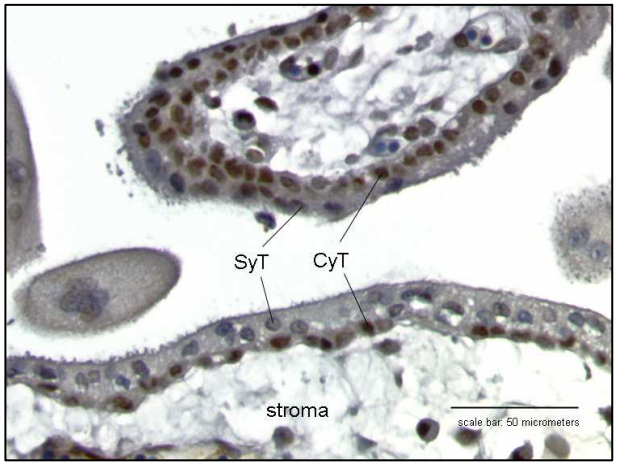


F

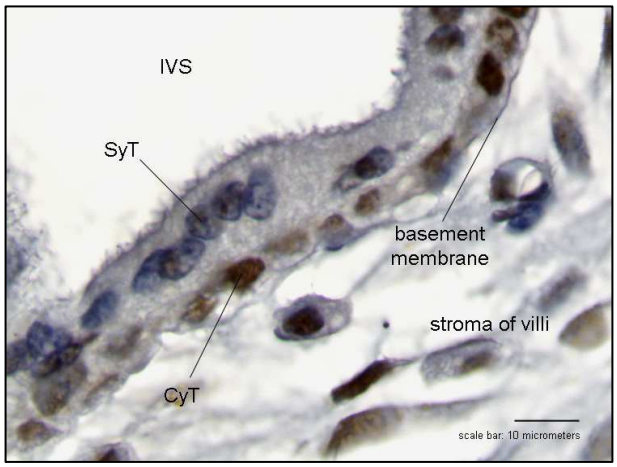
Figure 3.2.4.3: Annexin V and TF immunocytochemical staining in pre-eclamptic placentas at x 10 magnification [annexin V (**A**), TF (**B**)], x 20 magnification [annexin V (**C**), TF (**D**)] and x 40 magnification [annexin V (**E**), TF (**F**)].



A



B



C

Figure 3.2.5.1: M30 staining of first trimester placenta at x 10 magnification (**A**), x 40 magnification (**B**) and x100 magnification (**C**).

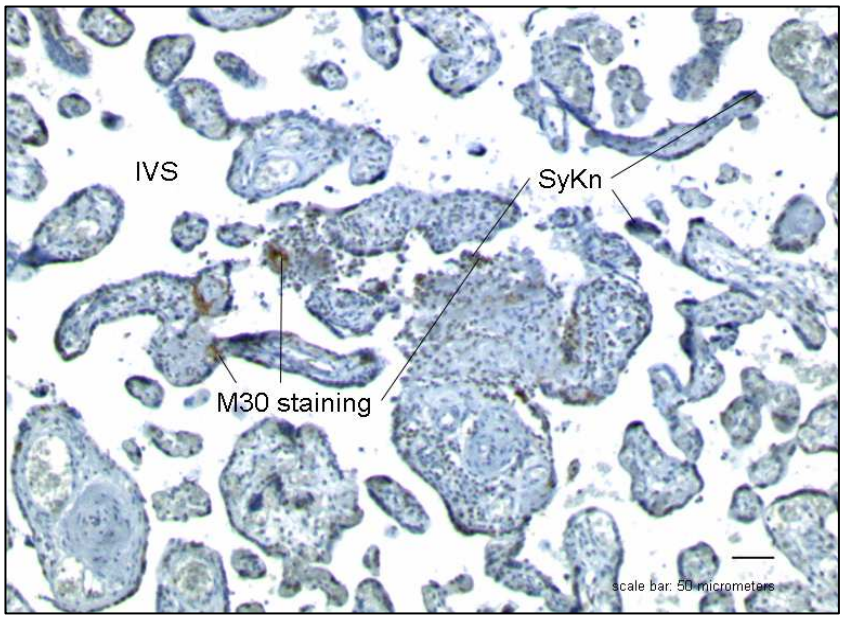
may represent non-specific staining of the nuclei of the proliferating cytotrophoblast cells. There was also some staining of the nucleus of some stromal cells which appear to be fibroblasts with their elongated spindle shaped cells.

In third trimester healthy placentas, M30 staining was present at the trophoblast layer. However there appeared to be a lesser degree of M30 trophoblast staining in third trimester placentas compared to first trimester placentas (figure 3.2.5.2 A, B). At higher magnification (figure 3.2.5.2 B), M30 staining was found to be present mainly at the nuclei of cytotrophoblast cells with very little M30 staining of the cytoplasm.

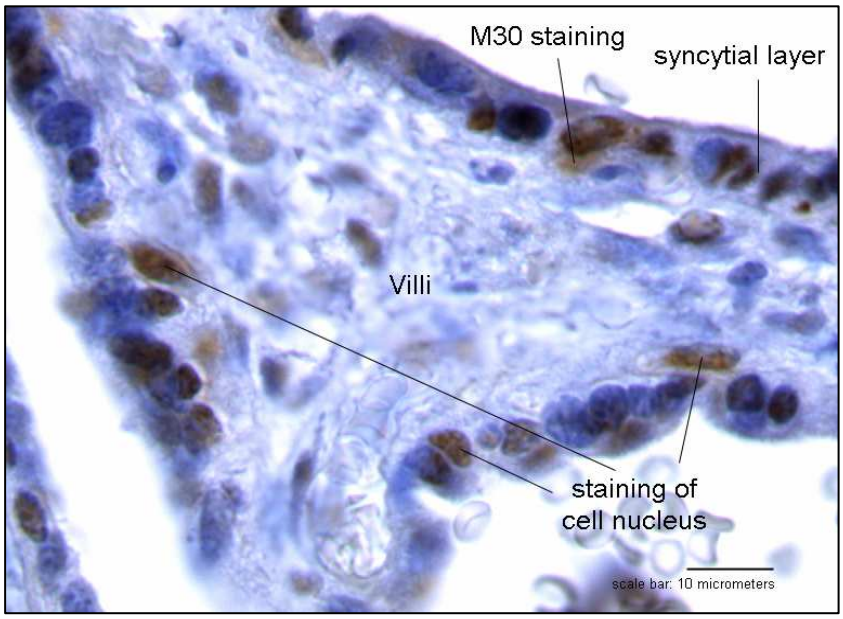
3.3 Discussion

It is known that initial deposition of fibrin leads to a cascade reaction that leads to elongation of the fibrin polymer chain leading to the formation of a blood clot. I was interested in identifying areas of fibrin deposition in healthy first trimester, healthy third trimester, IUGR and pre-eclamptic placentas as perivillous fibrin deposition on the surface of the trophoblast can act as a barrier between feto-maternal exchange, which can ultimately affect fetal wellbeing.

Perivillous fibrin deposition was found to be present in healthy first and third trimester, IUGR and pre-eclamptic placentas but there appeared to be a greater amount of fibrin deposition in pre-eclamptic and IUGR placentas compared to healthy first and third trimester placentas. Perivillous fibrin deposition was found to be present on many syncytial knots (which are composed of ageing syncytiotrophoblast nuclei prior to extrusion as part of the process of villous trophoblast apoptosis and turnover(52)) and Nelson *et al* has shown apoptotic changes in the syncytiotrophoblast in areas of fibrinoid deposit(34). It is known that apoptotic cells expose phosphatidylserine on the cell surface which is pro-coagulant and can lead to lead to coagulation and subsequent fibrin deposition. Therefore, this deposition of fibrin on syncytial knots may reflect syncytiotrophoblast apoptosis in these knots. This impression of greater amount of fibrin deposition in IUGR and pre-eclamptic samples was not quantitated however. Quantitation may have been achieved by simply directly classifying each fibrin deposit in an area of the slide as small, medium, or



A



B

Figure 3.2.5.2: M30 staining of third trimester healthy placenta at x 10 magnification (**A**) and x100 magnification (**B**).

large and the total value added up. This should be done with at least two independent observers to reduce operator error. Alternatively stereology could be employed for a more quantitative answer.

The results from immunocytochemical staining showed that annexin V was present on the surface of syncytiotrophoblasts but annexin V staining was found to be discontinuous in some parts. This pattern was present in all healthy, IUGR and pre-eclamptic sample groups. It was noted that when compared to healthy placentas, IUGR and pre-eclamptic placentas appeared to have greater areas of absent annexin V staining (denudation). Some areas of annexin V denudation appeared to be located at areas of the placental villi where there was no clear appearance of normal syncytial cell morphology and where the underlying stromal tissue also appeared disorganised with no clear features of stromal cells or blood vessels, thus appearing rather amorphous. It is possible these amorphous areas represented areas of villi infarction as infarcted areas of placental villi would not be able to sustain its trophoblast layer thus leading to syncytial death with the resultant loss of annexin V expression. However, in some areas of annexin V denudation on the syncytial surface, there was normal appearance of underlying villi cellular architecture. Here, the lack of annexin V may possibly be due to syncytiotrophoblast damage or apoptosis thus leading to reduced annexin V expression. These mechanisms would be in keeping with the greater amount of placental infarction and trophoblast damage in the conditions of IUGR and pre-eclampsia.

As annexin V prevents coagulation, I was interested to discover if there was a correlation between the distribution of annexin V expression on the syncytiotrophoblast surface and areas of TF expression and areas of fibrin deposition. Back-to-back slides of annexin V and fibrin revealed that in areas of annexin V denudation, there was often fibrin deposition in the same area in the corresponding slide (inverse localisation). This fibrin deposition also often extended into the underlying areas of amorphous-looking stroma in the villi. Perivillous fibrin deposition of the intervillous space also occurred in some of these areas. This pattern was consistent in all healthy, IUGR and pre-eclamptic samples although it was noted that in contrast to healthy pregnant placentas, IUGR and pre-eclamptic placentas seemed to have greater areas of annexin V denudation with associated greater areas of fibrin staining in the corresponding slides. This would be in keeping with the findings of Shu *et al* who found plasma level of the fibrin degradation products and thrombin-

antithrombin III complex from pre-eclamptic patients were elevated as the expression of annexin V in the placenta was reduced(48).

As TF is a potent initiator of coagulation, one might expect that there would be greater expression of TF in the areas where there is absent annexin V staining. Certainly, high levels of TF as well as TF mRNA have been found in the placentas of pre-eclamptic women compared to healthy pregnant controls(29). However there were only very sporadic areas of TF staining found (3 to 6 small areas of TF staining per slide) in the placental samples of the healthy pregnant, IUGR and pre-eclamptic pregnancies despite the large amounts of perivillous fibrin deposition in IUGR and pre-eclamptic placental samples. A simple explanation is that there was only very little TF expression in the placenta. Estelles *et al* did not find any appreciable expression of TF in either healthy or pre-eclamptic placentas although they found a five-fold increase in the amounts of TF mRNA in pre-eclamptic compared to healthy placentas(29). Perhaps placental TF only becomes expressed in more severe pre-eclampsia. It is also possible that as TF is such a potent initiator of coagulation, even slight expression of tissue factor in the placenta would immediately lead to the deposition of fibrin, thereby preventing the primary antibody directed towards TF from attaching. As a result of sub-optimal TF staining, a comparison of back-to-back staining between TF and fibrin was not possible during this project. It would have been interesting to see if there was an increased deposition of fibrin even on these very small areas of TF expression in the placenta.

It is possible that annexin V prevents coagulation on the trophoblast surface and this may be the mechanism by which the placenta prevents excessive fibrin deposition on its syncytial surface thereby facilitating effective feto-maternal transfer. An absence of annexin V on the surface of the syncytium may lead to fibrin deposition leading to reduced feto-maternal exchange, subsequent trophoblast death and followed by underlying villi death and necrosis. This might explain the areas of disorganised villi architecture underlying areas of annexin V denudation. On the other hand, these amorphous areas may represent areas of villi infarction as a result of placental vessel thrombosis, villi hypoxia and subsequent syncytial death with subsequent loss of annexin V synthesis and expression on the syncytiotrophoblast. IUGR and pre-eclamptic placentas had greater areas of annexin V denudation with associated greater fibrin deposition in those areas when compared to placentas from healthy pregnant individuals. It is likely that the greater syncytial damage in

the conditions of IUGR and pre-eclampsia is reflected in loss of annexin V expression. IUGR and pre-eclampsia are also associated with greater placental infarction and this may lead to villi ischemia, loss of annexin V synthesis and subsequent perivillous fibrin deposition. Another possibility is there may be reduced expression of annexin V in the conditions of IUGR and pre-eclampsia compared to healthy pregnancy. A way of investigating this possibility would be by measuring and comparing total annexin V mRNA in the placenta of healthy, IUGR and pre-eclamptic pregnancies.

M30 was used as a marker to identify cellular apoptosis within the trophoblast layer in first and third trimester samples in healthy pregnancies. In first trimester samples, there was abundant M30 staining of cytotrophoblast nuclei but little staining of the cytotrophoblast cytoplasm. While M30 was utilised as a marker to identify apoptotic cells, it is known that M30 can also stain the nuclei of actively proliferating cells(51). The high number of cytotrophoblast cell nuclei stained by M30 in the first trimester compared to third trimester placenta samples suggests that there was a large degree of cytotrophoblast cell proliferation occurring in the first trimester. This is in keeping with the function of cytotrophoblast cells being the stem cells of the syncytium, actively proliferating and differentiating into the syncytiotrophoblast. Staining of trophoblastic sprouts was also seen, possibly reflecting the developing placenta where the villi are actively growing and the placental sprouts are remodelling and forming new villi. In the third trimester, there were less cytotrophoblasts present and less staining of cytotrophoblast nuclei compared to the first trimester samples. This may simply reflect a lower degree of trophoblast proliferation in the third trimester compared to the first trimester. The uncertainty over the M30 staining meant that it was not possible to investigate any relationship between apoptosis and fibrin deposition or annexin V expression in the trophoblast. Perhaps another marker of apoptosis may be employed and this may be scope for future work.

The main aim of this chapter was to investigate the role of annexin V and apoptosis in coagulation in pre-eclamptic placentas. IUGR and pre-eclamptic placentas appeared to have greater amounts of fibrin deposition in the perivillous spaces compared to healthy pregnancies and this is in keeping with current literature. However, I have discovered that there is a relationship of inverse localisation between annexin V and fibrin staining on the surface of the syncytiotrophoblasts in healthy pregnant, IUGR and pre-eclamptic pregnancies. This suggests that annexin V on the surface of the trophoblast may confer

anticoagulant properties to the placental villi. With regard to TF, there were only a few sporadic areas of TF staining in the placentas of healthy pregnant, IUGR and pre-eclamptic pregnancies and there was no clear relationship identified between annexin V expression and TF expression in these placentas. M30 staining for apoptotic cells was not satisfactory and I was therefore unable to identify if there was any association between cellular apoptosis and annexin V, TF expression and fibrin deposition in the placenta.

There was the impression of a greater amount of fibrin deposition on the trophoblasts of pre-eclamptic pregnancies compared to healthy pregnancies. Microparticles are prothrombotic and the level of microparticles in the systemic circulation has previously been found to be raised in various prothrombotic conditions such as acute coronary syndrome, diabetes, paroxysmal nocturnal haemoglobinuria and severe hypertension. Microparticle levels have also been found to be raised in late miscarriages(74), suggesting they may play a part in placental thrombosis(276). It is possible that there may also be greater levels or procoagulant activity of microparticles in pre-eclampsia which may contribute to the procoagulant state of pre-eclampsia. In the next chapter, I set out to develop an assay to measure the pro-coagulant activity of microparticles in maternal blood in pre-eclamptic and healthy pregnant women.

Chapter 4 Microparticle procoagulant activity and placental debris in pre-eclampsia

4.1 Introduction

In mammalian cells, cell membrane phospholipids are asymmetrically distributed with PS normally maintained at the inner leaflet of cell membranes by an active process. When the cell undergoes activation or apoptosis, PS becomes externalised onto the outer surface of the cell membrane by a 'phospholipid scramblase' pathway. The cell membrane then forms blebs which are released into the blood circulation as microparticles(277). Phosphatidylserine is a phospholipid and is known to provide a surface for the conversion of prothrombin to thrombin by various clotting factors(278;279). Phosphatidylserine now located on the external surface of microparticles, would be in a position to act as a surface upon which these clotting factors assemble, forming the prothrombinase complex which then catalyses the conversion of circulating prothrombin to thrombin.

Microparticles are procoagulant in nature and levels of circulating microparticles in blood have previously been quantified using flow cytometry. Flow cytometry has several advantages. It can identify the subpopulation of cells from which the microparticles are derived by using an appropriate fluorescent antibody (for example fluorescein isothyocyanate [FITC]-conjugated monoclonal antibody) specific to that cell population. The total amount of microparticles in the sample can also be measured using FITC-conjugated annexin V which binds to all microparticles in the sample. In pre-eclampsia, microparticles from different cell subpopulations such as endothelial, T-helper, T-suppressor, granulocyte and platelets have been measured and all have been found to be raised compared to healthy pregnant controls(80;87;89), although one study found lower levels of platelet-derived microparticles(86). Despite differences in microparticle levels in these cell subpopulations, no difference was found in the total amount of microparticles (from all cells of origin) between pre-eclamptic and healthy control groups(86;87). While flow cytometry is a useful technique to quantify the amount of microparticles in a sample, it has a disadvantage in that it cannot measure the actual procoagulant activity of microparticles in the sample.

One of the primary aims of this thesis is to measure the procoagulant activity of microparticles in the maternal blood of pre-eclamptic and healthy pregnant controls as well as to relate microparticle pro-coagulant activity to markers of coagulation, endothelial function and placental function. While the amounts of microparticles have previously been measured by other researchers, I was interested in measuring the procoagulant activity of microparticles. Aupeix *et al*(65) used a plate-based prothrombinase assay to measure procoagulant activity of microparticles in HIV patients. This assay worked by measuring the amount of thrombin generated from prothrombin via the phosphatidylserine (PS) component of microparticles. The amount of thrombin generated was then used as a surrogate end-point to determine the procoagulant potential of microparticles in the sample. This assay measured the procoagulant activity of all microparticles in the blood sample, regardless of its cell of origin and whether they were of placental or maternal origin.

Aside from the determination of the procoagulant activity of microparticles in maternal blood, I was also interested in measuring the amount of placental-derived microparticles in maternal blood. The syncytiotrophoblast layer of the placenta is in direct contact with maternal blood. In healthy pregnancy, constant placental remodelling and renewing of the syncytiotrophoblast layer results in apoptosis of syncytiotrophoblasts (9) and this has been proposed to result in the shedding of syncytiotrophoblast membrane fragments(STBMs) into the maternal side of placental circulation(76). Microparticles are also released from apoptotic cells and therefore, it is possible that STBMs are placentally-derived microparticles. As microparticles are known to be procoagulant, so may STBMs. In comparison to healthy pregnancy, there is greater placental apoptosis in pre-eclampsia. This may lead to greater amounts of microparticles of syncytiotrophoblast origin which may contribute to the overall procoagulant state of pre-eclampsia.

Fetal CRH is synthesised by the placenta in large amounts and its synthesis has been localised to the trophoblast(98). When microparticles bleb off from the cell membrane, they are released as little packets which contain cytoplasm from the cell of origin. Microparticles from the trophoblast would therefore contain within them fetal CRH mRNA. As maternal CRH mRNA is undetectable in the maternal systemic circulation(98), any measurable CRH mRNA in maternal circulation would originate from the placenta. However it is possible that not just microparticles but cellular fragments released from

damaged trophoblasts, for example, may also contain fetal CRH mRNA. Thus any measure of fetal CRHmRNA would include a measure of these cell fragments. It would therefore be appropriate to collectively term both microparticles and cellular fragments as 'placental cellular debris'. The amount of fetal CRH mRNA was measured as a ratio of CRH to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). At the time of commencement of this project, measurement of placental-derived microparticles had not been done although subsequently Goswami *et al*(81) measured syncytiotrophoblast microparticle levels using an anti-trophoblast antibody (NDOG2). This antibody was not commercially available however.

4.2 Principle of the prothrombinase assay

The principle of this prothrombinase assay is the capture of PS-expressing microparticles using a specific binding protein and quantification of the procoagulant activity of these PS-expressing microparticles using a chromogenic substrate. The method of Aupeix *et al*(65) was used in the development of this prothrombinase assay. In their assay, results were expressed as nanomolar PS equivalents with reference to a standard curve created from synthesised phosphatidylcholine: phosphatidylserine (PC:PS) vesicles of known composition. I prepared synthetic PC:PS vesicles according to the method of Pigault *et al*(275), as detailed in Chapter 2 of this thesis. This method resulted in the production of vesicles with a concentration of 1mM phosphatidylserine in solution, i.e: 1mM phosphatidylserine equivalents (PS equivalents).

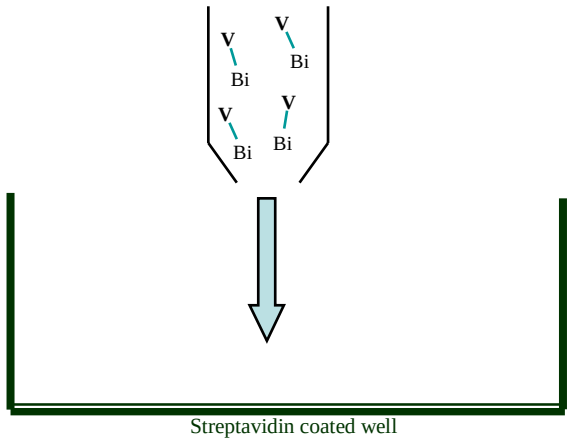
4.3 Steps of the prothrombinase assay

The different steps of the prothrombinase assay were as follows, with reference to figure 4.3.1. Biotinylated annexin V was added to the streptavidin-coated wells of a microtiter plate and incubated for 30 minutes (Step 1). Biotinylated annexin V became bound to streptavidin in the wells with the result that the wells became lined with annexin V (Step 2). Excess biotinylated annexin V was then washed out and 'Inhibitor mix' was then added into the well (Step 3). 'Inhibitor mix' is a mixture of Phe-Pro-Arg-Chloromethylketone [PPACK (a rapid inhibitor of thrombin)] and 1-5 Dansyl-Glu-Gly-Arg-

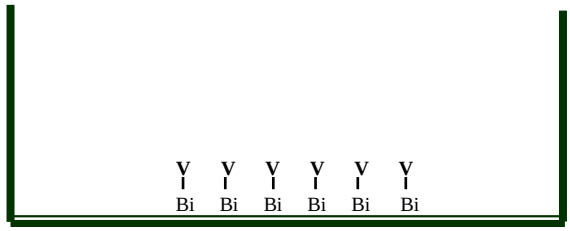
Chloromethylketone [DnsGGACK (an inhibitor of factor Xa)] and 'inhibitor mix' functions to inactivate any endogenous thrombin or factor Xa (which converts prothrombin to thrombin) that may be present in the sample.

The sample to be measured was then added into the well (Step 4) and incubated for 30 minutes, allowing any PS-containing particles in the sample to be captured by annexin V lining the wells (Step 5). The wells were then washed out. 'Master mix' (a mix of factor Va, factor Xa and calcium chloride in buffer) was then added into the well (Step 6). The activated factor V, activated factor X and calcium in the 'master mix' assembles on the phospholipid surface of the captured microparticle, forming the prothrombinase complex. Prothrombin was then added into the wells (Step 7) and was converted by the prothrombinase complex assembled on the surface of captured microparticles to thrombin during incubation over 30 minutes. The conversion of prothrombin to thrombin was then halted by the addition of ethylenediaminetetraacetic acid (EDTA) which chelates calcium, which is essential to the functioning of the prothrombinase complex (Step 8). The amount of thrombin generated by the prothrombinase complex would be a surrogate measure of the procoagulant activity of captured microparticles in the sample.

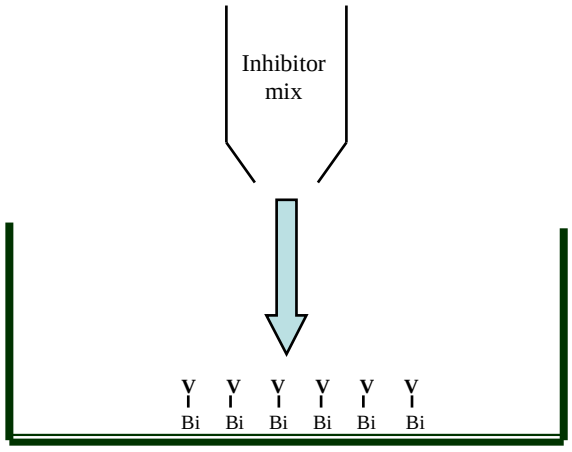
The amount of thrombin generated was quantified by the addition of chromozym TH (a chromogen that is converted to a yellow-coloured end product by thrombin) into the well (Step 9). The plate was immediately put into a microtitration plate reader to quantify the change in optical density of chromozym TH over time (OD/time) at 405nM wavelength (Step 10) over the course of 20 minutes. The rate of conversion of chromozym TH to its coloured end product is related to the amount of thrombin present, which is in turn related to the amount of prothrombinase complex formed by captured microparticles. In this way, the procoagulant activity of the microparticles in the sample could be measured. The rate of colour change of chromozym TH in an unknown sample is compared to a standard curve created by synthetic phosphatidylcholine: phosphatidylserine (PC: PS) vesicles of known concentration.



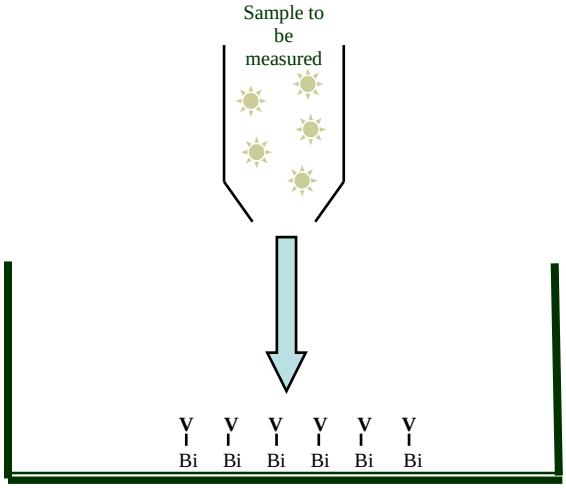
Step 1: Addition of biotinylated annexin V (Bi-V) into streptavidin coated well.



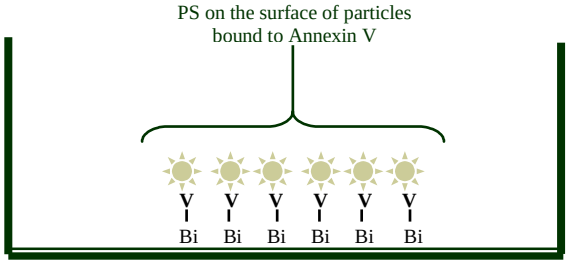
Step 2: Annexin V lining the well.



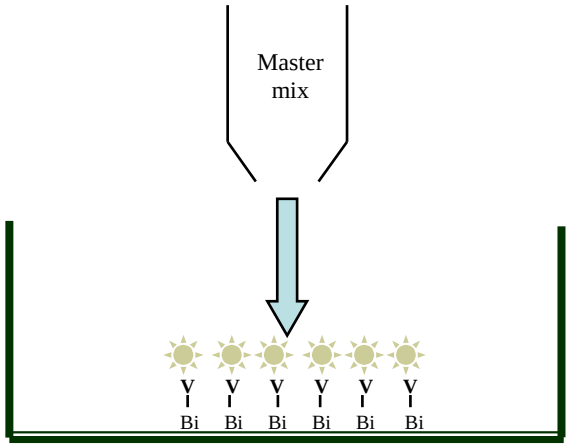
Step 3: 'Inhibitor mix' added.



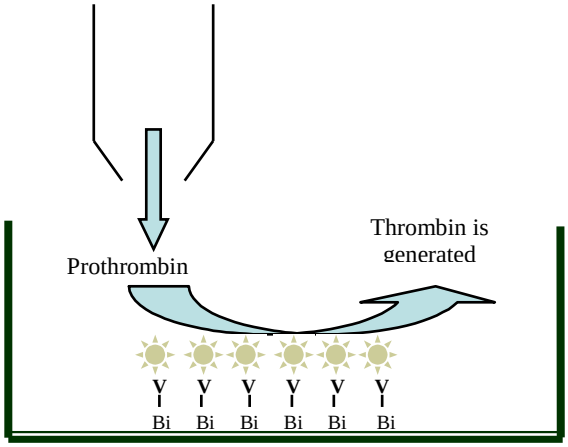
Step 4: Addition of the sample to be measured (eg. patient's plasma).



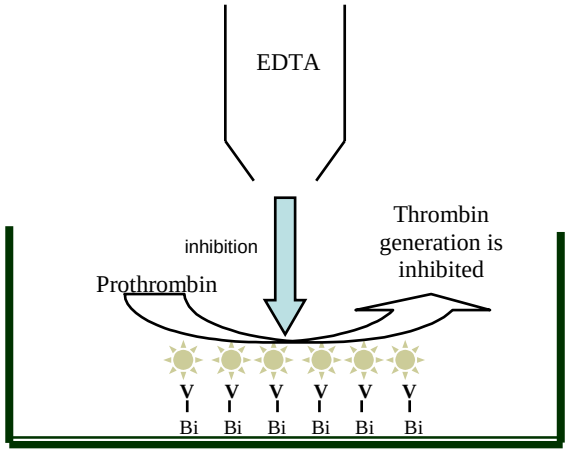
Step 5: Annexin V lining the well captured PS-expressing microparticles from the sample.



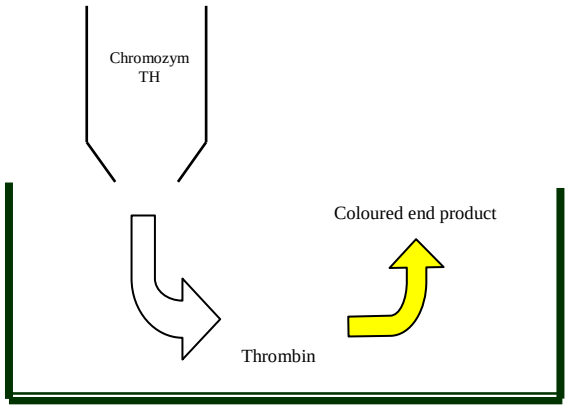
Step 6: Addition of 'master mix' (a mix of factor Va, factor Xa and calcium chloride in buffer) which together with the captured PS formed the prothrombinase complex.



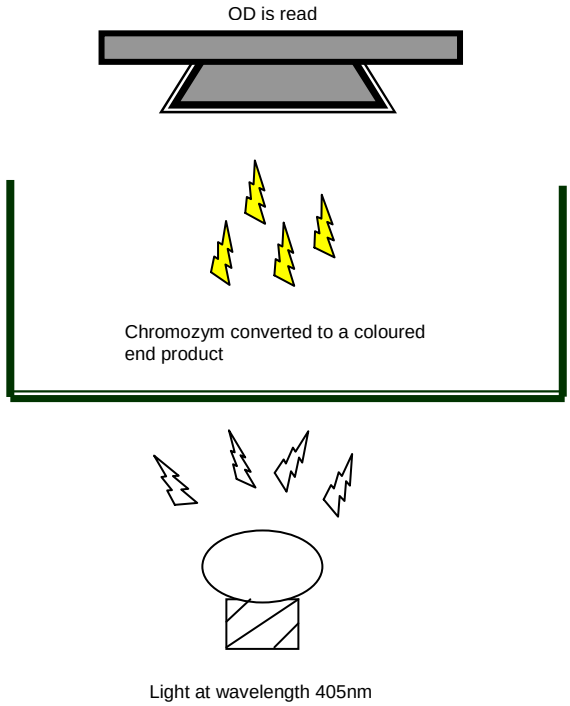
Step 7: Prothrombin was converted to thrombin by the prothrombinase complex.



Step 8: Addition of EDTA to stop the conversion of prothrombin to thrombin after 30 minutes.



Step 9: Chromozym TH is added and was converted to a coloured end-product by the generated thrombin. For purposes of clarity, annexin V-Biotin and captured PS particles are not shown in the diagram.



Step 10: Intensity of the end-product of chromozym TH was quantified in a plate reader equipped with kinetics software. Colour change was recorded at set time intervals.

Figure 4.3.1: The steps of the prothrombinase assay

4.4 Hypotheses and objectives

There is greater maternal endothelial activation, platelet activation and trophoblast apoptosis in pre-eclampsia and I hypothesise that this may lead to greater microparticle generation. This may in turn lead to greater microparticle procoagulant activity in maternal blood. I also hypothesised that greater trophoblast apoptosis and possibly trophoblast damage in pre-eclampsia may lead to greater levels of cellular debris of placental origin in maternal blood and this debris may contribute to the overall procoagulant activity of total microparticles in maternal blood.

Objectives:

1) To set up and validate a prothrombinase assay to measure microparticle procoagulant activity in blood.

2) To recruit healthy individuals and type II diabetics (with and without diabetic complications) in order to pilot the prothrombinase assay.

3) To recruit pre-eclamptic and healthy pregnant matched controls to measure and compare their microparticle procoagulant activity in maternal blood.

4) To measure the amount of fetal CRH mRNA in maternal blood in pre-eclamptic and healthy pregnant matched controls as a measure of placental cell debris.

4.5 Development of the prothrombinase assay

4.5.1 Concentration: activity relationship

The concentration-activity relationship was first determined for the standard vesicles. Several dilution series of synthetic PC: PS vesicles were performed to identify a linear relationship between the change in OD/minute [measured as Absorbance Units/minute

(AU/min)] with increasing concentrations of synthetic vesicles. It was found that a linear relationship between concentration and activity existed in the concentration range of between 0-50nM PS equivalents (figure 4.5.1).

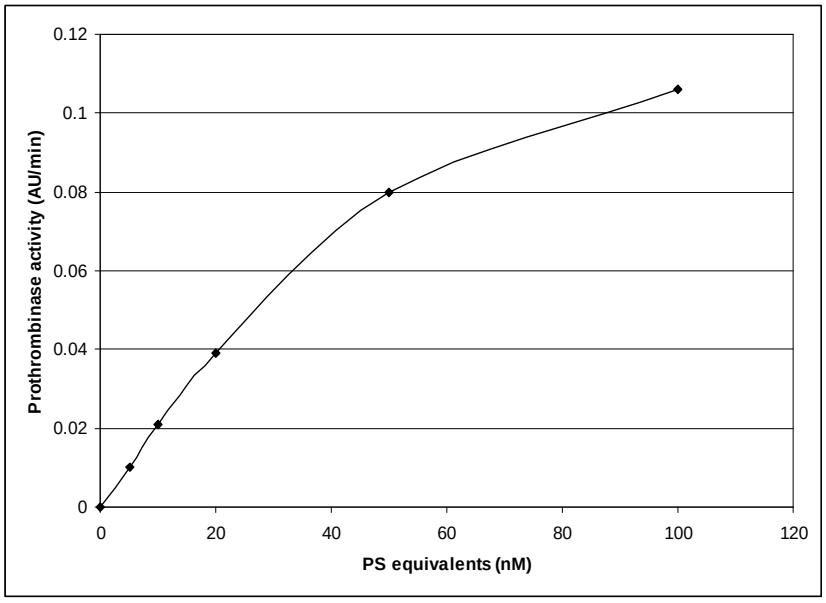


Figure 4.5.1: Concentration: activity relationship of synthetic PS: PC vesicles

4.5.2 Assay validation

In order to validate the assay, inter-vesicle preparation, intra-assay and inter-assay reproducibilities were assessed. The inter-vesicle preparation variability was calculated using 3 independently prepared synthetic vesicle preparations (svA, svB and svC) prepared and analysed on the same day using the same plate. The vesicles were analysed at a concentration range from 0nM to 50nM PS equivalents. All 3 preparations gave a similar curve (figure 4.5.2.1). Analysis of the slopes of the trendlines of all samples gave a coefficient of variation of 9.09% (mean 0.0011, SD 0.001 AU/min/nM).

The intra-assay variability was analysed by measuring samples of synthetic PC:PS vesicles at 20nM PS equivalents in 8 consecutive wells. This was performed on 3 different preparations (svA, svB and svC) all measured in the same assay. The results are as shown

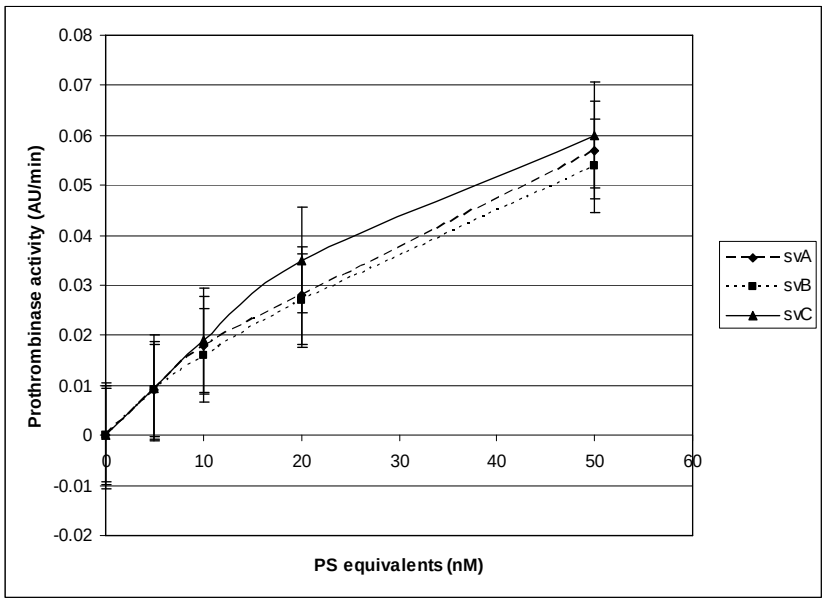


Figure 4.5.2.1: Inter-vesicle preparation variability of 3 separately prepared samples (svA, svB and svC). Standard error bars shown.

in table 4.5.2.1. The coefficients of variation of the samples were 2.76% (svA), 3.77% (svB) and 1.21% (svC) respectively, giving an average coefficient of variation of 2.85% between the 3 preparations.

In order to obtain the inter-assay variability, three separate prothrombinase assays were carried on the same sample synthetic vesicle preparation (svB) on different days. The results are as in figure 4.5.2.2. It was found that the prothrombinase activity of these vesicles appeared to decline whilst in storage in a 4°C fridge. These assays were carried out 38 days, 43 days and 112 days after the vesicles were synthesized. A comparison of the slopes of the trendlines showed that there was a large drop in vesicle activity when stored for 112 days when compared to storage at 38 and 43 days [the gradient dropping from 0.001 AU/min/nM (at 38 and 43 days storage) to 0.0004 AU/min/nM (at 112 days storage); a drop of 2.5 fold]. The inter-assay CV was 45.8%. These results suggested that there was degeneration of vesicle activity whilst in storage.

	Prothrombinase activity (AU/min)		
Well	Preparation svA	Preparation svB	Preparation svC
1	0.040	0.038	0.038
2	0.038	0.038	0.038
3	0.038	0.038	0.038
4	0.038	0.036	0.038
5	0.037	0.038	0.039
6	0.038	0.036	0.039
7	0.038	0.036	0.038
8	0.040	0.040	0.038
Std deviation	0.00106	0.00141	0.00046
Mean	0.0384	0.0375	0.0383
Coefficient of variation (%)	**2.76**	**3.77**	**1.21**

Table 4.5.2.1: Measurement of intra-assay variation at 20nM PS equivalents.

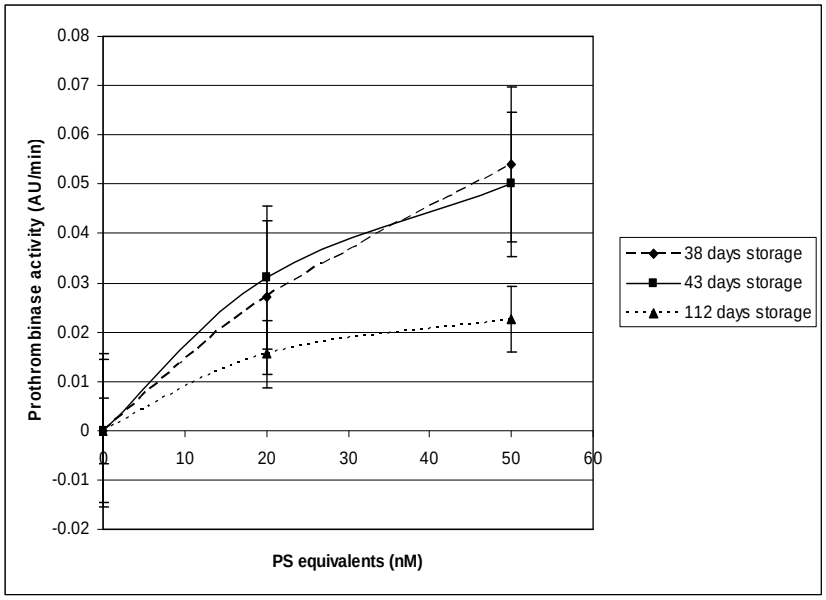


Figure 4.5.2.2: Analysis of inter-assay variability. Note the progressive drop in vesicle activity of sample svB from 38 days storage to 112 days storage. Standard error bars shown.

4.5.3 Synthetic PC: PC vesicle stability

The results so far suggested that these PC:PS synthetic vesicles could be synthesized relatively consistently but there appeared to be a decline in vesicle procoagulant activity over time whilst in storage. A possible reason for the reduction in activity could be degradation of the synthetic PC:PS vesicles whilst in storage. In order to test this hypothesis, a new sample of PC:PS vesicles were made (labelled svD). Three different methods of storage (fridge, step-freezing and flash-freezing) were carried out using sample svD to identify if any one method was superior in maintaining vesicle activity. Fridge storage was in a 4°C fridge. In step-freezing, the sample was put in a 4° C fridge for an hour, followed by -20°C freezer for an hour and then finally stored in a -80°C freezer. In flash-freezing, the freshly made sample was immediately stored in a -80°C freezer. All 3 samples were analysed on the same day of preparation (day 1), after 2 days, after 10 days, after 16 days and after 30 days in storage. The results showed that there was a drop in vesicle activity in all 3 methods of storage compared to the fresh sample. The drop was greatest within the first 2 days with little further degradation from 2 to 30 days storage. In all 3 methods of storage, the slope of the activity vs. concentration curve went from 0.0029 AU/min/nM for the day 1 sample, dropping to between 0.0012 and 0.0015 AU/min/nM for the stored samples (table 4.5.3.1). This showed a drop in activity of between 1.9 to 2.4 fold. It was evident that none of these 3 methods of storage were able to preserve the activity of these vesicles (figures 4.5.3.1 to 4.5.3.3).

Storage (days)	**Fridge (AU/min/nM)**	**Step freezing (AU/min/nM)**	**Flash freezing (AU/min/nM)**
1	0.0029	0.0029	0.0029
2	0.0015	0.0014	0.0015
10	0.0014	0.0013	0.0014
16	0.0013	0.0012	0.0012
30	0.0014	0.0012	0.0014

Table 4.5.3.1: Values of the slopes of the concentration-activity curves for synthetic PS:PC vesicles by fridge storage, by step-freezing and flash freezing.

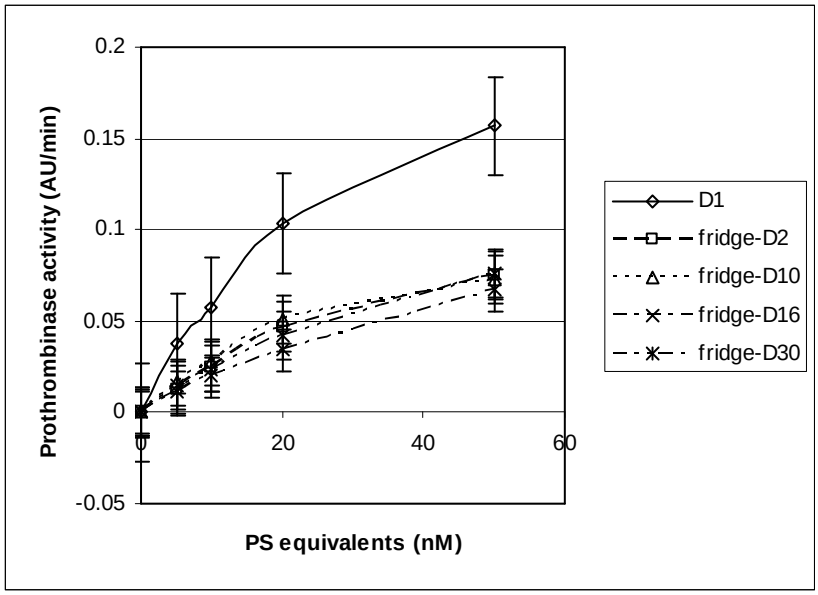


Figure 4.5.3.1: Synthetic vesicle preparation (svD) analysed on day 1(D1) and after 2(D2), 10(D10), 16(D16) and 30(D30) days in storage in a 4°C fridge. Standard error bars shown.

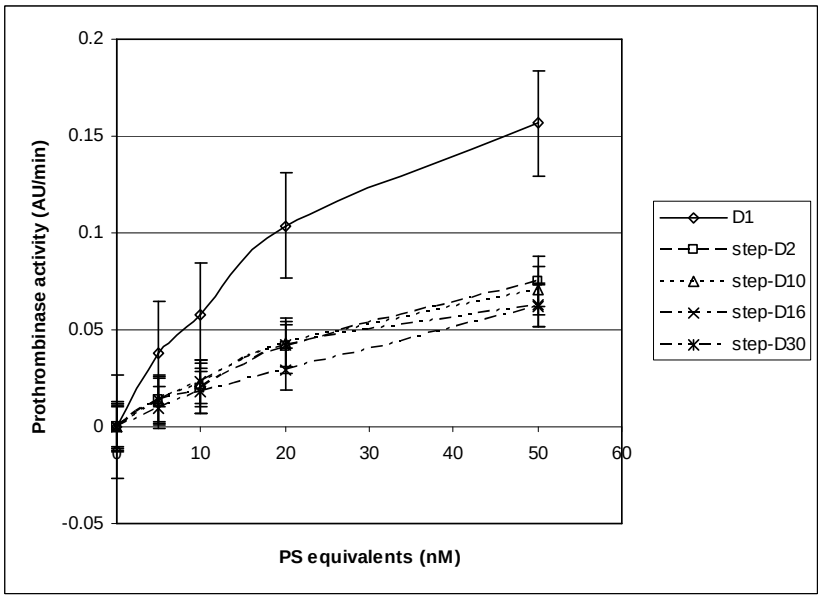


Figure 4.5.3.2: Synthetic vesicle preparation (svD) analysed on day 1(D1) and after 2(D2), 10(D10), 16(D16) and 30(D30) days in storage by step freezing. Standard error bars shown.

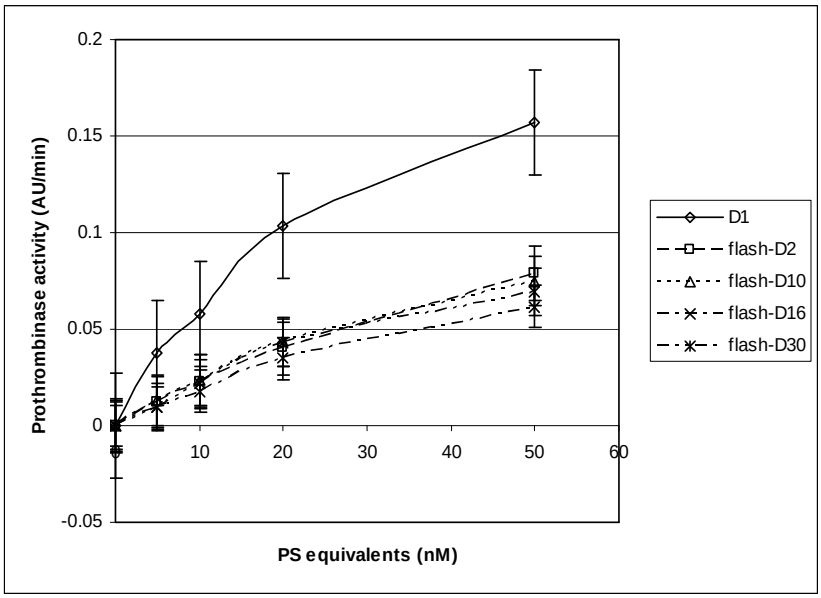


Figure 4.5.3.3: Synthetic vesicle preparation (svD) analysed on day 1(D1) and after 2(D2), 10(D10), 16(D16) and 30(D30) days in storage by flash freezing. Standard error bars shown.

4.5.4 BeWo culture-derived microparticle preparations as a standard

Naturally occurring microparticles express phosphatidylserine on their surface and microparticles are known to be released from cells undergoing apoptosis. There was the possibility that 'natural' microparticles could be generated from cultured cells *in vitro*, then isolated and stored to create a standard preparation. These 'naturally-derived' microparticles could potentially be more stable in storage as they would contain various proteins and lipids in their cell membrane which may aid surface stability.

A BeWo choriocarcinoma cell line was cultured and apoptosis was initiated by addition of tumour necrosis factor α (TNF-α) 10ng/ml and interferon γ (IFN-γ) 10ng/ml and incubated for 24 hours. The cell culture medium was removed and centrifuged at 3000g to remove cellular debris. This solution was then immediately analysed by the prothrombinase assay to measure prothrombinase activity. Two cultures were initially prepared: BeA and BeB. The change in AU/min of the culture medium was found to be under the required range when compared to the values obtained using the synthetic PC: PS vesicles. Clearly, these samples were not going to provide the appropriate range of PS equivalents.

In order to increase the specific activity of these preparations, it was decided to concentrate the culture medium. Two further cultures were made: BeC and BeD and the culture medium was concentrated 10 times by sucrose dialysis. Serial dilutions were performed and the OD/min of each dilution was charted. It was found that samples BeC and BeD had a higher activity compared to non-concentrated samples (BeA and BeB). However the change in OD/min of BeC and BeD varied greatly. The change in OD/min of sample BeC was 0.538 AU/min/µl which was above the range of the standard curve produced from the synthetic PC:PS vesicles. In comparison, the change in OD/min of sample BeD was only 0.127 AU/min/µl. Clearly the microparticles pro-coagulant activities produced by these 2 separate BeWo cultures were very different.

In order to test for stability in storage, the concentrated culture medium of BeC was stored at 4°C and measured after 7 days. Sample BeC showed a loss of activity when measured after 7 days storage (figure 4.5.4.1) and calculation of the initial slope of the trendline showed that there was a 3.1-fold drop in activity (0.0173 AU/min/µl [day 1] to 0.0056 AU/min/µl [day 7]). Therefore it appeared that creation of microparticles using a BeWo culture was not a satisfactory way of producing vesicles for the purpose of creating a standard as there was high inter-preparation variability as well as degradation in storage.

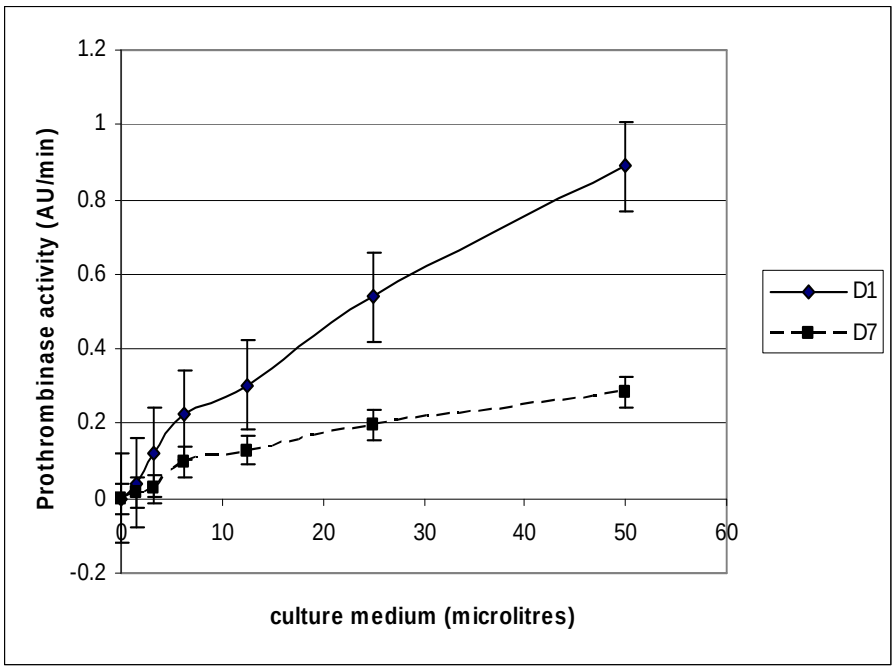


Figure 4.5.4.1: Loss of activity of sample BeC when measured after 7 days storage (D7) compared to when fresh (D1). Standard error bars shown.

4.5.5 Pilot studies of prothrombinase assay

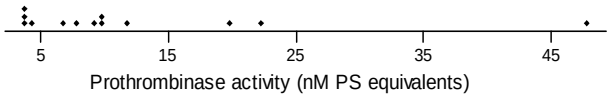
The aim of developing the prothrombinase assay was to allow us to measure the degree of microparticle pro-coagulant activity in the maternal blood of pre-eclamptic and healthy control patients. Various attempts at producing suitable vesicles for the purposes of a standard curve showed that the most satisfactory method of producing consistent vesicles was by the creation of synthetic PC:PS vesicles but there was the drawback of vesicle degradation in a very short time regardless of method of storage. Due to time limitation, rather than continue trying to find a solution to the problem of vesicle degradation, it was decided to use freshly prepared on-the-day synthetic PC:PS vesicles as a standard when analysing blood samples. For purposes of piloting the assay, I recruited healthy volunteers from the laboratory to enable me to identify an approximate range of microparticle pro-coagulant activity in the healthy population. I also recruited groups reported to have had higher levels of microparticle procoagulant activity for purposes of informing the power calculation of the pre-eclamptic/control analysis. A literature search at the time revealed that plasma microparticle levels had been measured in uncomplicated and complicated type II diabetics and greater amounts of microparticles were found in type II diabetic patients with complications. Using flow cytometry, Omoto *et al* (273) showed that there were significantly increased levels of platelet and monocyte-derived microparticles in type II diabetics compared to healthy non-diabetic controls. When microparticle levels were compared between uncomplicated diabetics and diabetics with complications of the disease, increased levels of microparticles were found in patients with complications such as retinopathy (approximately 100% increase) and nephropathy (approximately 160% increase)(273). Using flow cytometry, Ogata *et al* (280;281) also compared the levels of platelet-derived microparticles in type II diabetic patients and discovered a 10 % increase in microparticle levels in patients who had diabetic complications. The available literature therefore indicated that plasma microparticle levels were increased in type II diabetes and further increased in diabetic patients with complications. This suggests that microparticle procoagulant activity may be greater in complicated type II diabetics compared to uncomplicated type II diabetics.

4.5.5.1 Microparticle pro-coagulant activity in healthy persons

Healthy male and female volunteers from the lab were recruited. There were 13 individuals recruited, all without any medical illnesses and they were not on any medication. The mean was 12.2 (SD 12.2) nM PS equivalents with a 95% confidence interval of 12.2 +/- 6.6. The results are as per table 4.5.5.1 and figure 4.5.5.1.

	Mean	**SD**	**95 % confidence interval**
Prothrombinase activity (nM PS equivalents)	12.2	12.2	5.6 to 18.8

Table 4.5.5.1: Prothrombinase activity of healthy individuals in nM PS equivalents.



F**igure 4.5.5.1:** Dotplot of prothrombinase activity in normal healthy individuals in nM PS equivalents.

4.5.5.2 Microparticle pro-coagulant activity in type II diabetic patients

Two groups of type II diabetic patients were recruited: well controlled diabetics (n=10) and diabetics with microvascular complications (n=6). The results are as per table 4.5.5.2 and figure 4.5.5.2. While there was an 80.5% increase in total pro-coagulant activity in the group with microvascular complications compared to the well controlled group, the difference was not statistically significant (p value= 0.12).

	Diabetics with complications (n=6)	**Diabetics without complications (n=10)**	**P value**
Prothrombinase activity (nM PS equivalents)	14.8 (8.7)	8.2 (5.3)	0.12*

Table 4.5.5.2: Comparison of prothrombinase activity between diabetics with complications and diabetics without complications measured in nM PS equivalents. The results are shown as: mean (SD). *P value was measured by a two sample T-test using log transformed values.

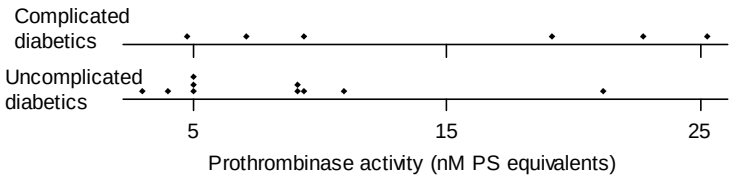


Figure 4.5.5.2: Dot plot of prothrombinase activity between complicated and uncomplicated diabetics in nM PS equivalents.

Analysis of type II diabetic patients was used to inform the power calculation for the measurement of the pre-eclamptic and control groups and a sample size of 32 cases and 32 controls predicted 90% power to detect a difference of 53% in procoagulant activity with a 65% standard deviation.

4.6 Results

4.6.1 Microparticle pro-coagulant activity in pre-eclamptic and healthy pregnant controls

Thirty two pre-eclamptic and 32 healthy control patients were recruited to the study, matched for age, body mass index (BMI), gestation at sampling and parity. Their characteristics are as per table 4.6.1.1. It can be seen that there was no statistical difference between the pre-eclamptic and control groups with regard to age, BMI, gestation at sampling and parity, reflecting that they were well-matched. Patients with pre-eclampsia delivered earlier and had smaller babies as expected. There were also significantly fewer smokers in the pre-eclamptic group.

Microparticle pro-coagulant activity was measured in pre-eclamptic and healthy control groups. The samples were analysed in a prothrombinase assay along with their matched controls on the same plate using freshly made on-the-day synthetic PC: PS vesicles as a standard curve. The two-sample T-test for continuous variables was used to test for statistical difference between the pre-eclamptic and healthy pregnant control groups. The results showed that with regard to microparticle pro-coagulant activity, there was no significant difference in microparticle prothrombinase activity between the pre-eclamptic and the healthy pregnant control groups [pre-eclampsia mean 107 (SD 137) nM PS equivalents vs. control mean 108 (SD 85) nm PS equivalents, p=0.36] . When early-onset pre-eclamptic (under 34 weeks' gestation) samples were considered, microparticle pro-coagulant activity was also not significantly different between the pre-eclamptic and control group [115.3 (59.0) vs. 120.2 (105.0), p=0.87]. These results are as shown in table 4.6.1.2, figure 4.6.1.1 and figure 4.6.1.2.

Characteristic	Pre-eclamptic (n=32)	Healthy pregnant controls (n=32)	P value
Age (years)	29.4 (6.3)	29.6 (5.7)	0.88
BMI (kg/m^2)	26.8 (4.5)	26.5 (4.4)	0.78
Number of smokers	1 (3)	8 (25)	0.012
Gestation at sampling (weeks)	35.8 (3.3)	36.0 (4.1)	0.89
Primiparous [number and (% of total)]	22 (69%)	24 (75%)	0.58
Gestation at delivery (weeks)	36.3 (3.0)	39.5 (0.9)	<0.001
Mean birth weight centile	23.2 (27.8)	52.3 (31.2)	<0.001

Table 4.6.1.1: Patient characteristics of pre-eclamptic and healthy pregnant controls showing mean (standard deviation) and p values. P value was measured by a two sample T-test. Statistical significance was set at $p<0.05$.

Characteristic	Pre-eclamptic	Healthy pregnant controls	P value
All pre-eclamptic samples	n=32	n=32	
Prothrombinase activity * (nM PS equivalents)	107 (137)	108 (85)	0.36
Early onset pre-eclamptic samples (under 34 weeks gestation)	n=8	n=10	
Prothrombinase activity * (nM PS equivalents)	115.3 (59.0)	120.2 (105.0)	0.87

Table 4.6.1.2: Prothrombinase activity of pre-eclamptic patients and healthy pregnant control patients showing mean (standard deviation) and p values**.** P value was measured by a two sample T-test using log transformed values*. Statistical significance was set at $p<0.05$.

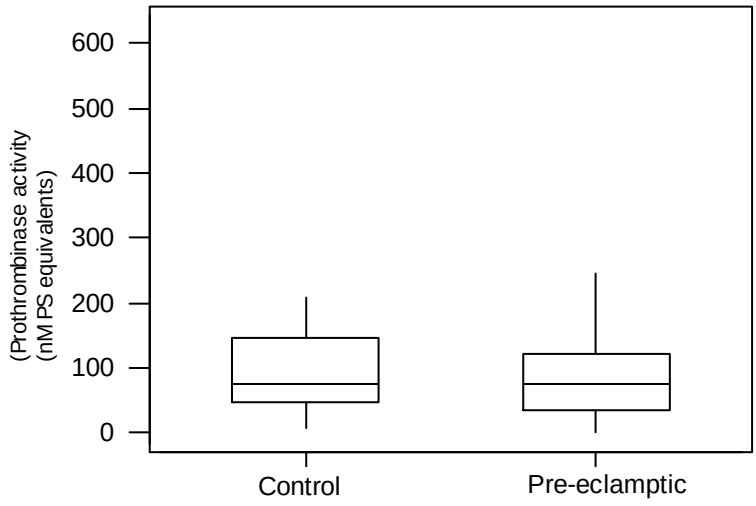


Figure 4.6.1.1: Interquartile range boxplot of prothrombinase activity of all pre-eclamptic and matched controls measured in nM PS equivalents.

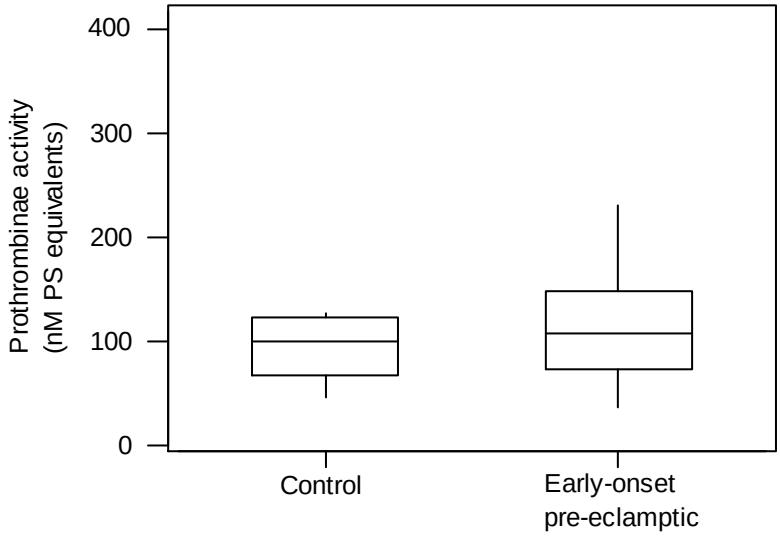


Figure 4.6.1.2: Interquartile range boxplot of prothrombinase activity of early-onset pre-eclamptic and matched controls measured in nM PS equivalents.

A *post hoc* analysis of power for the pre-eclamptic and controls revealed a sample size of 32 cases and 32 controls predicted 90% power to detect a difference of 67% in procoagulant activity with a 81% standard deviation.

4.6.2 Measurement of fetal corticotrophin-releasing hormone (CRH) mRNA in maternal blood

There was significantly greater amounts of fetal CRH mRNA in maternal peripheral blood in pre-eclamptic patients compared to healthy pregnant matched controls [pre-eclamptic mean 0.75 (SD 2.77) vs. healthy control mean 0.20 (SD 0.74), p=0.014] as shown in table 4.6.2 and figure 4.6.2. This suggests that there were significantly greater amounts of placental debris in the maternal systemic circulation in pre-eclampsia compared to healthy pregnancy. As the CRH/GAPDH mRNA ratio included some zero values, the Wilcoxon rank sum test was used to test the differences between groups. Fetal CRH mRNA levels were not found to be correlated to microparticle prothrombinase activity in either the healthy pregnant or pre-eclamptic groups. When fetal CRH mRNA levels were compared between pre-eclamptic and healthy control pregnancies before and after 36 weeks gestation, a significant increase in fetal CRH mRNA levels were found in pregnancies after 36 weeks gestation in both pre-eclamptic and healthy pregnant controls. Table 4.6.3 and figure 4.6.3.

Characteristic	**Pre-eclamptic (n=32)**	**Healthy pregnant controls (n=32)**	**P value**
Fetal CRH mRNA relative to GAPDH	0.75 (2.77)	0.20 (0.74)	0.014

Table 4.6.2: Fetal CRH mRNA levels of pre-eclamptic and healthy pregnant controls. Mean (standard deviation) and p-values. The difference between control and pre-eclampsia groups was tested using a Wilcoxon signed-rank test for CRH/GAPDH mRNA ratio.

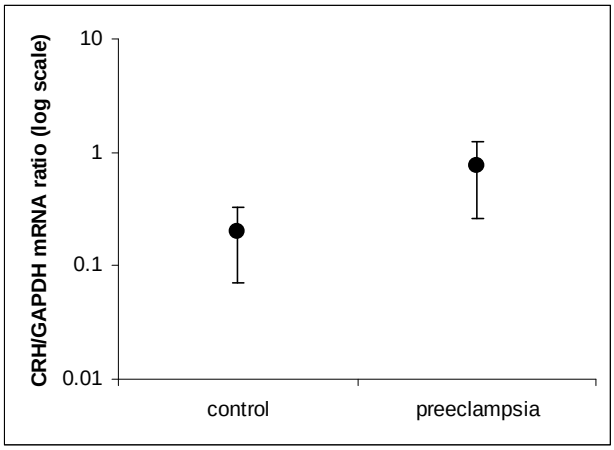


Figure 4.6.2: Fetal CRH mRNA ratio of pre-eclamptic and matched controls measured as CRH relative to GAPDH. Means and standard errors are shown**.** CRH/GAPDH mRNA ratio is plotted on a logarithmic scale.

	Healthy control < 36 weeks	**Healthy control > 36 weeks**	**Pre-eclamptic < 36 weeks**	**Pre-eclamptic > 36 weeks**
Fetal CRH mRNA relative to GAPDH	0.0 (0.0)	0.30 (0.20)	0.39 (0.23)	1.14 (0.82)

Table 4.6.3: Fetal CRH mRNA levels of pre-eclamptic and healthy pregnant controls under and over 36 weeks' gestation. Mean (standard error).

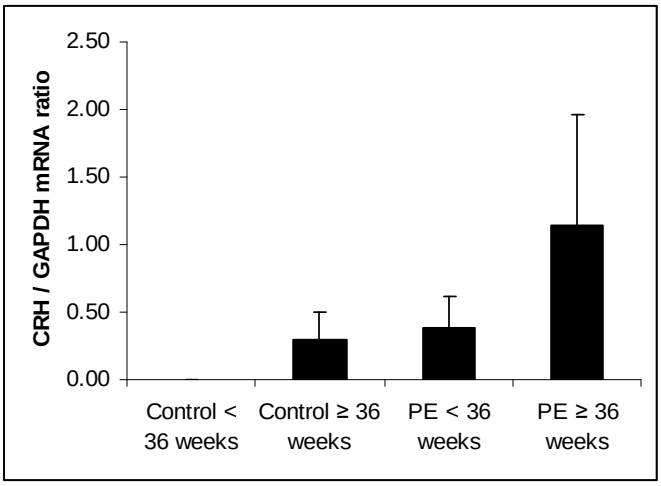


Figure 4.6.3: Fetal CRH mRNA ratio of pre-eclamptic and matched controls under and above 36 weeks gestation measured as CRH relative to GAPDH. Means and standard errors are shown**.**

4.7 Discussion

One of the main aims of this thesis was to measure microparticle procoagulant activity in the maternal circulation in a case-control study of pre-eclamptic patients and healthy pregnant matched controls to identify if there was any significant difference in microparticle pro-coagulant activity between the two groups. Phosphatidylserine (PS) is a component of cell membranes and it is normally located on the inner leaflet of the membrane. PS has procoagulant properties in that it provides a surface for the conversion of prothrombin to thrombin by clotting factors. When a cell is activated or undergoes apoptosis, PS is externalised to the outer surface of the cell membrane, the cell membrane is blebbed off and released as microparticles into the circulation(282). These circulating microparticles in plasma would now potentially be in a position to affect coagulation in maternal circulation.

The prothrombinase assay was chosen as a method to measure the procoagulant activity of microparticles in maternal blood. The reason this assay was chosen was that it was designed to physically capture microparticles in plasma and quantify its procoagulant activity by using its thrombin generating ability as a surrogate measure. This seemed to be a most direct assessment of microparticle procoagulant activity.

The prothrombinase assay required a standard curve measuring the prothrombinase activity in relation to the concentration of PS present. I followed the method of Aupiex *et al*(65) who used synthetic phosphatidylserine : phosphatidylcholine (PC:PS) vesicles to act as a standard in their prothrombinase assay. Their method of synthesis of these vesicles was based on the method of Pigault *et al*(275) who created synthetic unilamellar vesicles of 67%PC: 33% PS (mol/mol). Various dilutional series identified a linear relationship between the prothrombinase activity and concentration of PS in these vesicles between the range of 0 to 50nM PS equivalents. This activity: concentration relationship now allowed me to relate the prothrombinase activity of the blood samples to the standard curve, allowing the measure of the procoagulant activity of PS (and therefore the procoagulant activity of microparticles) in the blood sample. To validate the assay, inter-vesicle preparations and intra-assay coefficients of variations were measured and found to be

reasonable, at 9.09% (inter-vesicle preparations) and an average of 2.6% (intra-assay variability). However inter-assay variability was not satisfactory with a coefficient of variation of 45.8%.

It was hoped that once the activity: concentration relationship of a batch of synthetic vesicles was established, this batch could be stored for future use in later experiments i.e. used as a quality control. However in the course of our experiments, there appeared to be degradation of procoagulant activity of these synthetic vesicles over time with the greatest loss of activity occurring within the first day in storage and this accounted for the high inter-assay CV. Three different methods of storage were attempted (fridge at 4°C, step freezing and flash freezing) to identify if there was a method of storage that maintained vesicle activity. Unfortunately, degradation of microparticle pro-coagulant activity occurred with all 3 methods. It was possible the loss of procoagulant activity was the result of vesicle instability, as these vesicles were essentially phospholipid complexes in buffer solution without any additional stabilising factors such as proteins that are present in cell membranes. The production of 'natural' microparticles from cultured BeWo cells undergoing induced apoptosis was then attempted to see if they maintained their procoagulant activity in storage. It was thought that these naturally-occurring microparticles would contain various membrane proteins that would help maintain stability in solution. Unfortunately there was no consistency in the procoagulant activity of these natural microparticles between batches and these 'natural' microparticles also lost their procoagulant activity in storage. It was therefore decided to use freshly made on-the-day synthetic PC:PS vesicles as a standard when performing the prothrombinase assay on blood samples.

To pilot the prothrombinase assay, 2 types of sample populations were chosen; healthy individuals and type II diabetics (complicated and uncomplicated). A literature search revealed that microparticle levels have previously been measured in type II diabetic populations. With regard to the healthy sample population, I recruited 13 healthy individuals and determined microparticle procoagulant activity in the healthy population to have a mean of 12.2 (SD 12.2) nM PS equivalents.

With regard to the type II diabetic sample population, 10 type II diabetics without complications and six type II diabetics with complications were recruited from the diabetic outpatient clinic. They were not on any anti-thrombotic medication such as aspirin, heparin or warfarin. Their microparticle procoagulant activity was measured and the results showed greater procoagulant activity in the blood of type II diabetics with complications [mean 14.8 (SD 8.7) nM PS equivalents] compared to uncomplicated diabetics [mean 8.2 (SD 5.3) nM PS equivalents] but this difference was not statistically significant. However, the reason the type II diabetic study was undertaken was to estimate the power calculation for the pre-eclamptic and healthy pregnant control study. The type II diabetic study in itself was not a primary aim of this project.

The measure of microparticle prothrombinase activity in pre-eclamptic patients was one of the primary aims of this project. A search of the literature revealed that microparticles had been measured in the blood of non-pregnant, healthy pregnant and pre-eclamptic patients and quantitated using flow cytometry. However, some results were conflicting. Using flow cytometry, Bretelle *et al* showed that platelet and endothelial-derived microparticles were raised in healthy pregnant women compared to healthy non-pregnant women(86). In contrast however, Van Wijk *et al* did not find any significant difference in endothelial-derived microparticles between healthy pregnant and healthy non-pregnant women(87). When microparticle levels were measured in pre-eclamptic patients, it was found there were greater numbers of microparticles derived from endothelial, T-helper, T-suppressor and granulocyte cells in the plasma of pre-eclamptic patients compared to healthy pregnant controls(80;87;89) and the highest proportions of microparticles in both groups were found to be platelet-derived microparticles(87). In contrast, Bretelle *et al* found a lower amount of platelet-derived microparticles in pre-eclamptic pregnancies compared to healthy pregnant controls(86). Despite differences in microparticle levels in these cell subpopulations, no statistically significant difference has been found in the total number of microparticles between pre-eclamptic and healthy pregnant control groups(86;87).

In contrast to measuring the amount of microparticles in pre-eclamptic patients, I was interested in comparing microparticle procoagulant activity in maternal blood between pre-eclamptic and healthy pregnant women. A prothrombinase assay was selected for this purpose. It is important to emphasise here that the prothrombinase assay measures the procoagulant activity of microparticles and not the amount of microparticles in the sample.

The prothrombinase assay measures the procoagulant activity of all microparticles in the sample regardless of cell of origin or whether they were of maternal or fetal origin. I recruited 32 pre-eclamptic and 32 healthy pregnant controls. They were matched for age, body mass index (BMI), gestation at sampling and parity. The results showed a mean value of 107 (SD 137) nM PS equivalents for pre-eclamptic patients and a mean value of 108 (SD 85) nM PS equivalents for healthy pregnant controls with a p-value of 0.36. This indicated that there was no statistically significant difference in microparticle procoagulant activity between the pre-eclamptic and matched healthy pregnant control groups. Goswami *et al* measured and found STBM levels to be significantly increased in early onset-pre-eclamptics (under 34 weeks' gestation) compared to healthy pregnant controls(81). It was possible that in early-onset pre-eclampsia, higher levels of syncytiotrophoblast-derived microparticles may lead to greater total microparticle procoagulant activity in maternal blood. However in my samples, microparticle procoagulant activity was not found to be significantly different between early-onset pre-eclamptics and matched control groups [early onset pre-eclampsia mean 115.3 (SD 59.0) nM PS equivalents vs. control mean 120.2 (SD105) nM PS equivalents, p value 0.87]. After I had commenced work on this project, Bretelle *et al*(86) published a paper where they measured microparticle procoagulant activity in maternal blood using a prothrombinase assay and found that total microparticle procoagulant activity was significantly higher in healthy pregnant women compared to non-pregnant controls. However Bretelle *et al* did not find any significant difference in microparticle procoagulant activity either between pre-eclamptic and healthy pregnant control groups. My results are thus in keeping with the findings of Bretelle *et al.*

I was interested in comparing the amount of placentally-derived cell debris in the maternal blood of pre-eclamptic and healthy control patients. Goswami *et al*(81) had previously measured syncytiotrophoblast-derived microparticle levels in the blood of pre-eclamptic patients by means of ELISA using an anti-trophoblast antibody (NDOG2) to capture the microparticles. However, the anti-trophoblast antibody (NDOG2) as used by Goswami *et al*(81) was not commercially available. Fetal CRH mRNA in maternal blood can only be derived from the fetoplacental unit and measurement of fetal CRH mRNA in maternal plasma would therefore act as a direct measure of placental-derived cell debris in the maternal circulation. Several studies have shown that in comparison to healthy pregnancy, a 4-10-fold increase in fetal CRH mRNA levels occurs in pre-eclampsia (101;106;107). Therefore, a measure of the level of circulating fetal CRH mRNA in maternal plasma was proposed. To allow direct comparison with previous reports(106), GAPDH was used as a

control gene and we demonstrated consistent levels of GAPDH mRNA expression in pre-eclamptic and healthy control plasma. The source of fetal CRH mRNA in maternal circulation had previously been proposed to be syncytiotrophoblast microvilli cell debris shed from the placenta into the maternal circulation(78) and syncytiotrophoblast microvilli cell debris has previously been detected in maternal blood using trophoblast-specific antibodies(80) as well as male-specific offspring DNA(283). Using real time PCR (Taqman), my results showed statistically greater amounts of fetal CRH mRNA in the plasma of pre-eclamptic pregnancies compared to the healthy pregnant matched control group (Table 4.6.2).

With greater placental thrombosis and inflammation in pre-eclampsia, it is possible that the placenta may be a significant source of procoagulant microparticles. Fetal mRNA levels have been found to be raised in pre-eclampsia(101;107) suggesting that there may be greater trophoblast shedding into the maternal systemic circulation in pre-eclampsia. I detected a significant increase in the amount of fetal CRH mRNA in pre-eclamptic samples compared to healthy pregnant samples but there was no increase in total microparticle procoagulant activity in maternal serum as might be expected if the amount of trophoblast shedding was considerable and had contributed significantly to the overall procoagulant activity. A possible reason for this is the amount of microparticles of placental origin may be small in comparison to the total amount of microparticles circulating in maternal plasma, so any increase of placental microparticles (while statistically significant in pre-eclampsia compared to healthy pregnancy) may not have had much impact on the overall procoagulant activity of the total microparticles in maternal plasma. When pre-eclamptic and healthy control samples were measured as 2 separate subgroups (patients recruited under 36 weeks' gestation and patients recruited after 36 weeks' gestation), there was higher mean fetal CRH mRNA levels in the both pre-eclamptic and healthy pregnant groups over 36 weeks' gestation. This increase in fetal CRH mRNA levels suggests that as pregnancy progresses towards term, there is greater trophoblast apoptosis or release of placental cell debris.

Microparticles are known to bind to fibrinogen and platelets(284) and in Chapter 3, there appeared to be greater fibrin deposition in pre-eclamptic placentas compared to healthy pregnant controls. It is possible that the majority of placental-derived microparticles may have become bound to perivillous fibrin in the placenta soon after release, thereby reducing

the amount of placental microparticles entering the maternal systemic circulation. These placental-derived microparticles may also have become bound to annexin V on the surface of the placental syncytiotrophoblast soon after release. Lastly it is possible that placental-derived microparticles may only exhibit very minimal pro-coagulant properties so while there may be greater amounts of placental-derived microparticles in pre-eclampsia, their contribution to total microparticle procoagulant activity is minimal.

The aims of this chapter were to determine if there was greater microparticle procoagulant activity in pre-eclampsia and also to determine if there were greater amounts of placental-derived microparticles in pre-eclampsia. A prothrombinase assay was set up and validated in order to measure the procoagulant activity of microparticles. The results did not show any increase in total microparticle pro-coagulant activity in maternal serum between pre-eclamptic women and healthy pregnant controls. Fetal CRH mRNA was measured to determine the amount of placental-derived microparticles and was found to be raised in pre-eclamptic patients compared to healthy pregnant controls and this is consistent with current literature. However I did not find any correlation between fetal CRH mRNA and total microparticle pro-coagulant activity suggesting that placental-derived microparticles in maternal blood did not lead to any significant contribution to total microparticle procoagulant activity.

The condition of pre-eclampsia is associated with changes in coagulation, endothelial function and placental function in comparison to healthy pregnancy. As microparticles have procoagulant properties and have been identified to originate from the endothelium and placenta, I hypothesise that there may be a relationship between microparticle procoagulant activity as well as placental cell debris with coagulation, endothelial function and placental function in the maternal circulation. I set out to investigate this in the next chapter.

Chapter 5 Markers of coagulation activation, endothelial activation and placental function in relation to microparticle procoagulant activity and placental debris in pre-eclampsia

5.1 Introduction

Healthy pregnancy is a state of coagulation activation in preparation to cope with maternal blood loss at delivery(5;285). This coagulation activation involves an increase in procoagulant factors and suppression of fibrinolytic pathways. High levels of TF the primary initiator of coagulation have been found in the placenta and in syncytiotrophoblasts(25;27). Procoagulant factors such as FVII, FXIIa and vWF have been found to be higher in maternal plasma (5;148;152;157;203;204). There is also an increased resistance to endogenous anticoagulant mechanisms such as (APC)(142;159). Thrombin generation is higher in pregnancy leading to increased fibrin production(140;142-144). There is suppression of fibrinolysis. Activation of plasmin, the key enzyme that degrades fibrin is inhibited by raised levels of both PAI-1 and PAI-2, the latter being produced by the placenta(172).

In pre-eclampsia, there are further changes in the coagulation system when compared to healthy pregnancy. There are increases in maternal plasma levels of TF, FVII, FXIIa, vWF, thrombin, F1+2 and TFPI. Higher PAI-1 levels have been found and this may suppress fibrinolysis. Markers of endothelial activation; sICAM-1 and sICAM-2 have also been found to be higher in pre-eclampsia. PAI-2, a marker of placental function has been found to be lower in pre-eclampsia. These changes are discussed in more detail in chapter 1.4 of this thesis.

As the only known cure for pre-eclampsia is delivery of the placental unit (regardless of gestational age), it is possible there are factors released by the placenta that provoke maternal procoagulant activity and endothelial activation. A number of potential factors have been identified, including the release of placental debris into the maternal circulation. Cell membrane fragments carrying specific placental trophoblast markers and soluble

mRNA and DNA of placental origin have been found in maternal plasma (91-93;95-97) and their amounts are increased in pre-eclampsia(106;107). Microparticles formed as a result of blebbing of cell membranes in apoptosis have been studied in pre-eclampsia because of their procoagulant properties(88). Changes (both positive and negative) in populations of microparticles derived from different cell types have been observed in pre-eclampsia(86;87;89). The concept of trophoblast membrane fragments circulating in maternal plasma is particularly intriguing with regard to coagulation activation as phosphatidylserine(PS) exposed on the surface of apoptotic cell membranes is known to be prothrombotic(278;279).

5.1.1 Markers of the coagulation cascade

With regard to markers of the coagulation cascade, the following factors were measured in maternal serum: TF, TFPI, TAT, F1+2, FVIIc, FXIIa and the APC ratio.

TF is a potent initiator of coagulation and is expressed on the surface of subendothelial cells thus forming a boundary around blood vessels. TF can also be found in soluble form in plasma. The coagulant activity of FVIIa can be measured in a bioassay to give the Factor VII coagulant activity (FVIIc). Increased levels of soluble TF and increased FVIIc activity in blood reflect an increased potential to initiate the coagulation cascade. TFPI antagonises the effects of tissue factor by inactivating the factor VIIa-TF complex and is therefore anticoagulant in nature. TFPI has been shown to be positively associated with markers of endothelial cell function; t-PA, thrombomodulin and vWF. Therefore increased levels of TFPI may also be associated with endothelial cell activation(123).

Once thrombin is formed, it circulates bound to endogenous serine protease inhibitors such as anti-thrombin III (AT) resulting in an inactive complex, the thrombin-antithrombin complex (TAT). A measurement of TAT levels can therefore be used to reflect the amount of thrombin in circulation. During the conversion of prothrombin to thrombin, prothrombin fragment 1+2 (F1+2) is released. Therefore, F1+2 can be used as a surrogate marker of the amount of thrombin generated.

The precise role of FXIIa is not well defined as FXIIa may have both pro- and anti-coagulant effects. It has a procoagulant effect in the intrinsic coagulation network and has an anticoagulant effect in that it leads to production of plasmin which is involved in blood clot dissolution. APC is anticoagulant in nature as it inhibits the activity of activated factor V and activated factor VIII thereby inhibiting the extrinsic coagulation pathway. The activity of protein C can be measured as the APC ratio. A lower ratio corresponds to APC resistance and a reduction on the activity of the endogenous anti-coagulant pathway.

Plasminogen activator inhibitor type-1 (PAI-1) inhibits the process of fibrinolysis by binding and inactivating the 3 main proteases involved in fibrinolysis [tissue plasminogen activator (tPA), urinary plasminogen activator (uPA) and plasmin]. PAI-1 is therefore procoagulant in nature. PAI-1 levels have been found to be significantly raised in pre-eclampsia compared to healthy pregnant controls.

5.1.2 Markers of endothelial function

The following markers of endothelial function were measured: sICAM-1, sVCAM-1 and vWF. ICAM-1 and VCAM-1 belong to the immunoglobulin super family of Cell Adhesion Molecules (CAMs) and are involved in the leukocyte-endothelial cell adhesion cascade where circulating leukocytes interact with CAMs on the surface of the endothelium leading to leukocyte adherence and transmigration into the interstitial tissue. In inflammation, proinflammatory cytokines activate endothelial cells and leukocytes leading to shedding of CAMs from their surface into the systemic circulation resulting in the presence of sICAM-1 and sVCAM-1 in plasma. sICAM-1 and sVCAM-1 may therefore serve as markers of endothelial activation and vascular inflammation. Von Willebrand factor is synthesized predominantly within vascular endothelial cells. Von Willebrand factor has two functions in haemostasis. It serves as a carrier for factor VIII and also functions to mediate platelet adhesion to vascular subendothelium. Thrombin formation at the site of injury stimulates further vWF release with the net effect of platelet plugging of the injury site. This physiological release of vWF by endothelial cells allows it to be a useful marker of endothelial activation.

5.1.3 Marker of placental function

PAI-2 is produced by placental villous syncytiotrophoblasts and is released during cellular activation or apoptosis. PAI-1 has been found to be raised in pre-eclampsia while PAI-2 has been found to be reduced in conditions of reduced placental function such as severe pre-eclampsia and intrauterine growth restriction. An increased PAI-1/PAI-2 ratio has been found in pre-eclampsia and therefore, the PAI-1/PAI-2 ratio can be a useful marker for pre-eclampsia(216).

5.2 Hypothesis

In chapter 4, it was shown that microparticle procoagulant activity was not significantly different between pre-eclamptic and healthy pregnant controls. However there was evidence that there was a greater amount of placental cellular debris in the maternal peripheral circulation in pre-eclampsia.

As microparticles have procoagulant properties and have been identified to originate from circulating blood cells, the endothelium and placenta, I hypothesise that there may be a relationship between microparticle procoagulant activity as well placental cell debris with coagulation, endothelial function and placental function in the maternal circulation in pre-eclamptic and healthy pregnancies.

Objectives:

1) To measure levels of coagulation markers (TF, TFPI, TAT, F1+2, FVIIc, FXIIa, APC ratio, PAI-1), markers of endothelial function (sICAM-1, sVCAM-1 and vWF) and placental function (PAI-1/PAI-2 ratio) in the same population of pre-eclamptic and healthy pregnant control patients as in chapter 4.

2) To correlate microparticle prothrombinase activity as well as fetal CRH mRNA levels in pre-eclamptic and healthy pregnant controls to these measures of coagulation activation, endothelial function and placental function.

5.3 Results

5.3.1 Patient characteristics

Both the pre-eclamptic and healthy control samples were from the same population as that used for the assessment of microparticle pro-coagulant activity in chapter 4. The pre-eclamptic and healthy pregnant controls groups were matched for age, BMI, parity and gestation at sampling (table 4.6.1.1). There were 8 smokers in the healthy control group compared to a single smoker in the pre-eclamptic group. This would be consistent with previous studies showing an inverse association of smoking with the incidence of pre-eclampsia(286). Pre-eclamptic patients delivered on average 3.2 weeks earlier and had lower birth weight babies compared to their matched healthy controls.

5.3.2 Maternal plasma markers of coagulation activation, endothelial function and placental function

The results are summarised in table 5.3.2. When compared to healthy pregnant controls, patients with pre-eclampsia had significantly higher levels of total TFPI [pre-eclampsia mean 38.7 (SD 8.2) vs. control mean 33.0 (SD 6.8) ng/ml, p=0.004], F1+2 [3.62 (4.40) vs. 1.97 (0.60) nmol/l, p=0.008], FXIIa [1.40 (0.43) vs. 1.21 (0.22) ng/ml, p=0.033] and PAI-1 102.7 (32.6) vs. 80.0 (27.1) ng/ml, p=0.003] Maternal plasma markers of endothelial function were higher in pre-eclamptic patients compared to controls: sVCAM-1 [732 (136) vs. 597.6 (85.5) ng/ml, p<0.001] and vWF [3399 (772) vs. 2579 (1044) mU/ml, p=0.001]. The PAI-1/PAI-2 ratio was significantly raised in the pre-eclamptic group compared to the healthy pregnant control group [0.29 (0.17) vs. 0.15 (0.13), p=0.001].

Parameter	Pre-eclampsia (n=32)	Control (n=32)	P value
Markers of coagulation activation			
Tissue factor (pg/ml)	166 (27)	161 (19)	0.37
Tissue factor pathway inhibitor (ng/ml)	38.7 (8.2)	33.0 (6.8)	**0.004**
TAT * (ug/l)	20.5 (30.3)	13.0 (5.2)	0.19
F1 +2 † (nmol/l)	3.62 (4.40)	1.97 (0.60)	**0.008**
FVIIc (IU/dl)	240 (44)	247 (44)	0.56
FXIIa (ng/ml)	1.40 (0.43)	1.21 (0.22)	**0.033**
APC ratio	2.74 (0.45)	2.81 (0.43)	0.55
PAI-1 † (ng/ml)	102.7 (32.6)	80.0 (27.1)	**0.003**
Markers of endothelial function			
sICAM-1 (ng/ml)	199.7 (41.3)	192.1 (57.5)	0.57
sVCAM-1 * (ng/ml)	732(136)	597.6 (85.5)	**<0.001**
vWF (mU/ml)	3399 (772)	2579 (1044)	**0.001**
Markers of placental function			
PAI-1/PAI-2 ratio †	0.29 (0.17)	0.15 (0.13)	**0.001**
Microparticle assessment			
Prothrombinase activity † (nM PS equivalents)	107 (137)	108 (85)	0.36
Fetal mRNA ‡ (CRH relative to GAPDH ratio)	0.75 (2.77)	0.20 (0.74)	**0.014**

Table 5.3.2: Maternal third trimester plasma markers of coagulation activation, endothelial activation, placental function, microparticle prothrombinase activity and fetal CRH mRNA levels. Mean (Standard Deviation) shown. † log transformed data, *square root transformed data, ‡ Wilcoxon rank sum test. Statistical significance set at $p<0.05$ and are highlighted in bold.

5.3.3 Correlation between measures of placental debris and maternal plasma markers of coagulation activation, endothelial function and placental function

The results are summarised in figures 5.3.3.1 to 5.3.3.3. Fetal CRH mRNA levels were found to be correlated with TFPI levels in pre-eclamptic (r=0.38, p=0.031) and healthy pregnant control (r=0.37, p=0.039) patients (figure 5.3.3.1). Fetal CRH mRNA levels were found to correlate with maternal plasma FVIIc activity in pre-eclamptic (r=0.43, p=0.017) but not in healthy pregnant patients (r=0.25, p=0.18) (figure 5.3.3.2). Microparticle procoagulant activity was inversely correlated with plasma F1+2 levels in the pre-eclamptic group(r= -0.64, p=0.001) (figure 5.3.3.3). There was no significant correlation between fetal CRH mRNA levels and microparticle prothrombinase activity in either the pre-eclamptic or control groups.

5.4 Discussion

I wished to relate maternal plasma levels of fetal CRH mRNA and maternal plasma microparticle procoagulant activity to markers of coagulation activation, endothelial activation and placental function in pre-eclamptic and healthy pregnant controls. The changes in markers of coagulation activation and endothelial activation in my results were consistent with the pattern expected for pre-eclampsia (88;126;158;172;173;181;208). In the pre-eclamptic group, there was a higher expression of TFPI (possibly to compensate for increased TF expression on the maternal vasculature or on placental debris), higher prothrombin fragment 1+2 levels (increased conversion of prothrombin to thrombin), higher PAI-1 (suppressed fibrinolysis) and higher levels of FXIIa which has both procoagulant (activates FXI) and anticoagulant (leads to increased plasmin production) effects. Higher concentrations of markers of endothelial activation sVCAM-1 and vWF were also found.

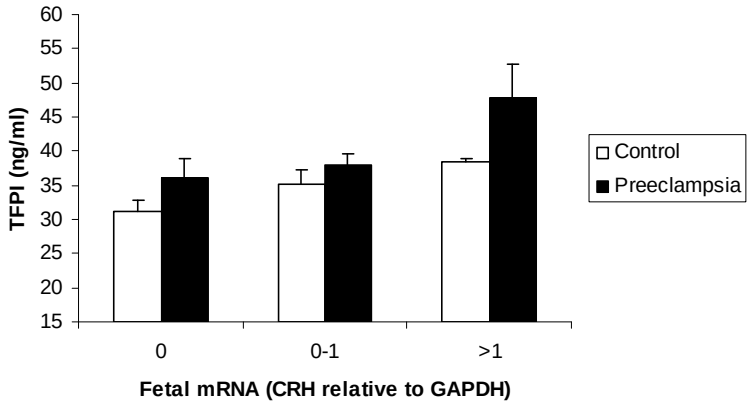


Figure 5.3.3.1: Relationship between TFPI and fetal CRH mRNA in pre-eclamptic and healthy control groups. Standard error bars shown.

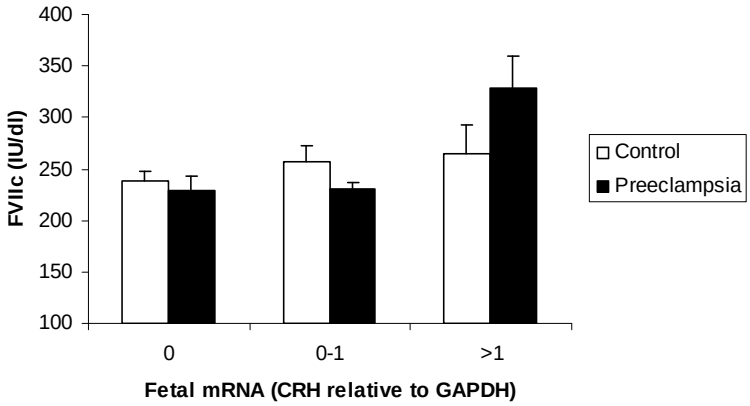


Figure 5.3.3.2: Relationship between factor VII coagulant activity and fetal CRH mRNA in pre-eclamptic and healthy control groups. Standard error bars shown.

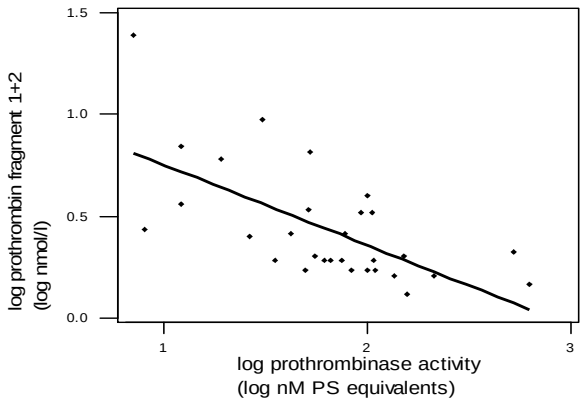


Figure 5.3.3.3: Correlation between log prothrombin fragment 1+2 and log prothrombinase activity in pre-eclamptic patients (r= -0.64, p=0.001).

Fetal CRH mRNA levels in maternal plasma correlated with TFPI levels in both control and pre-eclamptic women. The simplest explanation is that placental TFPI is shed along with the fetal CRH mRNA and is just another marker of placental debris(27;124). It is also possible that the circulating fetal mRNA, or associated material, induces TF expression on the maternal endothelial cell surface but without affecting soluble levels of TF in maternal plasma, hence inducing an up-regulation of TFPI expression. In the pre-eclampsia group, fetal CRH mRNA levels correlated with FVII coagulant activity. It is possible that placental debris may carry exposed TF on its surface leading to initiation of coagulation. Alternatively, placental cell debris might provide a platform for thrombin generation via the TF/FVII-dependent coagulation activation pathway as a result of the enhanced interaction of clotting factors with exposed phosphatidylserine residues on the debris surface. Factor VII coagulant activity was particularly elevated at high levels of fetal CRH mRNA. This would suggest that, above a certain circulating level, fetal mRNA initiates coagulation activation via the TF/FVII-dependent coagulation activation pathway. This response may however be limited by the increase in TFPI.

In pre-eclampsia, the microparticle procoagulant activity was inversely correlated with prothrombin fragment 1 +2. This is a paradox since these particles which are assayed by their ability to promote thrombin generation *in vitro* are found to be negatively associated with thrombin generation *in vivo.* This observation would be consistent with microparticles in the pre-eclamptic patient group interfering with the coagulation pathway *in vivo*. Berckmans *et al*(68) who observed a similar inverse relationship between the microparticle number and thrombin generating capacity in plasma and TAT levels has suggested that microparticles may perform an anti-coagulant function by promoting the generation of low amounts of thrombin thus activating protein C. There is evidence that microparticles have anticoagulant functions as platelet-derived microparticles catalysed FVa inactivation by APC(75). Van Wijk *et al* (88) observed that the TF/FVII-dependent coagulation activation pathway induced by microparticles was active in pre-eclampsia whereas thrombin generation by microparticles was not enhanced. These authors argue that the source of the microparticles may affect their pro- or anti-coagulant properties. They concluded that their data, which looked at a composite of particles derived from many cell sources (both fetal and maternal), did not support a role for circulating microparticles in coagulation activation. My data on phosphatidylserine-exposing microparticles is in agreement with these observations, but the positive correlations between fetal CRH mRNA levels with TFPI and FVIIc would suggest that cell debris derived from the placenta might indeed

have procoagulant activity. Further experimental evidence is required to support this concept.

It is not yet clear whether elevated circulating levels of fetal mRNA or DNA in maternal plasma are specific to pre-eclampsia or intrauterine growth restriction(287;288) but increased levels of circulating fetal DNA have been observed in high altitude pregnancies(289). Bretelle *et al* found no difference in total annexin V positive microparticles or procoagulant activity between healthy and growth restricted pregnancies(86). Recently it has been shown that mRNA-containing microparticles, derived from endothelial progenitor cells, shuttled a specific subset of cellular mRNA to human endothelial cells *in vitro* thus promoting angiogenic effects(63). Heyl *et al* detected increased amounts of VCAM-1 and ICAM-1 on the surface of cultured human umbilical vein endothelial cells after stimulation with sera from pre-eclamptic pregnancies suggesting that there was endothelial cell activation as a result of exposure to pre-eclamptic sera(195). This raises the possibility that microparticles may have very specific cellular effects via targeted delivery of mRNA.

In summary, pre-eclamptic patients had higher levels of TFPI, F1+2, FXIIa, sVCAM-I, vWF, PAI-1 and PAI-1/PAI-2 ratio compared to healthy controls. These changes in markers of coagulation activation, endothelial function and placental function were consistent with the pattern expected for pre-eclampsia. However, the main aim of this chapter however was to identify any relationships between microparticle procoagulant activity and placental cell debris with these markers. I have discovered that placental cell debris via the positive correlation with FVIIc may exhibit procoagulant properties in pre-eclampsia.

Pre-eclampsia is a prothrombotic state and platelets play a major role in coagulation. In coagulation, activated platelets release thromboxane, a potent platelet aggregator at the site of platelet adhesion. Thromboxane (an eicosanoid) is synthesised *in vivo* by platelets as well as by trophoblasts from PUFAs. The levels of thromboxane are significantly raised in pre-eclampsia. As thromboxanes are synthesised from PUFAs, it possible that there may be changes in the maternal fatty acid profile in pre-eclampsia that may influence the synthesis of thromboxane. In the next chapter, I set out to investigate this possibility.

Chapter 6 Erythrocyte membrane fatty acid composition in relation to coagulation activation, endothelial function and placental function

6.1 Introduction

6.1.1 Eicosanoids in coagulation and vasoactive properties

Platelets are small anucleate cells in the systemic circulation that play a critical role in thrombosis. Platelet activation involves the release of thromboxane (an eicosanoid) that activates other platelets leading to a positive feedback loop. Thromboxane is synthesised by platelets as well as by placental trophoblasts. Thromboxanes are synthesised *in vivo* in from polyunsaturated fatty acids (PUFAs) liberated from cell membrane phospholipids. Prostacyclin (PGI_2) is also an eicosanoid. Prostacyclin is synthesised mainly by the vascular endothelium and also the placenta. Prostacyclin is a major endothelium-derived inhibitor of platelet activation and inhibits platelet adhesion and thrombus formation. Thromboxane is a vasoconstrictor while prostacyclin is a vasodilator. Thus, thromboxane and prostacyclin have antagonistic effects.

6.1.2 Thromboxane and prostacyclin in pregnancy and pre-eclampsia

In healthy pregnancy, there is production of equal amounts of thromboxane and prostacyclin(218). However in pre-eclampsia, pre-eclamptic trophoblasts have been found to produce over three times as much thromboxane but less than 50% as much prostacyclin compared to healthy placentas (218;222). Greater amounts of thromboxane B_2 (the metabolite of thromboxane A_2) has been found compared to 6-keto-prostaglandin F1α (the metabolite of prostacyclin) in the maternal plasma of pre-eclamptic pregnancies compared to healthy pregnant controls and this pattern has also been found in the urine of pregnant women who have gone on to develop pre-eclampsia. Plasma levels of prostacyclin are reduced in both mild and severe pre-eclampsia. In contrast, thromboxane levels were

unchanged in mild pre-eclampsia but significantly higher in severe pre-eclampsia, which is consistent with platelet activation in pre-eclampsia.

Thromboxane is a vasoactive substance. In pre-eclampsia, as a result of failure of uterine spiral arteriole conversion by extravillous trophoblasts into large capacitance vessels, spiral arterioles maintain their muscular coat which allows them to be influenced by the vasoconstrictive properties of thromboxane. Therefore, the increased thromboxane: prostacyclin ratio can lead to the features of pre-eclampsia; vascular hypertension, increased platelet aggregation and reduced uteroplacental blood flow [reviewed in (218)].

6.1.3 Thromboxane and prostacyclin synthesis from fatty acids

The eicosanoids thromboxane and prostacyclin are synthesised from their fatty acid precursors [illustrated in figure 1.5.3]. The 2-series eicosanoids have a greater potency of action than 3-series eicosanoids. The 2-series eicosanoids are synthesised from n-6 fatty acids while 3-series eicosanoids are synthesised from n-3 fatty acids. Therefore, the balance of n-6 to n-3 fatty acids in the body can determine the balance of which of these types of eicosanoids will be synthesised. As thromboxane A_2 has a greater potency of action compared to thromboxane A_3, greater thromboxane A_2 synthesis in platelets will lead to greater platelet activity leading to a procoagulant state.

6.1.4 Fatty acids

Fatty acids are precursors of eicosanoids. In PUFA metabolism in humans, the precursor of the n-6 series PUFAs is linoleic acid and the precursor of the n-3 series PUFAs is α-linolenic acid. These precursors are metabolised into their respective products by the sequential actions of desaturase and elongase enzymes. Desaturases and elongases are shared between the different groups of fatty acids which lead to competitive inhibition and the major determinant of which fatty acid group is preferentially synthesised is the amount of substrate present (see figure 1.5.4). It has been suggested that humans originally evolved on a diet where n-6 to n-3 fatty acid intake was in the ratio of 1:1. In the current western

diet, the n-6: n-3 ratio ranges from 10:1 to 25:1(244), leading to the majority of eicosanoids produced being of the potent 2-series prostaglandins (including thromboxane A_2). The can lead to a state which favours thrombosis and vasoconstriction.

6.1.4.1 Maternal fatty acid status in healthy pregnancy

Various changes in maternal plasma levels of fatty acids have been identified in healthy pregnant women versus non-pregnant women. Most studies measured fatty acid levels in plasma but this measure can be confounded by the subjects' fasting status. A more accurate measure of fatty acids may be obtained by analysing the fatty acid composition of erythrocyte cell membranes. The half-life of an erythrocyte if 120 days and so a measure of erythrocyte membrane fatty acid composition would be representative of the mother's fatty acid status over the preceding 3 months.

Various groups have measured erythrocyte fatty acid composition in healthy pregnancy(249-254). With regards to n-6 fatty acids, linoleic acid, dihomo-γ-linolenic acid (DHLA), arachidonic acid, and n-6 docosapentaenoic acid (n-6 DPA) were found to be higher in pregnancy compared to healthy non-pregnant controls. With regards to n-3 fatty acids, α-linolenic acid and DHA were found to be higher compared to healthy non-pregnant controls. Nervonic acid (24:1 n-9) and palmitoleic acid (16:1 n-7) were also found to be higher in healthy pregnancy compared to healthy non-pregnant controls. This general increase of fatty acids may be due to greater maternal mobilisation of fatty acids in healthy pregnancy. In the third trimester, some fatty acids such as arachidonic acid, EPA, n-6 DPA and DHA become reduced compared to the second trimester levels.

6.1.4.2 Maternal fatty acid status in pre-eclampsia

There is some evidence that n-3 fatty acids may be protective towards the development of pre-eclampsia. Individuals who had an increased intake of some n-3 PUFAs were associated with a lower risk of developing pre-eclampsia. Conversely, low levels of total n-3 fatty acids as well as low levels of EPA and DHA were associated with an increased risk

of pre-eclampsia. It was also found that pregnant women with the lowest ratio of n-3 to n-6 fatty acids in maternal erythrocytes had a higher risk of developing pre-eclampsia (259).

Fatty acids have been measured in the maternal plasma of pre-eclamptic pregnancies and there was a trend towards lower levels of total PUFAs, EPA and DHA in pre-eclampsia compared to healthy pregnant controls. Higher levels of arachidonic acid as a percentage of total fatty acids have been found in pre-eclampsia. While these studies measured fatty acid changes in maternal plasma in pre-eclampsia, there did not appear to be any studies looking at fatty acid changes in maternal erythrocyte cell membranes in pre-eclampsia.

6.2 Hypothesis

I hypothesised that there may be an imbalance of maternal levels of n6 and n3 fatty acids in pre-eclampsia. As fatty acids are the precursors in the synthesis of various eicosanoids, an imbalance in n6 and n3 fatty acids may lead to greater synthesis of potent thromboxanes and this may influence coagulation activation, endothelial activation, placental function and microparticle procoagulant activity in the maternal circulation.

Objectives:

1) To measure the fatty acid profile of pre-eclamptic and healthy pregnant control populations from chapter 5 of this thesis. To avoid the subjects fasting status as a confounder, erythrocyte cell membranes fatty acids were measured. Statistical significance was set at p=0.005 to account for multiple analyses.

2) To correlate these fatty acids to markers of coagulation, endothelial function, placental function as well as microparticle procoagulant activity (as per chapter 5) in both pre-eclamptic and healthy control samples.

6.3 Results

6.3.1 Patient groups

The patient groups are a subset from the sample population in chapter 5 of this thesis. Pre-eclamptic patients (n=26) and healthy pregnant control patients (n=25) were analysed. They were matched for age, body mass index (BMI), gestation at sampling and parity (0 or ≥1). The patient characteristics are shown in table 6.3.1. There were significantly less smokers in the pre-eclamptic group in keeping with the known inverse association between smoking and risk of pre-eclampsia(286). The pre-eclamptic patients also delivered significantly earlier and their babies had significantly lower mean birth weight centiles in keeping with the known characteristics of pre-eclamptic pregnancies.

6.3.2 Erythrocyte fatty acid composition

The following fatty acid groups were analysed: saturated fatty acids (SaFA), monounsaturated fatty acids (MUFA) and the n-3 and n-6 groups of polyunsaturated fatty acids (PUFA). These fatty acids were measured both as a percentage (%) of the total amount of fatty acids in the sample and also in absolute quantities (nmol/ml). The results are shown in tables 6.3.2.1 (percentage) and 6.3.2.2 (absolute quantity).

6.3.2.1 Measurement as a percentage of total fatty acids

In the saturated fatty acid group, there was a higher percentage of palmitic acid (16:0) in pre-eclamptic samples compared to healthy pregnant controls [pre-eclampsia mean 28.61 (SD 4.08) % vs. controls mean 24.26 (SD 3.96) %, $p<0.001$]. In the monounsaturated fatty acid (MUFA) group, there was a higher percentage of palmitoleic acid (16:1 n-7) in pre-eclamptic samples compared to healthy pregnant controls [0.90 (0.44) vs. 0.55 (0.36) %, $p=0.004$]. There was a higher percentage of oleic acid (18:1 n-9) in pre-eclamptic samples compared to healthy pregnant control samples [16.15 (1.62) vs. 14.70 (1.68) %, $p=0.003$].

Characteristic	Pre-eclamptic (n=26)	Healthy pregnant controls (n=25)	p-value
Age (years)	28.0 (6.3)	28.7 (6.0)	0.70
BMI (kg/m^2)	26.4 (5.0)	26.0 (4.5)	0.76
Number of Smokers n (%)	0	5	0.02
Gestation at sampling (weeks)	35.9 (3.4)	36.2 (4.1)	0.80
Primiparous (number of)	20	21	0.53
Gestation at delivery (weeks)	36.3 (3.2)	39.5 (1.0)	<0.001
Mean birth weight centile	20.5 (25.5)	46.6 (28.3)	0.001

Table 6.3.1: Patient characteristics of pre-eclamptic and healthy control groups in erythrocyte cell membrane fatty acid analysis. Mean (standard deviation). Statistical significance set at $P < 0.05$.

In the n-6 polyunsaturated fatty acid (PUFA) group, dihomogamma-linolenic acid (20:3 n-6) was found to be significantly lower in pre-eclamptic samples compared to healthy pregnant control samples [1.05 (0.64) vs. 1.70 (0.71) %, p=0.001]. Arachidonic acid (20:4 n-6) was found to be significantly lower in pre-eclamptic samples compared to healthy pregnant controls [7.19 (4.17) vs. 11.14 (3.88) %, p=0.001]. In the n-3 group of PUFAs, docosahexaenoic acid (22:6 n-3) was found to be significantly lower in pre-eclamptic samples compared to healthy pregnant controls [1.84 (1.73) vs. 2.89 (1.66) %, p=0.002].

Fatty acid	Pre-eclamptic (n=26)	Control (n=25)	Two-sample T test p value
SaFA			
12:0	0.15 (0.31)	0.02 (0.11)	0.05
14:0	0.65 (0.34)	0.55 (0.39)	0.33
16:0	28.61 (4.08)	24.26 (3.96)	**<0.001**
17:0	0.19 (0.28)	0.22 (0.25)	0.69
18:0	16.61 (2.37)	16.92 (3.16)	0.70
20:0	0.62 (0.23)	0.59 (0.210)	0.60
22:0	2.27 (0.64)	1.76 (0.88)	0.02
24:0*	5.34 (1.49)	4.53 (1.66)	0.04
MUFA			
16:1 n7	0.90 (0.44)	0.55 (0.36)	**0.004**
17:1 n7	0.05 (0.19)	0.004 (0.02)	0.20
18:1 n9	16.15 (1.62)	14.70 (1.68)	**0.003**
20:1 n9	0.26 (0.32)	0.37 (0.21)	0.14
22:1 n9	0.21 (0.41)	0.09 (0.30)	0.27
24:1 n9	6.58 (0.90)	6.03 (1.13)	0.06
PUFA n6			
18:2 n6	6.41 (1.72)	7.59 (1.55)	0.01
18:3 n6	0.01 (0.07)	0.08 (0.16)	0.07
20:2 n6	0.11 (0.21)	0.15 (0.20)	0.46
20:3 n6	1.05 (0.64)	1.70 (0.71)	**0.001**
20:4 n6	7.19 (4.17)	11.14 (3.88)	**0.001**
22:4 n6	1.71 (1.41)	2.05 (0.85)	0.30
22:5 n6	0.24 (0.36)	0.56 (0.35)	0.008
PUFA n3			
18:3 n3	0.06 (0.14)	0.17 (0.31)	0.11
20:5 n3	1.06 (0.77)	1.11 (0.64)	0.80
22:3 n3	0.31 (0.31)	0.31 (0.26)	1.00
22:5 n3	1.35 (1.02)	1.61 (0.68)	0.30
22:6 n3*	1.84 (1.73)	2.89 (1.66)	**0.002**

Table 6.3.2.1: Comparison of erythrocyte fatty acid composition (as a % of total fatty acids) in maternal blood between pre-eclamptic patients and healthy pregnant controls. * using log transformed values. **SaFA** (saturated fatty acids), **MUFA** (monounsaturated fatty acids) and **PUFA** (polyunsaturated fatty acids). Statistical significance is set at a p-value of 0.005 and highlighted in bold.

6.3.2.2 Measurement as absolute amounts of fatty acids

In the n-6 polyunsaturated fatty acid (PUFA) group, dihomogamma-linolenic acid (20:3 n-6) was found to be significantly lower in pre-eclamptic samples compared to healthy pregnant control samples [pre-eclampsia mean 19.89 (SD 15.06) nmol/ml vs. controls 33.52 (15.99) nmol/ml, p=0.003]. Arachidonic acid (20:4 n-6) was found to be significantly lower in pre-eclamptic samples compared to healthy pregnant controls [136.0 (108.8) vs. 224.9 (96.0) nmol/ml, p=0.003]. In the n-3 group of PUFAs, docosahexaenoic acid (22:6 n-3) was found to be significantly lower in pre-eclampsia compared to healthy pregnant controls in absolute amounts [34.50 (37.87) vs. 53.98 (31.74) nmol/ml, p=0.002].

6.3.3 Summary measures of erythrocyte fatty acid composition

The results of summary measures for fatty acids are shown in table 6.3.3**.** Total MUFA was significantly higher in pre-eclamptic patients compared to healthy pregnant controls (pre-eclamptic mean 23.09 (SD 2.20) % vs. controls mean 20.88 (SD 1.63) %, p<0.001]. There was significantly lower total PUFAs as a percentage of total fatty acids in pre-eclamptic compared to healthy controls [20.39 (8.78) vs. 28.06 (8.06) %, p=0.002].

The different fatty acid classes (n-9, n-7, n-6 and n-3) were measured as a percentage of total fatty acids in the pre-eclamptic and healthy pregnant control groups. There was a higher percentage of total n-9 fatty acids in pre-eclamptic vs. healthy pregnant control samples [pre-eclamptic mean 22.02 (SD 2.04) % vs. control mean 20.24 (SD 1.75) %, p=0.002]. There was also a higher percentage of total n-7 fatty acids in pre-eclamptic vs. healthy pregnant control samples [1.06 (0.60) vs. 0.63 (0.41) %, p=0.004]. In contrast, there was a lower percentage of total n-6 fatty acids in pre-eclamptic vs. healthy pregnant control samples [16.26 (7.10) vs. 22.61 (6.62) %, p=0.002]. There was no statistical difference in total n-3 PUFAs in the pre-eclamptic group compared to healthy pregnant controls [4.14 (2.02) vs. 5.48 (1.85) %, p=0.02]. The ratio of total n-6 to n-3 fatty acids (n-6/n-3) was compared and it was found that there was no significant difference between the pre-eclamptic and healthy pregnant control groups [4.41 (2.25) vs. 4.30 (1.31) %, p=0.83].

Fatty acid	Pre- eclamptic (n=26)	Control (n=25)	Two-sample T test p value
SaFA			
12:0	4.57 (9.18)	0.59 (2.94)	0.04
14:0	14.73 (6.78)	13.77 (8.80)	0.67
16:0	564.4 (108.7)	539.7 (104.1)	0.41
17:0	3.51 (5.08)	4.88 (5.67)	0.37
18:0	299.6 (68.8)	336.5 (60.3)	0.05
20:0	10.55 (3.86)	11.12 (4.41)	0.63
22:0	33.60 (10.16)	31.18 (15.11)	0.51
24:0	72.65 (19.34)	67.62 (12.78)	0.28
MUFA			
16:1 n7	18.19 (8.48)	12.20 (7.22)	0.01
17:1 n7	0.46 (1.99)	0.08 (0.38)	0.34
18:1 n9	294.2 (69.8)	305.3 (83.8)	0.61
20:1 n9	4.41 (5.72)	7.27 (4.65)	0.06
22:1 n9	2.93 (6.03)	1.26 (3.58)	0.23
24:1 n9	91.45 (20.02)	95.42 (26.32)	0.55
PUFA n6			
18:2 n6	122.1 (55.7)	162.70 (54.0)	0.01
18:3 n6	0.38 (1.92)	1.81 (3.73)	0.10
20:2 n6	2.16 (4.10)	3.25 (4.43)	0.37
20:3 n6	19.89 (15.06)	33.52 (15.99)	**0.003**
20:4 n6	136.0 (108.8)	224.9 (96.0)	**0.003**
22:4 n6	25.85 (22.01)	37.70 (17.86)	0.04
22:5 n6	5.38 (7.24)	10.44 (6.74)	0.01
PUFA n3			
18:3 n3	1.34 (3.29)	3.15 (5.02)	0.14
20:5 n3	15.92 (8.20)	20.07 (9.51)	0.10
22:3 n3	5.00 (4.92)	5.78 (4.90)	0.57
22:5 n3	18.90 (13.93)	29.42 (13.79)	0.01
22:6 n3*	34.50 (37.87)	53.98 (31.74)	**0.002**

Table 6.3.2.2: Comparison of erythrocyte fatty acid composition in absolute amounts (nmol/ml) in maternal blood between pre-eclamptic patients and healthy pregnant controls. * using log transformed values. **SaFA** (saturated fatty acids), **MUFA** (monounsaturated fatty acids), **PUFA** (polyunsaturated fatty acids). Statistical significance is set at a p-value of 0.005 and highlighted in bold.

Fatty acid	Pre-eclamptic (n=26)	Control (n=25)	Two-sample T test p value
% saturated	56.52 (7.41)	51.07 (7.49)	0.01
% unsaturated	43.48 (7.41)	48.93 (7.49)	0.01
% MUFA	23.09 (2.20)	20.88 (1.63)	**<0.001**
% PUFA	20.39 (8.78)	28.06 (8.06)	**0.002**
Total n-9	22.02 (2.04)	20.24 (1.75)	**0.002**
Total n-7	1.06 (0.60)	0.63 (0.41)	**0.004**
Total n-6	16.26 (7.10)	22.61 (6.62)	**0.002**
Total n-3	4.14 (2.02)	5.48 (1.85)	0.02
n-6/n-3 ratio	4.41 (2.25)	4.30 (1.31)	0.83
Unsaturated index	105.23 (32.45)	130.65 (32.46)	0.01
Average chain length	18.33 (0.23)	18.51 (0.28)	0.02
C20-22	20.81 (6.22)	26.01 (6.44)	**0.005**
20:4n6/20:3n6 ratio	6.11 (1.47)	6.91 (2.22)	0.15
22:6n3/22:5n3 ratio	1.40 (0.89)	1.89 (0.75)	0.047
18:1n9/18:0 ratio	0.99 (0.12)	0.89 (0.13)	0.01
18:0/16:0 ratio	0.53 (0.06)	0.63 (0.07)	**<0.001**

Table 6.3.3: Comparison of erythrocyte fatty acid composition (as a % of total fatty acids) between pre-eclamptic patients and healthy pregnant controls. Statistical significance is set at a p-value of 0.005 and are highlighted in bold. The 20:4n6/20:3n6 ratio is an index of Δ^5 desaturase activity, the 22:6n3/22:5n3 ratio is an index of Δ^6 desaturase activity, the 18:1n9/18:0 ratio is an index of stearoyl-CoA desaturase activity and the 18:0/16:0 ratio is an index of elongase activity.

Long chain fatty acids [chain length of 20 to 22 carbon atoms (C20-22)] were measured as a percentage of the total amount of fatty acids. It was found that there was a significantly lower percentage of long chain fatty acids in the pre-eclamptic group compared to the healthy pregnant control group [20.81 (6.22) vs. 26.01 (6.44) %, p=0.005].

In order to assess the activity of the various desaturase enzymes, the product: precursor ratios were calculated. There was no significant difference in Δ^5 desaturase activity between pre-eclamptic and healthy pregnant control patients as measured by the 20:4 n-6/20:3 n-6 ratio [pre-eclamptic mean 6.11 (SD 1.47) % vs. control mean 6.91 (SD 2.22) %, p=0.15]. There was no significant difference in Δ^6 desaturase activity in the pre-eclamptic group compared to the healthy pregnant control group as measured by the 22:6n3/22:5n3 ratio [1.40 (0.89) vs. 1.89 (0.75) %, p=0.047]. There was no significant difference in stearoyl-CoA desaturase activity in the pre-eclamptic group compared to the healthy pregnant group as measured by the 18:1 n-9/18:0 ratio [0.99 (0.12) vs. 0.89 (0.13) %, p=0.01]. The ratio of total 18:0 to 16:0 fatty acids was compared as an estimate of elongase activity and it was found that this ratio was significantly reduced in the pre-eclamptic group compared to the healthy pregnant control group [0.53 (0.06) vs. 0.63 (0.07) %, p<0.001].

6.3.4 Correlation of fatty acids to coagulation factors, markers of endothelial function, placental function and microparticle procoagulant activity

In healthy pregnant control patients, tissue factor pathway inhibitor (TFPI) was found to be positively correlated with the absolute amounts of 24:1 n-9 (nervonic acid) [r=0.40, p=0.05]. TFPI was also found to be positively correlated with the absolute amounts of 18:2 n6 (linoleic acid) [r=0.54, p<0.01] as well as its metabolites 20:3 n6 (dihomogamma-linolenic acid) [r=0.48, p=0.02] and 20:4 n6 (arachidonic acid) [r=0.52, p= 0.01]. These correlations are illustrated in figure 6.3.4.1 (A to D). Plasminogen activator inhibitor-1 (PAI-1), was found to be positively correlated with the absolute amounts of 18:2 n6 [r=0.44, p=0.03] and 20: 3 n6 [r=0.41, p= 0.04]. These correlations are illustrated in figure 6.3.4.2 (A and B). Plasminogen activator inhibitor-2 (PAI-2) was found to be positively correlated with the absolute amounts of 18:2 n6 [r=0.49, p=0.02]. This correlation is illustrated in figure 6.3.4.3.

In pre-eclamptic patients, activated factor XII (FXIIa) levels were found to be positively correlated with the absolute amounts of the monounsaturated fatty acid 24:1 n9 (nervonic acid) [r=0.418, p=0.03)]. FXIIa levels were also found to be positively correlated with the

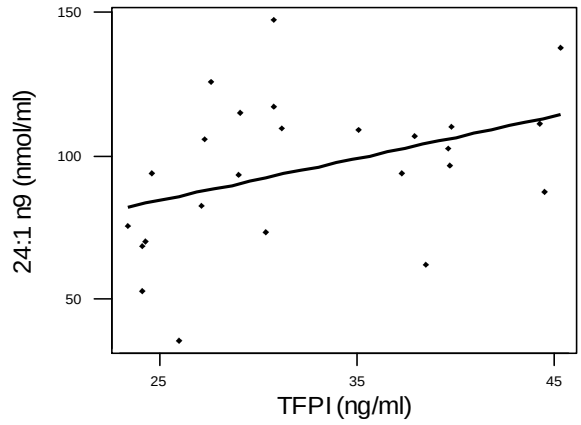
absolute amounts of polyunsaturated the fatty acids 18:2 n6 (linoleic acid) [r=0.41, p=0.04] and its metabolites 20:3 n6 (dihomogamma-linolenic acid) [r=0.44, p= 0.03], and 20:4 n6 (arachidonic acid) [r=0.44, p=0.03]. These correlations are illustrated in figure 6.3.4.4 (A to D).

There was no correlation with the results of other markers of coagulation activation (tissue factor, thrombin-antithrombin, factor 1+2, factor VII or APC ratio) endothelial function (soluble ICAM, soluble VCAM, vonWillebrands factor), placental function (PAI-1/PAI-2 ratio) or plasma microparticle pro-coagulant activity as measured in Chapter 5.

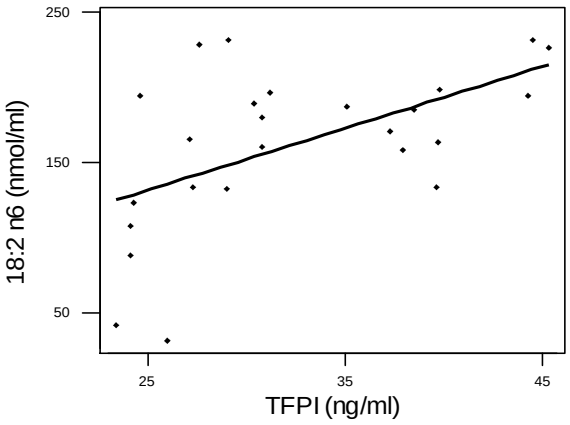
6.4 Discussion

This study of fatty acids was a cross-sectional case-control study examining the differences in fatty acid levels between pre-eclamptic and healthy pregnant controls. It must be remembered however, that the balance of fatty acids in body may be described as a flux, as the amount of fatty acids in the body is continually affected by dietary intake, synthesis from fatty acid precursors and utilisation of fatty acids to create various substances such as eicosanoids, phospholipids and triglycerides. Additionally, in pregnancy, the placenta removes fatty acids from maternal stores to supply the growing fetus. This cross-sectional study would only examine the amount of fatty acids in the mother at steady-state levels and cannot identify if the change in level of a particular fatty acid was due to increased or decreased synthesis or utilisation. More sophisticated labelling of fatty acids would be required to identify the fatty acids along its metabolic pathways.

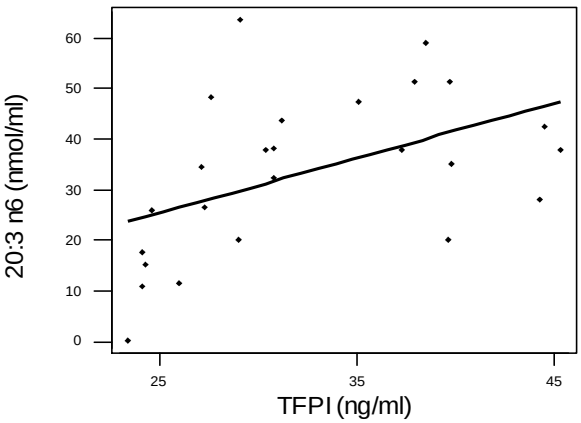
The measurement of a fatty acid as a concentration defines the actual amount of that particular fatty acid in the sample. The measurement of a fatty acid expressed as a percentage defines the proportion of that fatty acid out of the total amount of fatty acids in the sample. Therefore, the value of a fatty acid expressed as a percentage can be affected by changes in the amounts of other fatty acids present in that sample.



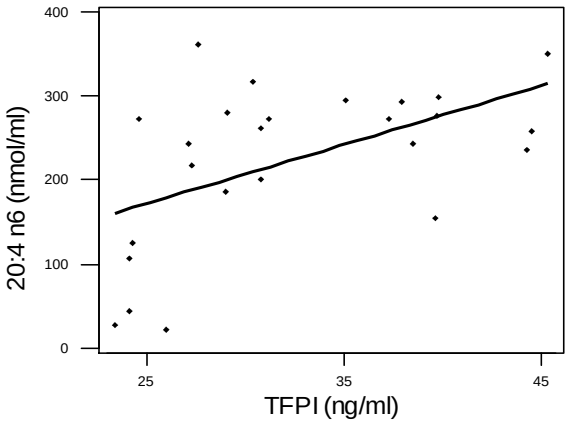
A r=0.397
p=0.05



B r=0.54
p <0.01

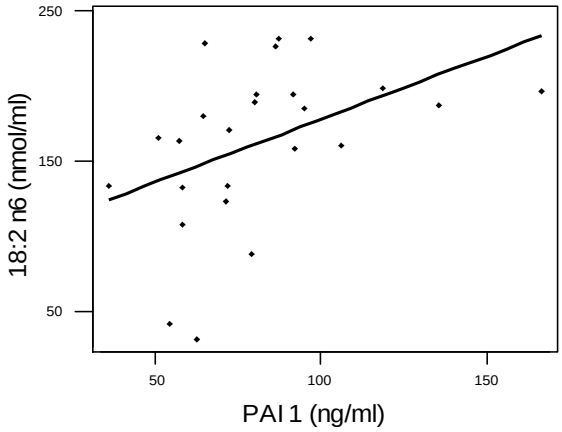


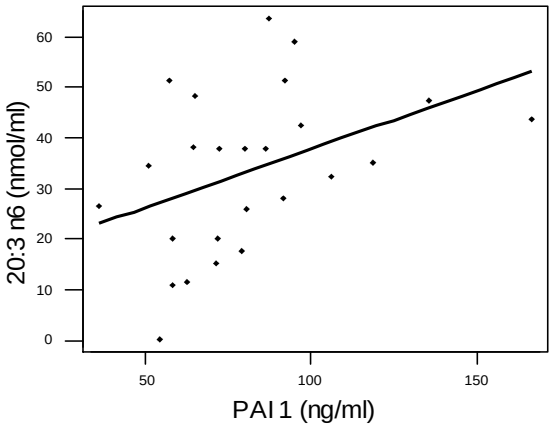
C r=0.48
p=0.02



D r=0.52
p=0.01

Figure 6.3.4.1: Correlation between fatty acids 24:1 n9 (**A**), 18:2 n6 (**B**), 20:3 n6 (**C**) and 20:4 n6 (**D**) with TFPI in healthy pregnant control patients.

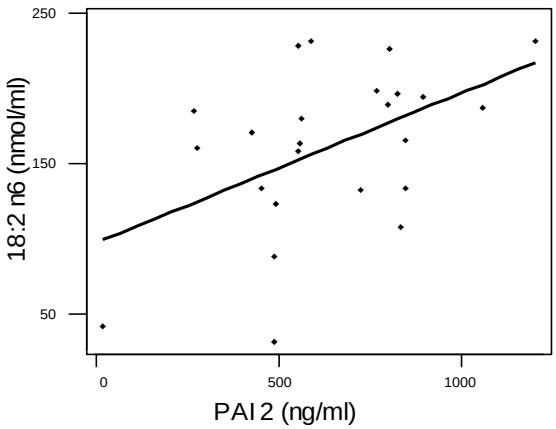




A r=0.44
p=0.03

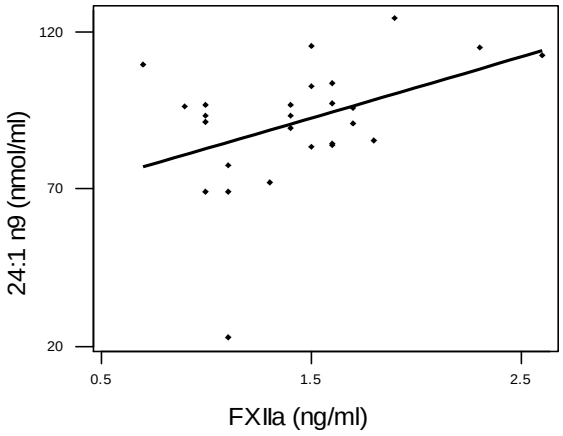
B r=0.41
p=0.04

Figure 6.3.4.2: Correlation between fatty acids 18:2 n6 (**A**) and 20:3n6 (**B**) with PAI-1 in healthy pregnant control patients

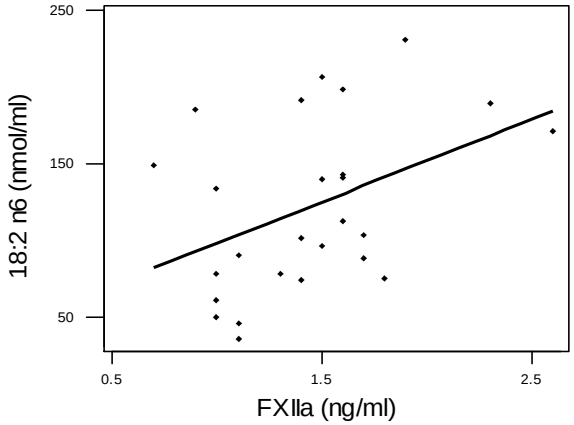


A r=0.49
p=0.02

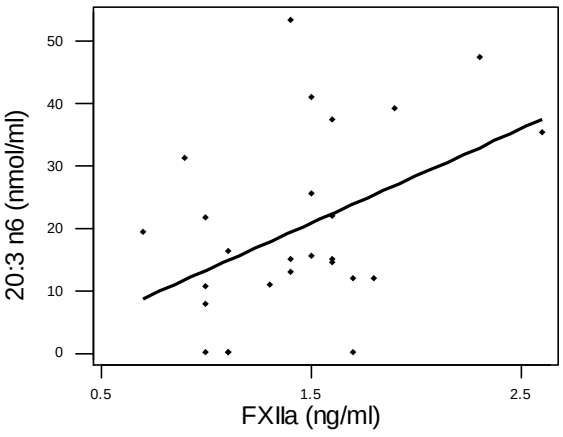
Figure 6.3.4.3: Correlation between fatty acid 18:2 n6 (**A**) with PAI-2 in healthy pregnant control patients



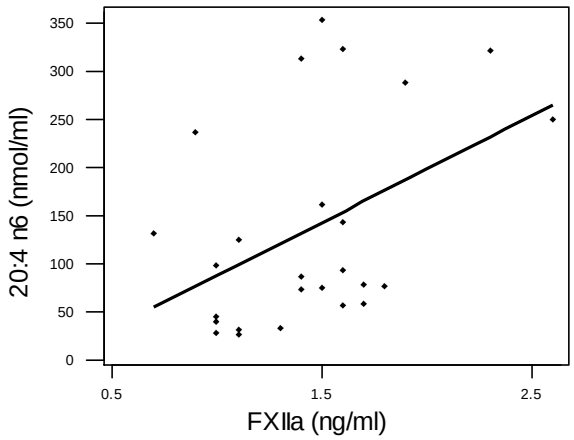
A r=0.418
p=0.03



B r=0.41
p=0.04



C r=0.44
p=0.03



D r=0.44
p=0.03

Figure 6.3.4.4: Correlation between fatty acids 24:1 n9 (**A**), 18:2 n6 (**B**), 20:3 n6 (**C**) and 20:4 n6 (**D**) with factor XIIa in pre-eclamptic patients

The percentage of palmitic acid (16:0) was significantly higher (by 18%) in the pre-eclamptic group compared to the healthy pregnant control group but there was no significant difference in the absolute amounts of palmitic acid between the pre-eclamptic and healthy pregnant groups (p=0.41). There was also a significantly higher percentage of 16:1 n-7 (by 64%) and 18:1 n-9 (by 9.9%) in the pre-eclamptic group compared to the healthy pregnant group. When measured in absolute amounts, there was a substantially higher amount of 16:1 n-7 in the pre-eclamptic group compared to the control group (by 49%). In the n-6 series of fatty acids, there were significantly lower percentages of 20:3 n-6 (by 38%) and 20:4 n-6 (by 35.5%) in the pre-eclamptic group compared to the healthy pregnant group and when measured in absolute amounts, both 20:3 n-6 and 20:4 n-6 were also significantly reduced (by 40.7% and by 39.5% respectively). In the n-3 fatty acid series, DHA (22:6 n-3) was lower both as a percentage of total fatty acids (by 36.3%) and in absolute amounts (by 36%) in pre-eclamptic samples. The pattern of fatty acids changes in pre-eclampsia are similar to the literature in some respects. Absolute amounts of palmitoleic acid were higher in pre-eclampsia in keeping with the findings of Lorentzen *et al*(260). There were lower absolute values of the long chain n-3 PUFAs 22:5 n-3 and 22:6 n-3 in pre-eclampsia in keeping with the findings of Wang *et al*(261). However in contrast to the findings of Ogburn *et al*(263), there was a lower percentage of arachidonic acid in pre-eclampsia. Although there are differences between my findings and that of other studies, it must be noted that the fatty acids in this study was measured from erythrocyte cell membranes while other studies looked at fatty acids in maternal serum, which is open to confounders such as maternal fasting status. The measure of fatty acids in erythrocyte cell membranes are a more accurate reflection of maternal fatty acid status.

These fatty acid changes are reflected in the summary indices. In pre-eclamptic samples, there were significantly higher percentages of total MUFAs (by 10.6%), total n-9 fatty acids (by 8.8%) and total n-7 fatty acids (by 68.3%) and this corresponds with the significantly higher percentages of 16:0, 18:1 n-9 and 16:1 n-7 in pre-eclampsia. In contrast, there were significantly lower percentages of total PUFAs (by 27.3%) total n-6 fatty acids (by 28.1%) and total long chain fatty acids (C20-22) (by 20%) which corresponds with the significantly lower percentages of 20:3 n-6, 20:4 n-6 as well as 22:6 n-3 in pre-eclampsia. When measured in absolute values, it was found that these same fatty acids 20:3 n-6, 20:4 n-6 and 22:6 n-3 were also significantly lower in pre-eclampsia. This suggests that the reduction in percentage of these three long chain PUFAs are due to a

reduction in absolute amounts of these fatty acids in pre-eclampsia and not necessarily as a result of higher amounts of other fatty acids.

One explanation for the higher percentages of palmitic acid, palmitoleic acid and oleic acid in pre-eclampsia may be greater mobilisation of these fatty acids from maternal stores. The predominant fatty acid in storage in adipocytes is palmitic acid and if there is increased maternal mobilisation of palmitic acid in pre-eclampsia, there will be a greater amount of substrate available for the synthesis of palmitoleic acid and oleic acid. Indeed, in the pre-eclampsia group, there was a 49% greater absolute amount of 16:1 n-7 along with a trend towards greater steroyl Co-A desaturase activity (by 11%) compared to healthy controls, suggesting that there may have been a higher amount of its substrate palmitic acid available.

There were significantly lower absolute amounts as well as percentages of dihomogamma-linolenic acid (20:3 n-6) and arachidonic acid (20:4 n-6) in pre-eclampsia. It is possible that this may be due to a dietary deficiency of 20:3 n-6 and 20:4 n-6 prior to and during pregnancy. Another reason why there may be lower absolute amounts of 20:3n-6 and 20:4 n-6 in pre-eclamptic patients may be due to a dietary deficiency of the essential fatty acid precursor linoleic acid. Pre-eclamptic women may have had a lower intake of linoleic acid prior to and during pregnancy compared to healthy controls and certainly, the absolute amounts of linoleic acid (18:2 n-6) was found to be lower in pre-eclamptic samples by 25%, but this difference was not statistically significant ($p=0.01$). However, there was no dietary data obtained from the pre-eclamptic and healthy control patients and so I am unable to address this hypothesis.

Pre-eclampsia is a procoagulant state and it is possible that the fatty acids 20:3 n-6 and 20:4 n-6 may have been utilised into the synthetic pathway of the potent procoagulant eicosanoid thromboxane A_2 (refer to figure 1.5.3 for the eicosanoid synthesis pathway from n-6 and n-3 fatty acids). Greater utilisation of these n-6 fatty acids into the synthesis may thus lead to a reduced maternal level of these fatty acids storage in erythrocytes. Certainly, this shift of balance towards the synthesis of more potent eicosanoids would be in keeping with the procoagulant state of pre-eclampsia and pre-eclamptic placentas have been found to produce up to three times as much thromboxane A_2 as healthy placentas

(221;222). It would be desirable to measure the amount of thromboxane produced by these pre-eclamptic patients, perhaps by measuring the plasma level of thromboxane metabolites (eg. thromboxane B_2) and correlate the concentrations of 20:3 n-6 and 20:4 n-6 with thromboxane B_2.

The n-3 fatty acid DHA (22:6n-3) has been found to be lower both as a percentage and in absolute amounts in pre-eclampsia. However, there was no significant difference in the other n-3 fatty acids either as a percentage or in absolute values between the pre-eclamptic and healthy controls nor was there any significant difference in Δ^6 desaturase or Δ^5 desaturase activity. DHA is abundant in neural and retinal tissues. It is possible that in comparison to healthy pregnancy, pre-eclamptic women had lower amounts of dietary 22:6 n-3 and as 22:6 n-3 is involved in the synthesis of the less potent thromboxane A_3 (refer to figure 1.5.4), a reduced dietary intake of 22:6 n-3 would lead to lower maternal stores which would lead to a lower amount of less potent thromboxane A_3 synthesis. Certainly, low levels of 22:6 n-3 as well as a low n-3:n-6 fatty acid ratio has been found to be associated with an increased risk of developing pre-eclampsia(258;259) and supplementary studies of fish oil (high in n-3 fatty acids) in pregnant women led to higher levels of the less potent thromboxane A_3 and lower levels of the more potent thromboxane A_2 (290). In the last trimester of pregnancy, the fetal requirements of 20:4 n-6 and 22:6 n-3 (DHA) are especially high as there is rapid development of fetal retinal as well as brain tissue(247;268) and this reduced amount of maternal 22:6 n-3 may be due to fetal accretion.

Fatty acids measured in the pre-eclamptic and healthy control group were correlated to markers of endothelial activation, placental function and microparticle pro-coagulant activity as measured in chapter 5. Total TFPI was found to be positively correlated with the absolute amounts of nervonic acid (24:1 n-9) in healthy pregnant controls. TFPI is expressed by cytotrophoblasts, syncytiotrophoblasts and the vascular endothelium(124). In chapter 5 of this thesis, TFPI was shown to be positively correlated with fetal CRH mRNA levels (a measure of placental debris) in maternal plasma suggesting that TFPI may simply be shed along with the placental debris. As healthy pregnancy progresses, Stewart *et al* (249) had shown an increase in absolute amounts of 24:1 n-9 ($p=0.0032$) as well as a positive correlation of 24:1 n-9 with placental weight. Therefore, this finding of a positive correlation of total TFPI with 24:1 n-9 may simply reflect greater TFPI shed along with

placental debris with the growing placenta as pregnancy progresses. Certainly in my samples, there was an increase in fetal CRH mRNA levels in healthy pregnancies above 36 weeks' gestation. TFPI was also positively correlated with the n-6 fatty acids 18:2 n-6, 20:3 n-6 and 20:4 n-6 in healthy pregnancy. As healthy pregnancy progresses, Stewart *et al* (249) has shown a trend towards an increase in amounts of 18:2 n-6 (p=0.041) and 20:3 n-6 (p=0.02). *In vitro* studies have shown that TFPI enhances lipoprotein lipase activity which increases triglyceride hydrolysis (291). This may explain the correlation of TFPI with 18:2 n-6, 20:3 n-6 and 20:4 n-6 as increased triglyceride hydrolysis may result in the release of 18:2 n-6 and the subsequent conversion of 18:2 n-6 to longer chain PUFAs.

PAI-1 is synthesised by various cell types including the placenta and adipose tissue and its levels have been found to be increased in obesity(292). In our healthy pregnant control group, PAI-1 was found to be positively correlated with absolute amounts of linoleic acid (18:2 n-6) and dihomogamma-linolenic acid (20:3 n-6). PUFAs have been found to be able to regulate the expression of genes by binding to nuclear receptor proteins and alter the transcription of target genes(293;294). Human umbilical vein endothelial cells cultured with DHA (22:6 n-3) and dihomo-γ-linolenic acid (20:3 n-6) have been shown to result in an increase in the amount of PAI-1 mRNA suggesting that 22:6 n-3 and 20:3 n-6 may directly induce PAI-1 transcription (295). *In vitro* experiments have also shown PAI-1 production from HepG2 cells when cultured with linoleic acid(296). It is possible that both linoleic acid and dihomo-γ-linolenic acid may both induce PAI-1 mRNA transcription thus leading to greater PAI-1 synthesis.

PAI-2 is produced by the placenta and is correlated with infant birth weight(172). In our healthy pregnant control group, PAI-2 was found to be positively correlated with absolute amounts of linoleic acid (18:2 n-6). Stewart *et al* (249)showed a trend towards an increase in maternal levels of linoleic acid as healthy pregnancy progressed (p=0.041). It is possible this correlation of linoleic acid with PAI-2 may simply be a reflection of placental growth.

In pre-eclamptic patients, activated factor XII (FXIIa) levels were found to be positively correlated with the monounsaturated fatty acid nervonic acid (24:1 n-9) as well as the polyunsaturated n-6 fatty acid 18:2 n-6 and its metabolites 20:3 n-6 and 20:4 n-6. In chapter 5, FXIIa levels were found to be significantly higher in pre-eclamptic patients

compared to healthy pregnant controls. Factor XIIa can be both pro- and anticoagulant in nature and there is little in the literature regarding FXIIa in relation to thromboxane and prostacyclines or the fatty acids 24:1 n-9, 18:2 n-6, 20:3 n-6 and 20:4 n-6. However it has been noted *in vitro* that FXIIa binding to the platelet glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation(297). It is possible that FXIIa levels may be raised in pre-eclampsia to counteract increased platelet activation and thus adhesion.

In summary, my findings of fatty acid patterns in pre-eclampsia are similar to what is in current literature but the measure of maternal fatty acids using erythrocyte cell membranes as opposed to plasma measures would mean that my results are not open to the influence of maternal fasting status. With regard to coagulation, the most relevant finding was the reduced amounts of total n-6, 20:3 n6 and 20:4 n-6 fatty acids which may be a reflection of greater utilisation of n-6 fatty acids into the synthesis of the potent thromboxane A_2 which may contribute to the procoagulant state of pre-eclampsia.

Chapter 7 Discussion

Pre-eclampsia affects approximately 2-7 % of all pregnancies in the United Kingdom and is a significant cause of maternal and fetal morbidity and mortality. The development of the syndrome of pre-eclampsia is multifactorial and may include a disorder in coagulation, endothelial function and placental function. The aetiology of pre-eclampsia is still unknown. However it is clear that there are both fetal and maternal factors involved. A two-stage model of pre-eclampsia has been proposed(298). The first stage of pre-eclampsia involves abnormal placentation and is considered the 'root cause'. Reduced placental perfusion is a feature in pre-eclampsia. In healthy pregnancy, extravillous trophoblasts invade into the myometrium converting uterine spiral arteries into large capacitance vessels which provide low resistance blood flow into the intervillous spaces of the placenta. These large capacitance vessels lose their elastic lamina and become unresponsive to vasoactive stimuli, allowing greater blood flow into the intervillous spaces. This remodelling of spiral arteries does not occur to any appreciable degree in pre-eclampsia; the spiral arteries maintain their muscular coat and continue to be responsive to vasoactive stimuli. This has the net effect of reduced placental perfusion. The second stage of pre-eclampsia involves the maternal syndrome which may result from abnormal placentation. There is activation of the coagulation cascade, leading to a procoagulant state. There is greater platelet activation, platelet consumption and widespread fibrin deposition with acute atherosis. In severe pre-eclampsia, disseminated intravascular coagulation may occur. Increased endothelial dysfunction, systemic inflammation and insulin resistance are also features of pre-eclampsia. There is also dyslipidemia with elevated triglycerides and free fatty acids. Certainly, obesity and diabetes are risk factors towards the development of pre-eclampsia, suggesting a metabolic component to the disease.

The fetus is wholly dependent on the mother for nutrition and to this end the placenta is responsible for effective feto-maternal transfer. The placental villi are constantly enveloped in maternal blood and mechanisms exist to prevent coagulation on the surface of syncytiotrophoblast cells to allow effective feto-maternal transfer. Annexin V is expressed on the surface of syncytiotrophoblasts. Annexin V has an affinity to phospholipids and trimerises on the syncytiotrophoblast cell membrane forming a lattice and effectively

shields the phospholipids exposed on cell membranes from contact by circulating coagulation factors. This prevents the formation of the prothrombinase complex and subsequent clot formation on the cell membrane. Annexin V therefore confers anticoagulant properties to the surface of syncytiotrophoblasts, in effect forming a 'non-stick' coating at the feto-maternal interface. Annexin V expression has been found to be decreased on trophoblasts of pre-eclamptic placentas(39;48) and certainly my results have suggested larger areas of discontinuity of annexin V staining in pre-eclamptic placentas compared to healthy control placentas.

In pre-eclampsia, there is increased perivillous fibrin deposition and certainly in chapter 3, there was the impression of greater perivillous fibrin deposition in pre-eclamptic samples compared to healthy pregnant samples although this was not quantitated. I hypothesised that there may be an association between annexin V expression and fibrin deposition on the trophoblast in pre-eclampsia as annexin V is anticoagulant in nature and may have a role in preventing fibrin deposition on the surface of the syncytiotrophoblast. Using immunocytochemistry, I noted that there was inverse localisation between annexin V and fibrin staining on the syncytiotrophoblast surface in healthy third trimester placentas. In pre-eclamptic and IUGR samples, there appeared to be larger areas of fibrin staining as would be expected but there still remained inverse localisation between annexin V and fibrin staining. This would be in keeping with the findings of Shu *et al* who found elevated plasma levels of fibrin degradation products and thrombin-antithrombin III complex from pre-eclamptic patients as the expression of annexin V in the placenta was reduced(48). There are several possibilities to explain this inverse localisation. It would be reasonable to presume that areas of healthy syncytiotrophoblast would express annexin V on its surface thus conferring anticoagulant properties to the surface of the syncytiotrophoblast. In contrast, areas of apoptotic or necrotic trophoblast may have reduced annexin V expression on the cell membrane along with externalisation of PS on to the surface of the cell membrane, leading to the assembly of procoagulant factors and subsequent fibrin deposition. Therefore, there would be greater annexin V staining on the surface of healthy syncytiotrophoblasts but little fibrin deposition. However, on the surface of apoptotic or necrotic syncytiotrophoblasts, there would be less annexin V staining and greater fibrin deposition. Another explanation for the appearance of this inverse localisation between annexin V and fibrin may be that fibrin has deposited on the surface of the syncytiotrophoblast regardless of the presence of annexin V expression and the deposition of fibrin on the trophoblast surface had physically prevented the primary antibody from

binding to annexin V. A possible method of resolving these two possibilities may be by using a substance that is able to dissolve fibrin *in vitro*. Ammodytase is a metalloprotease from viper venom and is able to dissolve fibrin clots by direct hydrolysis of the α and β-chains of fibrin(299). It may be possible to use ammodytase to dissolve the fibrin clots on the surface of the trophoblast which may then uncover any underlying annexin V. This slide may then be stained for annexin V. A counterpart back-to-back slide may then be prepared and stained for fibrin. If annexin V staining was found in the same location as fibrin staining in these back-to-back slides, this would suggest that fibrin had physically obstructed the primary antibody from attaching to annexin V.

Tissue factor is a potent initiator of coagulation and is expressed by placental syncytiotrophoblasts. I was interested in identifying if there was any relationship between annexin V which has anticoagulant properties and tissue factor which is the potent initiator of coagulation. Tissue factor mRNA levels have previously been found to be high in pre-eclamptic placentas. Back-to-back slides of the placentas of healthy third trimester, IUGR and pre-eclamptic pregnancies were prepared. Despite the positive control staining strongly for tissue factor, there were only very few small scattered areas of tissue factor staining in all the placentas. This made any attempt of identifying any relationship between annexin V and tissue factor difficult. The most likely reason for my results is that there were only very small amounts of tissue factor protein expressed in the placenta. However, Estelles *et al* found a five-fold increase in TF mRNA levels in pre-eclamptic compared to healthy placentas(29). It is possible that in pre-eclampsia, there is priming of coagulation in the placenta resulting in higher levels of TF mRNA synthesis and placental TF protein only becomes expressed in severe pre-eclampsia. Another possibility for this very slight staining of tissue factor is that as tissue factor is such a potent initiator of coagulation, any slight expression of tissue factor in the placenta immediately led to the deposition of fibrin on these areas, thereby preventing the primary antibody directed towards tissue factor from attaching. It is also possible that while the antibody worked on the positive controls, the primary antibody had not worked so well on the trophoblasts. Perhaps an alternative primary antibody may be tried out.

The placental syncytiotrophoblast layer envelops the placental villi and is in contact with maternal blood. Placental cytotrophoblast cells are the stem cells of syncytiotrophoblasts that proliferate and eventually fuse into the syncytiotrophoblast layer. As the placenta

develops, ageing syncytiotrophoblasts undergo apoptosis. As there is greater placental apoptosis in pre-eclampsia, I wished to identify areas of the placenta undergoing apoptosis in pre-eclamptic, IUGR, healthy third trimester and healthy first trimester placentas with the aim of relating these areas to fibrin deposition. It is possible areas of trophoblast apoptosis may have lower annexin V expression, which can then lead to deposition of fibrin. Austgulen *et al* (50) used M30 to detect apoptotic placental cells in healthy third trimester placentas and noted abundant staining at the syncytiotrophoblast layer, more so in areas where there was greater perivillous fibrinoid deposition. M30 CytoDEATH antibodies detect a caspase cleavage site within cytokeratin 18 in the cell cytoplasm and Austgulen *et al* noted that M30 was more sensitive and had less non-specific staining compared to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) staining. I initiated preliminary investigations by attempting to immunolocalise apoptotic cells in healthy first trimester and third trimester placentas using M30 CytoDEATH antibodies. Unfortunately, in all my samples, it was the majority of cytotrophoblast nuclei and most of the syncytiotrophoblast nuclei that was found to be heavily stained with M30, with very little staining of cell cytoplasm. It is known that M30 can non-specifically stain the nuclei of highly proliferating cells and both cytotrophoblasts and syncytiotrophoblasts actively proliferate. It was unfortunate that M30 did not perform as anticipated. Perhaps for future experiments, alternative markers for cellular apoptosis such as bcl-2 and caspase-3 may be tried as had been done by Aban *et al*(21).

During the process of cellular apoptosis, phosphatidylserine (which is normally located on the inner leaflet of cell plasma membranes by an active process) becomes externalised to the outer surface of the cell plasma membrane. Small blebs form on the surface of the cell membrane and are released into the circulation as microparticles. In the extrinsic coagulation pathway, prothrombin, activated factor X, activated factor V and calcium ions assemble on a phospholipid surface forming the prothrombinase complex. Phosphatidylserine (a phospholipid) exposed on the surface of apoptotic cells and microparticles may also allow for the assembly of the prothrombinase complex by providing a procoagulant surface. The amount of microparticles in the systemic circulation has been found to be raised in various prothrombotic conditions such as acute coronary syndrome, diabetes, heparin induced thrombocytopenia, paroxysmal nocturnal haemoglobinuria and severe hypertension, suggesting that microparticles may be procoagulant in nature. Using flow cytometry, microparticles have previously been identified from various cell sources, such as platelets, endothelium and leukocytes.

Earlier, it was proposed that there was a 2-stage model towards the development of the maternal syndrome of pre-eclampsia. It is tempting to speculate that the placenta releases a factor that directly leads to the maternal manifestation of pre-eclampsia. However as yet, this proposed 'Factor X' has yet to be identified. There is a high turnover of trophoblast in pregnancy as the placenta constantly remodels itself. Cytotrophoblast cells fuse to form syncytiotrophoblast cells which when they age, accumulate to form syncytial knots which then undergo apoptosis. In the placenta, the syncytiotrophoblast layer envelops the villous portion of the placenta which is the side of the placenta that is in direct contact with maternal blood. Syncytiotrophoblast membrane particles (STBMs) have been identified in the maternal circulation and are proposed to originate from apoptotic syncytial cells. In that regard, they may be considered as placental-derived microparticles. However, as there is the possibility that circulating placental cell membrane particles may also originate by cellular damage, it may be more accurate to describe circulating placental cell membrane particles collectively, as placental cellular debris. In pre-eclampsia, there is greater placental apoptosis compared to healthy pregnancy and it was an intriguing possibility that greater placental apoptosis may lead to the release of greater amounts of placentally-derived microparticles. Greater amounts of placentally-derived microparticles may contribute to an increase in total microparticle (derived from all cell types) levels in the maternal circulation which may in turn contribute to the procoagulant state of pre-eclampsia.

It is possible that in the maternal systemic circulation, circulating cell membrane particles (regardless of cell of origin) may expose phosphatidylserine on its surface and may therefore be procoagulant. I hypothesised that circulating microparticles in the maternal systemic circulation makes a significant contribution to the procoagulant state of pre-eclampsia. However, a simple measure of the amount of microparticles (which could be achieved by flow cytometry) in blood would not necessarily reflect the procoagulant activity of microparticles. To measure the procoagulant activity of microparticles, a prothrombinase assay was validated and utilised. In this assay, captured microparticles from the blood sample were incubated with clotting factors and the measure of thrombin generated was utilised as a measure of the procoagulant activity of the captured microparticles. This assay in fact replicates the events of coagulation in the body as coagulation *in vivo* also involves the assembly of various clotting factors on a phospholipid

surface. In the process of developing this assay for our use we encountered difficulties in the storage of the standard vesicles. Limitations of time precluded further attempts towards finding a suitable method to avoid degradation of these standard vesicles in storage. Therefore, all measures of microparticle prothrombinase activity in the blood samples were done using freshly prepared standard vesicles, directly comparing pre-eclamptic samples and controls in the same assay. The microparticle prothrombinase activity of maternal systemic blood was measured and there was no significant difference found in total microparticle prothrombinase activity between pre-eclamptic patients and healthy pregnant controls.

It must be remembered that the prothrombinase activity of total microparticles in maternal systemic blood would be a measure of the procoagulant activity of microparticles derived from both maternal and placental cells. As there is greater placental apoptosis in pre-eclampsia it would have been interesting to measure the procoagulant activity of placental-derived microparticles. However that would require an anti-trophoblast antibody to capture the placental-derived microparticles and such an antibody was not commercially available. Therefore a measure of fetal corticotrophin-releasing hormone (CRH) mRNA levels in maternal blood was undertaken as a method of simply quantifying the amount of placentally-derived cell debris in maternal blood. Fetal CRH is synthesised by the trophoblast and it is presumed that when trophoblast microparticles bleb off, they would contain within them mRNA representative of the genes expressed in those cells, including CRH mRNA. Maternal CRH mRNA is not present in the systemic circulation and so any measure of CRH mRNA in maternal blood would be of placental origin. When fetal CRH mRNA levels were measured, the levels were four-fold higher in pre-eclamptic samples compared to healthy pregnant controls. This suggests that there were greater amounts of placental-derived cell debris in maternal circulation in pre-eclampsia. However it must be remembered that we cannot presume that all fetal CRH mRNA in maternal blood comes from placentally-derived microparticles, as placental cell debris derived from damaged trophoblast, for example, may also contain fetal CRH mRNA. Thus, a measure of fetal CRH mRNA would encompass both placentally-derived microparticles and trophoblast cell debris, collectively termed ‘placental cell debris’.

It is interesting that the greater amounts of placentally-derived cell debris by a factor of four in pre-eclamptic patients did not contribute significantly to their total microparticle

procoagulant activity. It is possible that the higher amount of placentally-derived cell debris was too small a fraction of the total microparticles in maternal blood to make any significant difference. It is also possible that placentally-derived cell debris may not express much phosphatidylserine on its surface. Another possibility is that while the amounts of placentally-derived cell debris were low at the time of collection, perhaps over time, the levels may gradually increase as a consequence of gradually worsening placental dysfunction and increasing trophoblast apoptosis. Certainly, when we looked at our pre-eclamptic samples as 2 separate subgroups (patients recruited under 36 weeks' gestation and patients recruited after 36 weeks' gestation), there was a higher mean fetal CRH mRNA level in the group over 36 weeks' gestation, suggesting there may an increase in fetal CRH mRNA levels as pre-eclampsia progresses. As the condition of pre-eclampsia is known to worsen suddenly, it is tempting to hypothesise that a sudden increase in placentally-derived cell debris that may be the precipitant for a worsening of the condition. It would be desirable in future experiments to measure fetal CRH mRNA levels as pregnancy progresses and to correlate duration of pre-eclampsia to fetal CRH mRNA levels to get a more accurate representation of this relationship.

The measure of fetal CRH mRNA measures the amount of placental cell debris but does not measure the procoagulant activity of placental-derived cell debris. At the time of commencement of this project, there were no reports in the literature of isolating placental cell debris in maternal circulation although subsequently, Goswami *et al*(81) used an antitrophoblast antibody to measure STBM levels in pre-eclampsia. With some adjustments, it may be possible to modify our prothrombinase assay to use such an antibody to capture placentally-derived microparticles onto a plate in order to measure their procoagulant activity.

As microparticles are known to exhibit procoagulant properties, I wished to correlate microparticle procoagulant activity and placentally-derived cell debris to markers of coagulation activation, endothelial function and placental function in pre-eclamptic and healthy controls. The pattern of changes in markers of coagulation activation, endothelial activation and placental function I observed were in keeping with that expected for pre-eclampsia. Tissue factor pathway inhibitor (TFPI) was higher in pre-eclampsia possibly as a reflection of endothelial activation in pre-eclampsia or possibly in response to cell-surface tissue factor expression. There were higher prothrombin fragment 1+2 (F1+2)

[increased conversion of prothrombin to thrombin] levels in pre-eclampsia, reflecting greater conversion of prothrombin to thrombin. Higher levels of activated factor XII (FXIIa) which has both procoagulant activity (activated factor XI) and anticoagulant activity (increases plasmin) effects were found as well as higher PAI-1 levels (suppressed fibrinolysis) were found in pre-eclampsia. With regard to markers of endothelial activation, there were higher levels of soluble VCAM-1 and vonWillebrand factor in pre-eclamptic samples. The PAI-1/PAI-2 ratio was also raised in pre-eclampsia in keeping with current literature. Interestingly, the levels of soluble tissue factor (the potent initiator of coagulation), thrombin-antithrombin (a measure of the amount of thrombin) and the APC ratio (APC inactivates activated factor V and activated factor VIII) were not significantly different between pre-eclamptic and healthy controls. These results suggest that although there were higher levels of priming factors towards coagulation, there did not appear to be much evidence of greater amounts of clot formation between pre-eclamptic and healthy control groups.

TFPI was correlated with fetal CRH mRNA levels in both pre-eclamptic and healthy controls, possibly reflecting shedding of placental TFPI along with fetal CRH mRNA as part of placental debris. It is also possible that placental-derived cell debris may induce maternal endothelial cell-surface tissue factor expression thus inducing upregulation of TFPI synthesis. It is also possible that placental-derived cell debris may carry exposed tissue factor on its surface thus upregulating TFPI. Alternatively, as a result of phosphatidylserine exposed on the surface, placentally-derived cell debris may present a platform for thrombin generation via the tissue factor/activated factor VII-dependent coagulation activation pathway. In the pre-eclamptic group, factor VII coagulant activity correlated with fetal CRH mRNA levels and FVII coagulant activity was particularly elevated at high levels of fetal CRH mRNA, suggesting an association between factor VII coagulant activity and circulating placentally-derived cell debris. This is interesting because the measure of factor VII coagulant activity is not a measure of factor VII levels but a measure of the procoagulant ability of factor VII.

The pattern of changes in the measured coagulation factors in the pre-eclamptic samples seemed to show a state of priming towards the generation of clots. Factor VII coagulant activity was higher in pre-eclampsia and this positively correlated with the amount of placentally-derived cell debris in maternal circulation. There was also the suggestion that

levels of placentally-derived cell debris may increase as pre-eclampsia progresses. It is possible that all these factors in combination may lead to a state of coagulation priming in pre-eclampsia and all that is required is a trigger to set off a chain of coagulation dysfunction that may precipitate a worsening of pre-eclampsia. Placental-derived cell debris is associated with factor VII coagulant activity, and the surface of the cell debris may expose phosphatidylserine which would present a surface for the assembly of the prothrombinase complex. There is the possibility that levels of placental-derived cell debris may increase as pre-eclampsia progresses. It is tempting to speculate that placental-derived cell debris may be a candidate for 'Factor X' in the development of pre-eclampsia. More work is required to explore this intriguing possibility.

I hypothesised that there may be a relationship between maternal fatty acid status and coagulation in pre-eclampsia and healthy pregnancy as fatty acids are precursors of eicosanoid synthesis. The balance of n-6 to n-3 fatty acids is long known to affect the balance of eicosanoid synthesis, with n-6 fatty acids leading to the synthesis of more potent thromboxanes and prostacyclines such as thromboxane A_2 while n-3 fatty acids lead to the synthesis of less potent eicosanoids such as thromboxane A_3. Thromboxanes are involved in platelet activation and aggregation and greater amounts of thromboxane A_2 (being more potent) leads to greater degrees of platelet activation and aggregation and therefore enhanced procoagulant status. Prostacyclin is another eicosanoid that is synthesised by n-6 and n-3 fatty acids. Prostacyclin is mainly synthesised by the vascular endothelium and functions as a vasodilatory agent as well as a platelet inhibitor.

I correlated maternal fatty acids to markers of coagulation activation, endothelial function and placental function. Measurements of fatty acids in plasma can be subject to the subjects fasting status and therefore, to obtain an accurate representation of maternal fatty acid status in pre-eclamptic patients and healthy pregnant controls, erythrocyte fatty acid levels were measured. Pre-eclamptic and healthy control groups were matched for body mass index (BMI), an index of body fat. This matching was important to rule out any differences in fatty acid levels that are simply as a result of different BMI as fatty acids are stored in adipose tissue and so increased amounts of adipose tissue may provide higher amounts of fatty acid precursors. I was interested in identifying any differences in fatty acid levels between pre-eclamptic and healthy pregnant conditions and to relate the fatty acids to markers of coagulation activation, endothelial function and placental function as

fatty acids are associated with eicosanoid synthesis and so may affect the maternal coagulation status.

Fatty acids were measured in absolute values and also as a percentage of total fatty acids. The results of the measurements in pre-eclampsia showed a shift of the fatty acid pattern towards a lower percentage of total PUFAs and a higher percentage of total monounsaturated fatty acids (MUFAs), in keeping with previously identified fatty acid patterns measured in the maternal serum of pre-eclamptic patients. Pre-eclamptic patients had a lower percentage of total long-chain fatty acids. Pre-eclamptic patients also had a lower percentage of total n-6 fatty acids and a trend towards lower total n-3 fatty acids compared to the control group. Dihomo-γ-linolenic acid and arachidonic acid, both precursors to the potent thromboxane A_2 were lower in pre-eclampsia. It is possible that this may represent greater utilisation of these n-6 fatty acids in order to synthesise thromboxane A_2 and this would be in keeping with the procoagulant state of pre-eclampsia. Pre-eclampsia is associated with a procoagulant state as well as maternal hypertension and placental ischemia. Thromboxane A_2 is associated with increased platelet activation which may contribute to the prothrombotic state of pre-eclampsia. Thromboxane is also a vasoactive substance which stimulates vasoconstriction and this may have a role in the development of maternal hypertension in pre-eclampsia. In the pre-eclamptic placenta, there is failure of extravillous trophoblast invasion into the muscular coat of the spiral arterioles resulting in reduced conversion of uterine spiral arteries into large capacitance vessels. The muscular coat of unconverted spiral arterioles would remain under the influence of vasoactive agents and high levels of thromboxane A_2 can promote vasoconstriction thus leading to placental ischemia. Indeed, the ratio of thromboxane A_2 to prostacyclin (a vasodilator) has been found to higher in pre-eclamptic placentas compared to healthy placentas.

There was a lower absolute amount as well as percentage of the n-3 fatty acid docosahexaenoic acid (DHA) in pre-eclampsia compared to healthy controls. DHA is important for neural and retinal development. It is difficult to determine if the pre-eclamptic women had a lower intake of DHA or if the fetus has accreted greater amounts of DHA as a survival mechanism in pre-eclampsia. One method of assessing this would be by measuring the DHA ratio between maternal blood and umbilical cord blood to identify any differences between pre-eclamptic and healthy pregnant controls. Another possible

reason for the lower amounts of DHA in pre-eclampsia was a reduced conversion of docosapentaenoic acid to DHA by Δ^6 desaturase. Certainly, long-chain PUFAs in erythrocyte phospholipids have been found to be are associated with insulin resistance in type II diabetics, which may be as a result of reduced insulin-mediated activation of Δ^6 desaturases(300) and indeed, pre-eclampsia is also a state of insulin resistance (187;301).

In pre-eclampsia, there were also higher levels of palmitic acid and its precursor palmitoleic acid. Palmitic acid is the main fatty acid stored in adipose tissue and a high maternal BMI is a risk factor towards developing pre-eclampsia. However, as our pre-eclamptic and healthy control samples were well-matched, both cases and controls should have relatively similar amounts of body fat. This higher level of palmitic acid in pre-eclamptic patients may reflect greater mobilisation of fatty acids from maternal stores in pre-eclampsia and this may be contributory to the dyslipidemia in pre-eclampsia. Obesity is associated with insulin resistance and so is pre-eclampsia, suggesting a metabolic component in the maternal manifestation of pre-eclampsia.

PAI-1 is synthesised by the placenta as well as by adipose tissue and PAI-1 levels are increased in obesity. PAI-1 was positively correlated with the n-6 fatty acids linoleic acid and dihomo-γ-linolenic acid in healthy pregnancy. Human umbilical vein endothelial cells cultured with DHA and dihomo-γ-linolenic acid (20:3 n-6) have been shown to result in an increase in the amount of PAI-1 mRNA suggesting that some fatty acids may directly induce PAI-1 transcription (295). This suggests that fatty acids may be able to alter the haemostatic balance by increasing PAI-1 levels. An *in vitro* culture of placental cells with linoleic acid and dihomo-γ-linolenic acid measuring PAI-1 mRNA levels would be a method to investigate this possibility.

Clinically, as the balance of n-6 to n-3 fatty acids in the mother may affect the synthesis of either more potent eicosanoids or less potent eicosanoids and possibly other coagulation factors, perhaps supplementation to restore the balance of n-6 to n-3 fatty acids may be a potential therapeutic option in pregnancies at risk of excessive thrombosis. For example, perhaps a method of reducing the synthesis of the potent eicosanoid thromboxane A_2 is by n-3 fatty acid supplementation into the diet. This may lead to competition for desaturases

and elongases in fatty acid metabolism which may lead to a reduction in the synthesis of the potent thromboxane A_2. Further studies are warranted.

My thesis provides evidence of an increased coagulation potential in pre-eclampsia as reflected by the raised levels of markers of coagulation activation. Total microparticle procoagulant activity in maternal blood has not been shown to be raised in pre-eclampsia but the four-fold rise in placental-derived cell debris levels in maternal plasma in pre-eclampsia may be significant. The coagulation potential of placental-derived cell debris should be further elucidated. It is possible that pre-eclampsia is a state of enhanced coagulation potential awaiting a precipitous event and certainly, the change of maternal state from pre-eclampsia to eclampsia can be very sudden indeed. The balance of maternal fatty acids is disrupted in pre-eclampsia and there is a shift of fatty acid balance towards one which may contribute to greater platelet activity and coagulation potential. My results present a case that pre-eclampsia is a state of increased coagulation potential resulting from multiple pathways.

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