Platelet Biology in Utero-placental Disease

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Platelet Biology in Utero-placental Disease

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfillment of the degree of Doctor of Philosophy

Supervisors: Professor Fergal Malone and Professor Dermot Kenny

July 2016
Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI examination in consideration of the award of a higher degree (PhD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed________________________________________________________________________

Sieglinde Maria Müllers, MB BCh BAO MRCPI

Student number: 14138417

Date________________________________________________________________________
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Abdominal circumference</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AEDF</td>
<td>Absent end diastolic flow</td>
</tr>
<tr>
<td>APLS</td>
<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>AVM</td>
<td>Accelerated villous maturation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>β-FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>β-TG</td>
<td>beta thromboglobulin</td>
</tr>
<tr>
<td>BPD</td>
<td>Bi-parietal diameter</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAmp</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>Dextrose</td>
</tr>
<tr>
<td>CD40</td>
<td>Platelet activation marker</td>
</tr>
<tr>
<td>CD63</td>
<td>Platelet activation marker</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRL</td>
<td>Crown rump length</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------------------------------------</td>
</tr>
<tr>
<td>CCR1</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CT</td>
<td>Closure Time</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>dH₂O</td>
<td>deionised water</td>
</tr>
<tr>
<td>DiOC₆</td>
<td>lipophilic dye (platelets)</td>
</tr>
<tr>
<td>DPFA</td>
<td>Dynamic Platelet Function Assay</td>
</tr>
<tr>
<td>DVI</td>
<td>Distal villous immaturity</td>
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<tr>
<td>DVH</td>
<td>Distal villous hypoplasia</td>
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<tr>
<td>EBL</td>
<td>Estimated blood loss</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>half maximal concentration</td>
</tr>
<tr>
<td>EFW</td>
<td>Estimated fetal weight</td>
</tr>
<tr>
<td>ENT</td>
<td>Endovascular trophoblasts</td>
</tr>
<tr>
<td>EO</td>
<td>Early-onset</td>
</tr>
<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
</tr>
<tr>
<td>Epi</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>EVT</td>
<td>Extravillous trophoblasts</td>
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<tr>
<td>FAU</td>
<td>Fetal assessment unit</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FL</td>
<td>Femur length</td>
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<td>Full Form</td>
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<tr>
<td>FXI</td>
<td>Factor XI</td>
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<tr>
<td>GH</td>
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</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>HC</td>
<td>Head circumference</td>
</tr>
<tr>
<td>HELLP</td>
<td>Hemolysis Elevated Liver enzymes Low Platelets</td>
</tr>
<tr>
<td>HIT</td>
<td>Heparin-induced thrombocytopenia</td>
</tr>
<tr>
<td>HT</td>
<td>Hypertension</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IMS</td>
<td>Invaginated membrane system</td>
</tr>
<tr>
<td>ITP</td>
<td>Immune thrombocytopenia purpura</td>
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<td>IUGR</td>
<td>Intra-uterine growth restriction</td>
</tr>
<tr>
<td>JNL</td>
<td>artificial solution of salts, dextrose and pH buffers</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>LO</td>
<td>Late-onset</td>
</tr>
<tr>
<td>LTA</td>
<td>Light transmission aggregometry</td>
</tr>
<tr>
<td>MACE</td>
<td>Major adverse cardiovascular event</td>
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<td>Mother and babies: Reducing risk through Audits and confidential enquiries across the UK and Ireland</td>
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<tr>
<td>MK</td>
<td>Megakaryocytes</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MOD</td>
<td>Mode of delivery</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>MPV</td>
<td>Mean platelet volume</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
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<tr>
<td>nTracks</td>
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<td>nTrans</td>
<td>No. of translocating platelets</td>
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<tr>
<td>nStasis</td>
<td>No. of static platelets</td>
</tr>
<tr>
<td>nVel</td>
<td>Velocity of translocating platelets</td>
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<tr>
<td>P2Y</td>
<td>Purinergic receptor Y</td>
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<td>PAF</td>
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<td>PFA-100</td>
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<td>PH₂</td>
<td>Prostaglandin H2</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
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<tr>
<td>PBWR</td>
<td>Placental birth weight ratio</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PET</td>
<td>Pre-eclampsia</td>
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<td>PGI₂</td>
<td>Prostacyclin</td>
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<tr>
<td>PIGF</td>
<td>Placental derived growth factor</td>
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<td>PMMA</td>
<td>Polymethymethacrylate</td>
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<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>POC</td>
<td>Point-of-care (test)</td>
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<tr>
<td>PPH</td>
<td>Postpartum hemorrhage</td>
</tr>
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<td>PPP</td>
<td>Platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PSA</td>
<td>Pressure sensitive adhesive</td>
</tr>
<tr>
<td>PSG</td>
<td>Pregnancy-specific glycoprotein</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>s-Flt 1</td>
<td>Soluble fms-like tyrosine kinase</td>
</tr>
<tr>
<td>SFH</td>
<td>Symphysio-fundal height</td>
</tr>
<tr>
<td>SPA</td>
<td>Spontaneous platelet aggregation</td>
</tr>
<tr>
<td>s-Pselectin</td>
<td>soluble P-selectin</td>
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<tr>
<td>STBEVs</td>
<td>Synctiotrophoblast extracellular vesicles</td>
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<td>TF</td>
<td>Tissue factor</td>
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<td>Thrombomodulin</td>
</tr>
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<td>Thrombopoeitin</td>
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<tr>
<td>TRA</td>
<td>Thrombin receptor antagonists</td>
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<td>TRAP</td>
<td>Thrombin receptor activating peptide</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>UAD</td>
<td>Umbilical artery Doppler</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VUE</td>
<td>Villitis of unknown aetiology</td>
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<td>VWF</td>
<td>von Willebrand Factor</td>
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Summary

The link between platelets and utero-placental disease has been an important research question for the last number of decades. The primary aim of this thesis was to comprehensively investigate platelet biology in utero-placental disease using a number of different platelet function assays, in three clinical entities of utero-placental disease: Gestational Hypertension (GH), Pre-eclampsia (PET) and intrauterine growth restriction (IUGR). A secondary aim was to correlate platelet function results with clinical outcomes.

For the first assay I found that spontaneous platelet aggregation was reduced in utero-placental disease compared with normal pregnancy controls. The second assay tested was a more comprehensive assay of agonist-induced aggregation and I found that platelet aggregation in response to incremental concentrations of a range of different agonists was also reduced in utero-placental disease compared with normal pregnancy controls. The final assay, the Dynamic Platelet Function Assay (DPFA) measured platelet behaviour over von Willebrand factor (VWF) under arterial shear-flow. I found significant differences in a number of novel platelet parameters in utero-placental disease. I also correlated platelet function with placental histopathology results and identified novel findings of altered platelet biology based on placental histopathology sub-type. I finally investigated differences in DPFA platelet function based on blood group type and found that pregnant patients with blood group O had significantly reduced platelet behaviour on VWF, which could possibly indicate and increased risk of obstetric haemorrhage.

Platelet biology appears to be altered in utero-placental disease, as assessed using three different platelet function assays. The continued development of the final assay, the DPFA, into a point-of-care test may enable more large-scale prospective studies of platelet function in pregnancy as a predictive tool and measure of aspirin response.
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Published Peer-reviewed manuscripts


Published Abstracts and Poster Presentations


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Health Research Board (HRB) Research Training Fellowship for Healthcare Professionals /2014 (shortlisted) ‘Platelet Biology in Utero-placental Disease’

Health Research Board (HRA) 2015, PROPHETS study: PRediction Of Preeclampsia using a sHEar-flow plaTelet aSSay
“Don’t only practice your art
but force your way into its secrets
for it and knowledge can
raise men to the divine”

Ludwig van Beethoven
Chapter 1. **Introduction**
1.1. Utero-placental disease- basic pathophysiology, clinical aspects and the role of platelets an introduction and overview

The following is an introduction to utero-placental disease, the impact of the condition, clinical features, and an up-to-date review of novel insights into the role platelet function in the condition as a background to the work of this thesis.

Utero-placental disease or placental insufficiency is a term encompassing pregnancy-specific conditions including pre-eclampsia (PET), hypertensive disorders and intrauterine growth restriction (IUGR), that occur as a direct consequence of defective placentation\textsuperscript{1,2}. Although the pathogenesis is multifactorial, a precipitating event is inadequate maternal spiral artery conversion occurring during the first and second waves of trophoblastic invasion\textsuperscript{3}. Platelet function has recently been shown to be involved in early placental development during this critical period of placental vascular remodelling\textsuperscript{4}.

PET and hypertensive conditions are common pregnancy complications of utero-placental disease and leading causes of maternal and perinatal morbidity and mortality\textsuperscript{5-7}. Irish mothers are now conceiving at a later age and have an increased prevalence of obesity, diabetes, in addition to complex medical conditions. These conditions are independently associated with an increased risk of developing PET and utero-placental disease\textsuperscript{5,6}. Defective placentation is the root cause of PET and can develop de novo in otherwise healthy ‘low-risk’ pregnancies. It is still not known why this happens to this otherwise healthy group of women. In some cases placentation can be defective from the outset, possibly due to microvascular disease already present in the older, obese and hypertensive woman. Both low and high-risk pregnancies can be affected\textsuperscript{6}.

IUGR is also a feature of utero-placental disease. The majority of cases of IUGR are deemed constitutional or ‘normal’ small pregnancies and are generally not at risk of adverse outcome\textsuperscript{8,9}. Approximately one third of the pregnancies affected by
fetal growth restriction are not recognised in the antenatal period, in part due to a lack of accurate predictive tools\textsuperscript{10,11}. It follows that undetected IUGR is a feature of approximately half of all stillbirths at post-mortem\textsuperscript{12}. This is unacceptably high. Current challenges lies in correctly identifying and managing pregnancies where fetal growth restriction is pathological, and thus reduce the burden of the associated perinatal morbidity and mortality\textsuperscript{13,14}.

Together, PET and IUGR affect between 5-10\% of pregnancies, therefore potentially implicating 15,000 pregnancies in Ireland per annum. There is currently no clinically useful test available to identify these conditions \textit{early} in pregnancy, at a time-point where treatment might be instituted to prevent disease\textsuperscript{10,15-17}. Representing a high-risk continuum not only from an antenatal management viewpoint, pregnancies complicated by PET and IUGR place a huge burden on neonatal care owing to the high proportion of iatrogenic premature deliveries and subsequent complications of prematurity\textsuperscript{6,7}. Furthermore this burden extends into adulthood, where cardiovascular disease and metabolic syndrome in the mother and child are associated with longer-term complications, which are linked to platelet dysfunction\textsuperscript{18-22}.

Researchers have long assumed a link between platelets and utero-placental disease. Firstly, an imbalance of prostaglandins in PET has been documented; thromboxane A\textsubscript{2} \textit{(promotes vasoconstriction and platelet aggregation)} appears to be increased, while prostacycllin \textit{(vasodilatory, inhibits platelet aggregation)} appears to be reduced\textsuperscript{23}. This imbalance is a marker of platelet activation. Secondly, in the most severe form of the disease, widespread disseminated intravascular coagulation (DIC) with resultant catastrophic end-organ thrombosis and failure can ensue if delivery does not occur in a timely manner\textsuperscript{5,24}. Thirdly, a fall in platelet count is a feature of the severe form of PET, HELLP \textit{(haemolysis, elevated liver enzymes, low platelets)} syndrome, and can indicate worsening disease in the mother\textsuperscript{25}. Sometimes this alteration in platelet count may even precede the clinical phase\textsuperscript{26,27}. Fourthly, placental micro-thrombus is a feature of utero-placental disease, and recent investigations into platelet behaviour in the
utero-placental micro-environment point toward increased platelet activation occurring as a consequence of defective placentation\textsuperscript{28,29}. Finally, pregnancies complicated by hypertensive disorders in the mother or growth restriction in the fetus are associated with long-term increased cardiovascular risk, metabolic syndrome and thromboembolic disease for both the mother and her offspring\textsuperscript{18-22}. These conditions are independently associated with increased platelet activation.

It follows that there is both direct and indirect evidence of a role for platelets in utero-placental disease, and the rationale for aspirin use in preventing this condition is ever more clear\textsuperscript{30,31}. These points will be elaborated on further. It is evident that platelets and utero-placental disease are intimately linked. Platelet dysfunction has also been linked to conditions outside of pregnancy including cancer, arthritis, diabetes and cardiovascular disease\textsuperscript{32-36}. There is a compelling rationale to understand platelet function more comprehensively in conditions in pregnancy linked to longer-term health problems.

The incidence of hypertensive conditions in pregnancy is increasing and is presenting earlier in pregnancy\textsuperscript{7,37}. This will place a huge burden on neonatal care provision, with substantive costs implicated due to the increasing rate of associated premature deliveries. Worldwide, mothers are still dying at an unacceptably high rate from complications of the condition\textsuperscript{38}. Between the years 2006-2008, PET was responsible for 22 maternal deaths in UK, directly attributable to eclampsia, where sub-standard care was identified as a factor in almost all cases\textsuperscript{39}. Although according to the recent MBRRACE report (Mothers and Babies: Reducing Risk through Audits and Confidential Enquires across the UK and Ireland, 2011-2013), there was a significant reduction in deaths due to PET and eclampsia where a total of 6 mothers died during 2011-2013\textsuperscript{40}. Still, every year there has been one maternal death in Ireland due to PET\textsuperscript{41}. 
Research aimed at uncovering further insights into the pathogenesis of utero-placental disease and strategies to develop screening tools in pregnancy may have wider health-care economic implications that extend from pregnancy into adulthood. The platelet is a cell implicated in this condition. Appropriate measures or assays of assessing platelet function may yield new diagnostic information in pregnancy.

We need to be doing more to prevent further maternal and perinatal fatalities. It could be argued that perhaps we have become far too ‘comfortable’ with managing the condition, where we continue to do what we have done for centuries: monitor blood pressure and assess urine for protein! (figure 1.1). Advances in our understanding of the mechanisms involved in the pathogenesis of utero-placental disease will continue to drive efforts towards effective screening tools and therapies. Assessment of platelet function may be one such tool.
Figure 1.1 an 18th century painting of a woman in the third trimester with suspected pre-eclampsia being reviewed by her physician. If you look closely you can see she appears to be in distress, possibly complaining of a headache or the ‘visual disturbances’ of pre-eclampsia. She also appears to have provided a urine sample in the porcelain jug beside her feet, a quantity of which appears to have been obtained in a beaker by the assistant in the background. Screening for pre-eclampsia in the 21st century is still largely based on these early practices of monitoring blood pressure and assessing urine for protein. Advances in our understanding of the mechanisms involved in the pathophysiology of pre-eclampsia will bring us one step closer to developing an accurate screening tool for this condition in pregnancy.
1.2. The rationale for platelet involvement in utero-placental disease

The concept of the involvement of platelets in utero-placental disease has been cemented for some time\textsuperscript{30}. Research into platelet function in pregnancy has been undertaken for decades after it became apparent that aspirin could prevent utero-placental disease\textsuperscript{31,42-43}. Some even suggested platelet function could predict the development of conditions related to utero-placental disease\textsuperscript{44-47}. However results to date of platelet function in pregnancy and utero-placental disease have been largely inconsistent and difficult to interpret. Part of the problem is that there has been a vast array of platelet function assays available, each assessing a different facet of platelet behaviour. Results of these tests correlate poorly with each other. In this thesis I will assess platelet function in utero-placental disease using standardised and novel, more physiological assays of platelet function.

There is now direct evidence of the involvement of platelets in the disease: platelets have recently been isolated within the maternal spiral arterioles where it is thought that they contribute to vascular remodelling of healthy placentation\textsuperscript{4,48-50}. Most recently, several research groups have identified the expression of extra-cellular vesicles (EVs) within the maternal circulation of pregnancies complicated by PET\textsuperscript{29,51,52}. These EVs are thought to be the result of shedding from the defective layer of syncyiotroblasts and have been shown to result in platelet activation within the maternal circulation, and may differentially affect maternal platelet function\textsuperscript{52}. Thus platelets and utero-placental disease are linked from early placental disease to the clinical presentation of the condition.

We continue to have two challenges in Obstetrics: (i) the early identification of patients at risk of developing utero-placental disease who are otherwise currently labelled as ‘low-risk’, and (ii) the optimisation of outcomes for high-risk patients on anti-platelet therapy in pregnancy. There is an opportunity to understand more comprehensively the contribution of platelet function in the development and
clinical course of conditions linked to utero-placental disease. More accurate and physiological measures of platelet function testing may be the key to improving pregnancy outcomes.

In summary, utero-placental disease is a complex problem and we have a limited understanding of the pathophysiology of the condition. As a result, our current approach to antenatal care and ‘screening’ is suboptimal, and mothers and babies continue to die or face the burden of long-term health problems as a direct consequence of the condition\textsuperscript{53}. It is not enough that we manage these women and their unborn on a short-term basis in pregnancy; the long-term cardiovascular complications and ‘fetal origins of adult disease’ are well documented. Thus utero-placental disease represents a growing health problem with implications for long-term child and adult health. PET and hypertensive disorders are on the increase; our mothers in Ireland are conceiving later in life and disease is occurring more often at preterm gestations. One treatment is at least partially effective in preventing the disease burden: the anti-platelet agent aspirin.

The primary aim of this thesis is to characterise platelet function in utero-placental disease using more physiologically relevant assays of platelet function. I will investigate differences in maternal platelet function using three platelet function assays in utero-placental disease compared with healthy pregnancy. I will correlate platelet function results with clinical severity of disease, and subsequently with placental histopathology results. The relationship between platelet function and placental histopathology has never been investigated before.
1.3. The clinical spectrum of Utero-placental disease

1.3.1. Hypertensive disorders in pregnancy

Pre-eclampsia (PET) is a unique condition to pregnancy, complicating between 5-8% of all pregnancies\(^6\). The first description of the condition is believed to be as early as the 5\(^{th}\) century BC by Hippocrates\(^54\). The term ‘eclampsia’ originates from the Greek term for lightening, thus describing a ‘pre-seizure’ event in the mother\(^54\).

No other condition in pregnancy is so common and yet so potentially dangerous to mother and baby.

Pregnancies complicated by hypertensive disorders contribute significantly to global maternal and perinatal morbidity and mortality. Worldwide it was directly responsible for over 29,000 maternal mortalities in 2013\(^55\). PET is the leading cause of neonatal intensive care admissions in the puerperium\(^56\). The incidence of PET and gestational hypertension (GH) has risen dramatically according to a recent study in the USA\(^7\). The healthcare costs associated with the increased requirement for very (28-31 weeks’) and extremely (<28 weeks’) preterm deliveries due to early-onset PET are colossal, where it is estimated to be approximately 40 and 100 times that of a term pregnancy, respectively\(^7\).

The standard approach to treatment consists of two elements: watchful waiting with medical management of the maternal hypertension or delivery of the fetus and placenta, an approach that we have been adopting for centuries. Delivery is the recommended approach where PET occurs near term\(^6\). The challenge arises when the condition occurs earlier in pregnancy, and delayed delivery is a reasonable and individualised approach employed in very preterm gestations, where each additional prolongation of gestational days impacts significantly on perinatal morbidity and mortality\(^57\). Unfortunately this prolongation can expose the mother to significant risks of morbidity and mortality, and a fine balance must be achieved between optimising perinatal outcome while minimising maternal risk.
If not promptly detected and managed, the complications include worsening hypertension and resultant eclampsia, hepatic rupture, cerebral infarction, severe end-organ damage and ultimately maternal and fetal death\textsuperscript{5,6,58}. Pre-eclampsia is a current global health problem, and is becoming more prevalent. For such a common pregnancy complication it is becoming more relevant than ever in obstetric care.

Perhaps the greatest challenge lies in the subsequent long-term management and primary and secondary prevention of conditions that have a clear association with a prior pregnancy complicated by utero-placental disease: cardiovascular disease, metabolic syndrome, ischaemic heart disease and thromboembolic disease\textsuperscript{18-20}. Most alarmingly, a population based cohort study of registry data of 626,272 births concluded early-onset PET necessitating preterm iatrogenic delivery in cases of idiopathic preeclampsia was found to be associated with an eightfold increase in death from cardiovascular disease\textsuperscript{20}. The current unmet need is a screening tool that will identify these otherwise ‘low-risk’ women who are actually at risk of developing PET and utero-placental disease\textsuperscript{53}.

In addition to maternal complications, hypertensive disorders result in the preterm delivery of 1/250 of all primigravid pregnancies, with 50% of severe early-onset preeclampsia culminating in preterm delivery, contributing significantly to perinatal morbidity and mortality\textsuperscript{6}. In addition, superimposed IUGR < 10\textsuperscript{th} centile complicates approximately 25% of cases of preterm delivery due to PET\textsuperscript{6}. The fetal origins of adult disease are well cemented\textsuperscript{21,22}.

What is clear is that the associated long-term diseases are linked with altered platelet function outside of pregnancy\textsuperscript{35,36,59}. It may be that pregnancy-specific changes in platelet function in pregnancies complicated by utero-placental disease may be associated with similar platelet derangements in related conditions that develop later in life. There is a rationale for a better understanding of the role of platelets in utero-placental disease to understand more comprehensively the mechanisms involved in the pathogenesis to optimise outcomes.
1.3.2. Definition of hypertensive disorders in pregnancy

The broad definition of PET is defined by new-onset hypertension ≥140/90 mmHg on two occasions measured using an appropriate cuff in the presence of proteinuria of ≥300mg/24 hours, correlating to ‘+1’ on a urine reagent strip occurring after 20 weeks’ gestation. The condition is further defined based on a number of clinical and biochemical criteria, including thrombocytopenia and liver function derangement; however the clinical presentation does not always conform to these strict definitions.

Gestational hypertension (GH) is defined as new onset hypertension ≥140/90 mmHg from 20 weeks’ gestation, whereas a diagnosis of essential or chronic hypertension requires this hypertensive range to occur < 20 weeks’ gestation. An emerging sub-group is that of ‘atypical PET’, where proteinuria may be initially absent although end-organ damage may already be a feature, and this can result in the pregnancy being incorrectly labelled as gestational hypertension. Correct diagnosis impacts significantly on subsequent management decisions, where generally a more aggressive approach is employed when a diagnosis of PET is made.

Although the current definition of PET takes into account any case presenting from 20 weeks’ gestation until delivery, this definition is far too broad. Evidence is emerging of a differing phenotype based on gestational age of presentation: early (<34 weeks’ gestation) and late-onset (> 34 weeks’ gestation) PET, where the former is typically a more severe clinical entity for both mother and fetus. Early-onset PET is thought to be related to defective first wave trophoblastic invasion with resulting haemodynamic changes of increased vascular resistance and reduced cardiac output occurring in a generally lower BMI cohort and at an earlier gestation compared to later onset PET. The latter is generally associated with maternal constitutional factors such as high BMI and is not as severe a disease as the early-onset sub-type. Obesity and endothelial dysfunction are
associated with platelet dysfunction. It may be that given these maternal characteristics are more prevalent in more severe early-onset disease platelet function may be different based on gestational age of detection and severity of disease. A specific aim of this thesis is to identify changes in platelet profiles in early versus late onset utero-placental disease across the three platelet assays that will comprise this thesis.

1.3.3. **Intrauterine growth restriction**

Intrauterine growth restriction (IUGR) can affect up to 10% of pregnancies, and is associated with significant neonatal and perinatal morbidity and mortality. When detected in pregnancy this requires intense serial fetal surveillance. Part of the current challenge lies in correctly identifying these pregnancies. There is a lack of adequate screening tests and guidance from randomised controlled trials. A serial measurement of estimated fetal weight (EFW) is neither recommended nor feasible in a low-risk population. The current identification of IUGR within most institutions is generally based on the symphysio-fundal height (SFH) measurement, which has poor sensitivity and specificity, and as a result approximately 30% of IUGR pregnancies are not correctly recognised. It follows that unrecognised IUGR is strongly associated with stillbirth. In addition to adverse composite outcomes of respiratory distress syndrome, intra-ventricular haemorrhage, and neonatal death, those that survive have an increased risk of later onset cardiovascular disease.
1.3.4. Definition of IUGR

There are a number of challenges in the current identification and management of pregnancies complicated by IUGR not least due to the varying definitions that exist\textsuperscript{63}. An accepted definition of IUGR is that of EFW less than 10\textsuperscript{th} centile, however approximately 70\% of such pregnancies are in fact ‘normal and physiologically small’ pregnancies that generally equate to a normal perinatal outcome\textsuperscript{63,64}. These pregnancies are often labelled ‘small-for-gestational-age’ or ‘SGA’, in which case there will not be evidence of utero-placental insufficiency, namely abnormal umbilical artery Doppler waveforms (UAD) with reduced amniotic fluid volume (AFI). A recent large prospective observational trial concluded the highest risk pregnancies to be those with an EFW <3\textsuperscript{rd} centile with or without abnormal UAD\textsuperscript{13}.

IUGR pregnancies can be further classified into early (<34 weeks’ gestation) and late (>34 weeks’ gestation) onset IUGR, with the highest proportion of adverse events occurring in early-onset cases\textsuperscript{8,14,63}. The current approach to the management of IUGR pregnancies includes more intense monitoring, and decisions to deliver are largely based on the presence of abnormal Doppler findings and the gestational age\textsuperscript{8,63}. A specific aim of this thesis will be to characterise platelet function in IUGR based on severity of disease including cases with an EFW <3\textsuperscript{rd} centile, early-onset IUGR and cases complicated with abnormal UAD.

The aetiology of IUGR is diverse, and like PET, includes fetal, genetic and environmental factors. Although common aetiologies include tobacco use, genetic factors, environmental factors, a significant proportion of pathological IUGR pregnancies are ‘placentally-driven’\textsuperscript{8,63}. Constitutional IUGR is not associated with placental abnormalities. The correct labelling of high risk IUGR pregnancies requires a combination of perinatal outcome and placental histopathology findings.
The clinical course of the ‘fetal condition’ of utero-placental disease, IUGR, tends to be well characterised. It could be said that the converse is true for the ‘maternal condition’, PET, where it can be sometimes difficult to predict the more severe cases that may suddenly develop complications. There appears to be great interest in the literature regarding platelet function changes in PET and not so much in IUGR. Perhaps it is the fact that IUGR is not typically associated with a maternal condition, and hence less emphasis has previously been placed on understanding maternal platelet changes in IUGR. However, it is clear that the two conditions are linked on the spectrum of utero-placental disease, with common placental features of utero-placental insufficiency evident in both\textsuperscript{3}. Aspirin has also been shown not only to prevent PET but also fetal growth restriction and it is possible that platelets are implicated in general in placental insufficiency, whatever the clinical end-point. While there is a growing body of evidence for the role of platelets in PET, some inconsistencies in previous platelet function studies exist and these will be elaborated on further in this chapter. There is very little information available regarding platelet function in IUGR, thus providing a rationale for examining this group more closely in this thesis.

The question remains as to why we haven’t tackled the problem by establishing an accurate screening tool or by developing useful therapies? The difficulty with establishing an accurate predictive test for utero-placental disease is that while risk factors for the condition have been well established, the underlying cause in apparently low-risk pregnancies remains unclear, and we have yet to fully optimise the outcomes even for those known to be at risk of developing disease\textsuperscript{65}. There are many different proposed causes for utero-placental disease\textsuperscript{3,30,66}. The placenta is necessary for the condition to occur, and the condition is effectively ‘cured’ when the fetus and placenta is delivered. Whatever the instigating factor, what is evident is that the placenta appears to be the uniting driving factor, and reduced placental perfusion appears to propagate a number of clinical end-points: release of multiple factors that trigger the maternal syndrome of PET\textsuperscript{66}, or reduced placental perfusion associated with fetal growth restriction. Platelet activation appears to be a key feature and recent evidence is now supporting this\textsuperscript{51,52}.
In an effort to understand more fully a mechanism for the involvement of platelets in utero-placental disease, the following will document the established and more recent novel haemostatic mechanisms thought to be involved, in particular for PET, including the role of platelets in placentation and the rationale for anti-platelet therapies in preventing utero-placental disease. This will serve as an introduction to a comprehensive literature review of studies of platelet function in utero-placental disease as a background and rationale for the work of this thesis.

1.4. Pathophysiology of utero-placental disease

The pathogenesis of utero-placental disease is multi-factorial. Whatever the initial insult, the common feature appears to be that of a defective hypoxic placenta. A placenta is necessary for the condition to ensue. There are many different proposed causes of PET and IUGR, including immune, genetic, environmental and cardiovascular factors\(^6,8,63\). One proposed mechanism is that of an altered immune-mediated response, particularly in women in their first pregnancy, where a first time pregnancy is a significant risk factor for PET\(^67\).

It can be clear in some cases at a first booking antenatal visit if a woman is at risk of developing utero-placental disease. Gestational hypertension, pre-eclampsia and intrauterine growth restriction are more likely to occur in pregnancies complicated by pre-existing medical conditions and endothelial dysfunction in the mother, such by diabetes (relative risk, RR 2-7)\(^5,6\), chronic hypertension (RR 10)\(^5,6\), renal disease (RR 20)\(^68\), antiphospholipid syndrome (APLS) and thrombophilia\(^69\). Fetal factors arising during the course of the pregnancy that can place the mother at increased risk of developing PET include multiple pregnancy\(^70\) (RR 4), and rare pregnancy complications such as hydatidiform mole\(^71\) and ‘Mirror Syndrome’\(^72\). PET is also associated with extremes of reproductive age (over 40, RR 3)\(^6\). Some of these predisposing factors are associated with changes in platelet function\(^35,36,62,73\).
A good example of the role of platelet activation associated with adverse pregnancy outcome is that of the autoimmune condition antiphospholipid syndrome (APLS). APLS is associated with the development of PET. Platelet activation and thrombus formation are key features in APLS, and this is likely antibody mediated. A successful pregnancy outcome in the condition is largely dependent on adequate anti-thrombotic and antiplatelet therapy, where the live-birth rate increases from 10-70% with treatment. Renal disease, APLS, thrombophilia and mirror syndrome are all associated with changes in platelet count, where thrombocytopenia is a feature. Age-related changes in platelet function have been recently established. This is further indirect evidence of a relationship between altered platelet function and utero-placental disease.

1.4.1. The balancing act of haemostasis in pregnancy and the involvement of platelets

Massive changes occur in the cardiovascular system in pregnancy. Cardiac output (CO) increases by 30-50% by the second trimester, and blood flow through the placenta at term is approximately 500ml/minute. This rapid placental blood flow must be abruptly stopped at delivery. This is largely achieved by uterine contractions, but changes in the haemostatic system are also believed to play a role. Overall, pregnancy represents a pro-thrombotic state, and this is felt to be an evolutionary advantage in preparation for the anticipation of haemorrhage at delivery. The major haematological changes include an increase in many clotting factors, decreased protein S and resistance to protein C. There is increased in-vivo thrombin generation and reduced fibrinolysis.

During pregnancy is it vital that the placental blood flow maintains its patency. The increase in haemostasis can predispose to thromboembolic disease in the mother in apparently low-risk pregnancies. Haemostatic changes have been shown to be further exaggerated in PET. Thrombosis in the placental bed could be detrimental to the fetus. If a thrombotic process occurs prematurely in the placenta.
this can result in utero-placental insufficiency, or at worst and if severe enough, fetal demise. As mentioned, this early assumption that platelets contributed to micro-thrombosis in the placenta of PET paved the way for large scale trials examining the antiplatelet agent, aspirin in pregnancy to determine if this therapy prevented platelet-mediated thrombus in the placenta.

1.4.2. Placental effects mediating the clinical end-point

The proposed cellular and biochemical changes in PET and resultant maternal syndrome are well established. Whatever the cause, the placenta appears to be ‘stressed’ and this leads to a number of biochemical responses in the mother and/or fetus. The clinical syndrome of raised blood pressure in pregnancy appears to represent a type of utero-placental response to ischaemia and oxidative stress.

Platelet function has recently been shown to be both pro and anti-angiogenic\textsuperscript{80}. It has been established that the hypoxic placenta appears to generate an imbalance in angiogenesis (\textbf{figure 1.2}). The increased release of the soluble protein sFlt-1 binds the growth factors vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), therefore resulting in a reduction in the availability of these factors\textsuperscript{81}. Many studies have documented the tipping of the balance in favour of \textit{anti-angiogenesis} in PET and IUGR\textsuperscript{17,82,83}. This again indirectly implicates platelets in utero-placental disease because platelets are the major source of VEGF in the circulation\textsuperscript{84}. The observed reduction in VEGF in pregnancies complicated by utero-placental disease may possibly signify a functional platelet defect in these pregnancies\textsuperscript{85}. The disordered VEGF signalling is believed to subsequently result in endothelial dysfunction and the maternal symptoms as a result of the vascular inflammation.
Preeclampsia is thought to represent a two-stage disease process, whereby anomalous maternal-fetal-placental cross talk occurring during placentation (stage 1) results in a shallowly implanted and inefficient placenta, and manifests in a varied phenotypic condition after 20 weeks’ gestation (stage 2). The resultant damaged endothelium has also been proposed as a nidus for leucocytes and highly activated platelets. An imbalance in prostaglandins thromboxane A₂ (vasoconstrictive) and prostacyclin (PGI₂) (vasodilatory) favour increases in thromboxane, further promoting reduced placental perfusion and platelet activation. Endothelial derived and platelet activation markers including platelet microparticles (MP’s) have also been shown to be enhanced in PET, further implicating endothelial damage in the condition.

There is evidence that platelets are indirectly activated in PET as part of a multifactorial response to the ischaemic placenta. There is new evidence to suggest that platelets may now be directly involved in the initial formation of the defective placenta, suggesting platelet behaviour is more intimately related to utero-placental disease than previously thought.
Figure 1.2 The biochemical response in PET involves an imbalance in angiogenic factors and appears to evoke platelet activation (adapted from a webinar by Chris Gardiner on preeclampsia and thromboembolism, ISTH, April 2016). The cause of PET is diverse, but whatever the instigating factor, placental distress and hypoxia ensue, with a resultant imbalance in angiogenesis, including increased sFlt-1 levels which bind the growth factors VEGF and PLGF, leading to a reduction in levels of these growth factors. Placental-derived extracellular vesicles (EV’s) have recently been shown to be released by the damaged placenta and this can in turn lead to platelet activation. The resultant damaged endothelium and vasoactive substance release by some factor(s) results in hypertension and the clinical manifestation of the condition.
1.5. The role of platelets in placentation

Looking more closely at the placental changes that occur in utero-placental disease, the common starting point appears to be a disruption of the vascular remodelling of the placenta, a process that occurs early in pregnancies destined to develop the condition. The following is an overview on the role of platelets in normal placentation.

1.5.1. Platelets and normal placentation

The human placenta is unique in that it is haemochorial. That is, maternal blood is in direct contact with the synctiotrophoblast layer of the embryo. The synctiotrophoblast has been coined the ‘gatekeeper’ between tissue and blood in the placenta. During placentation, embryo-derived trophoblasts differentiate into either syncytiotrophoblasts (within floating placental villi, principally involved in gas and nutrient transfer) or cytotrophoblasts (villous-anchoring sites near the maternal interface). Cytotrophoblasts then transform into extra-villous trophoblasts (EVT) which in turn differentiate into invasive endovascular trophoblasts (ENT). This vascular remodelling converts the maternal arteries into low resistance high-flow vessels to promote optimum blood flow to the developing fetus. This process is suboptimal in PET, such that the invasion barely occurs in the myometrial layer, and the vessels remain high-resistance vessels with reduced placental perfusion overall. The establishment of an optimally functioning fetal-placental unit is thus vital in achieving a normal pregnancy outcome.
Platelets are rich sources of growth factors and may have a role in promoting the development of a healthy placenta by release of such platelet-derived growth factors. The non-haemostatic role of platelets in angiogenesis is now well established.80,90. Several of platelet-derived growth factors, including fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1) and platelet-derived growth factor (PDGF), have been shown to protect early embryonic tissue from apoptosis.91 In humans, platelet-derived VEGF and bFGF are strongly expressed during placentation, with even more significant expression when a pregnancy is complicated by fetal growth restriction secondary to inadequate placentation.92-95.

Platelets have been recently implicated in these key early placental events, where they have been shown to promote the actual remodelling of cytotrophoblasts into the ENT that line maternal spiral arteries.4,48-50,96 (figure 1.3). The suggestion that platelet-derived chemokines drive the preferential infiltration of trophoblasts towards maternal vessels came from human studies confirming predominant trophoblastic expression of the chemokine receptor CCR1.48 A subsequent investigation by the same authors comparing human myometrial and placental tissues confirmed that the ligand for CCR1, RANTES, was released by platelets that were trapped and activated during vascular remodelling and that CCR1 was predominantly expressed in ENT.49.

In the aforementioned study immune-stained platelets within placental tissue at <10 weeks’ gestation were also shown to be localised specifically within maternal spiral arteries, and expressed the platelet activation marker P-selectin.49 In contrast, platelets were not found to be isolated in spiral arteries of hysterectomy specimens. In addition, long-term (>48 hours) co-culturing of platelets with EVT demonstrated typical morphological changes to ENT. Therefore activated platelets, concentrated within maternal spiral arteries, appear to promote invasion and remodelling, but do not necessarily aggregate. Interestingly there is data emerging on the role of platelets in cancer that is somewhat analogous to this.33,97 Platelets have been shown to interact with and alter cancer cells and may promote
Figure 1.3 Platelet-derived factors appear to drive the vascular remodelling of a healthy placenta mediated by a possible chemokine gradient produced by platelets in spiral arteries (reproduced from Sato 2003). In the human placenta, embryo-derived trophoblasts aggressively invade maternal spiral arteries and transform the arteries to low-resistance large-calibre vessels. This process, which ensures adequate placental perfusion, is called maternal vascular remodelling. Histological examination has shown deposition of maternal platelets in the trophoblast aggregates formed in the spiral arteries. Several lines of evidence suggest that these platelets are activated. Soluble factors released from the activated platelets, as a whole, enhanced invasive capacity of isolated trophoblasts in vitro. These findings suggest the importance of non-hemostatic platelet function in maternal vascular remodelling.
metastasis\textsuperscript{97}. The angiogenic function of platelets may thus be physiological or pathological.

It has further been suggested that although platelet activation may be occurring in the placenta, this does not appear to result in platelet-mediated thrombus formation in healthy pregnancies. The endovascular expression of the platelet inhibitors thrombo-modulin and tissue-and uro-kinase plasminogen activator, which are abundant in pregnancy, may mitigate unwanted thrombus formation\textsuperscript{98-100}. Interestingly, murine studies have revealed the importance of thrombo-modulin (TM) in early development, where TM knock-out mice have 40\% embryonic lethality, with 100\% lethality occurring when the TM co-receptor endothelial protein C receptor (EPCR) is absent\textsuperscript{101,102}.

The platelet activation marker P-selectin is partially responsible for the adhesion of leukocytes and platelets to the endothelium, and increased levels of P-selectin have been implicated in atherogenesis\textsuperscript{103-105}. It may follow that inappropriate or perhaps even exaggerated platelet behaviour early in placentation may partly contribute to the defective placentation of utero-placental disease\textsuperscript{106}. In an effort to identify possible signalling mechanisms in placentation that appear to extrapolate platelet-derived growth factors, Shanely et al investigated the role of the immunoglobulin superfamily, pregnancy-specific glycoproteins (PSG) during healthy placentation\textsuperscript{96}. PSG’s are secreted by the synctiotrophoblast and are expressed in abundance in normal pregnancy, with maternal serum levels reaching a peak at full term\textsuperscript{107,108}. PSG1 in human platelets was found to inhibit the platelet receptor αIIbβ3 and therefore inhibit fibrinogen-mediated thrombosis, further suggesting a selective pregnancy-specific platelet aggregometry inhibitory mechanism\textsuperscript{96}. Interestingly, in pregnancies complicated by utero-placental disease decreased levels of PSG have been documented, and this may contribute to thrombosis formation in the condition\textsuperscript{109,110}. 

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In summary, platelets promote angiogenesis and this function appears to be integral in the development of a healthy placenta as outlined in the various studies above. A number of more recent studies have perhaps provided the most convincing evidence to date for the role of platelet activation in the developing placenta and how this can result in defective placentation and the subsequent clinical manifestations of gestational hypertension, pre-eclampsia and intrauterine growth restriction.

1.5.2. Novel insights into the mechanism of platelet involvement in utero-placental disease

The sycytiotrophoblast has been shown to continually renew itself, much like skin. Extracellular vesicles originating from the syncytiotrophoblast layer (syncytiotrophoblast extracellular vesicles, STBEVs) are shed and are detectable in the maternal circulation in healthy pregnancy. It is believed that this shedding promotes maternal tolerance to the developing fetus\textsuperscript{111}. In PET, it has been recently shown that larger STBEVs are shed in a disorderly manner and can directly promote platelet activation and inflammation through increased expression of tissue factor\textsuperscript{51,52} (Figure 1.4). Small tissue factor bearing vesicles do not trigger thrombosis alone; they require negatively charged phospholipids, and these have been shown to be supplied by platelets in-vivo\textsuperscript{112}.
Figure 1.4. In PET the defective syncytiotrophoblast layer associated with placental hypoxia appears to shed large extracellular vesicles (EVs) that bind tissue factor-bearing platelets thus promoting platelet activation (adapted from a webinar by Chris Gardiner on preeclampsia and thromboembolism, ISTH, April 2016). EVs (yellow circles) bind tissue factor (TF) and subsequently lead to internalising of the tissue-factor bearing vesicles by the platelet with resulting platelet degranulation and subsequent platelet activation. Thrombin generation has been shown to play a role in this mechanism. It has been suggested by some authors that platelets are chronically activated and ‘exhausted’ in PET, and are therefore devoid of their granule content, rendering the platelet less responsive to aggregating agents. Platelet aggregation will be one of the assays tested in this thesis.
In the recent study by Tannetta et al, STBEVs were incubated with platelets and platelet binding with STBEVs was shown to increase over time. This was also found to be augmented by PAR-1 activation using the agonists to this platelet receptor: thrombin or thrombin-receptor activating peptide (TRAP). The STBEVs appear to become internalised in the platelets. STBEVs have also been shown to lead to platelet aggregation in PET, a response that was prevented by hirudin, therefore implicating the role of thrombin in platelet responses in PET. The mechanism behind the relationship between these extra-villous particles shed by what is a defective diseased placenta and platelets, warrant further investigation. There is a rationale for examining the signalling mechanisms involved in PAR-1 platelet signalling in utero-placental disease. A specific aim of this thesis will be to examine the platelet responsiveness involving the PAR receptor (by assessing platelet responses to the agonist thrombin or TRAP) in pregnancies complicated by PET and utero-placental disease.

1.6. The role of aspirin in preventing utero-placental disease

Pooled data from several large randomised trials have concluded that low-dose aspirin indirectly improves early placentation and prevents adverse pregnancy outcome when commenced early in pregnancy. A recent meta-analysis has demonstrated that low-dose aspirin commenced in early pregnancy (<16 weeks’ gestation) can reduce PET, IUGR, preterm birth and perinatal death by up to 50% in high-risk pregnancies. Aspirin inhibits platelet cyclooxygenase (COX) and consequently prostaglandin production and platelet aggregation. At low doses (doses <300mg) aspirin inhibits the vasoconstrictive effects of the prostaglandin thromboxane without inhibiting the beneficial vasodilatory effects of the prostaglandin prostacyclin (PGI₂). There is indirect evidence that aspirin improves placentation, and this makes sense in light of the more direct evidence of
platelet involvement in placentation described above. Aspirin is thought to promote effective placentation prior to the second wave of trophoblastic invasion however the role of aspirin and platelet function in pregnancy is not fully understood.

Current NICE guidance suggests aspirin should be prescribed to those with a history of early-onset severe PET/IUGR/HELLP syndrome in a prior pregnancy (in other words high-risk pregnancies), which implies a recognised prophylactic treatment is recommended based on clinical history alone, or comorbid medical conditions\(^6\). However PET is common in nulliparous women, where disease often remains quiescent sometime after placental pathology has already established; nulliparity is associated with a relative risk of 3 of developing PET. This suggests there is a considerable ‘at-risk’ ‘low-risk’ group, where therapy may be indicated, however without any current reliable screening tool to identify who may require it.

The benefit of aspirin to prevent PET when it is administered from first trimester suggests a platelet-mediated thrombotic tendency in this condition, and the assumption would be that platelets are activated in the condition. There is evidence now to suggest this is the case. When aspirin is administered at a gestational age after placental establishment (after 16 weeks’ gestation) it does not appear to be beneficial in preventing disease. What is not really clear is the effect of aspirin on platelet function in pregnancy. There are very limited studies of aspirin response in pregnancy\(^{114,115}\). Platelet inhibition of the COX pathway (through arachidonic acid (AA)) can be assessed with standard platelet aggregation assays; the pharmacological effect of aspirin can be assessed by measuring serum and urinary thromboxane levels. The difficulty in interpreting results and determining ‘aspirin response’ is that the various different platelet function assays correlate poorly with each other in terms of aspirin effect\(^{116}\). Moreover these two assays only describe part of the effect of aspirin.
There are also various different doses at less than 300mg available that constitute ‘low-dose’ aspirin, and many of the different trials of aspirin in pregnancy used differing doses. The bio-availability of aspirin in pregnancy and the effect of the continual changes in the cardiovascular system need also to be taken into account. The effect of BMI and compliance in pregnancy, where there may be a maternal fear of teratogenicity are all factors that may modulate a response to aspirin.

Even with implementing a strategy of aspirin prophylaxis as a ‘one-for-all’ prevention in high-risk pregnancies, a considerable proportion of these patients will still go on to develop adverse pregnancy outcome. There is a substantial lack of data regarding aspirin response in pregnancy, and the reason for this non-responsiveness is not clear but this may explain the suboptimal results in prevention of utero-placental disease. Given the widespread non-response to aspirin that is seen in patients with cardiovascular disease\textsuperscript{116,117}, it is entirely possible that the modest reduction in utero-placental disease observed with aspirin therapy may well be due to residual increased platelet reactivity in some pregnant patients, where aspirin may be less effective.

More accurate and physiological measures of platelet function testing may help uncover insights into aspirin response in pregnancy. However more physiological measures of platelet functional testing warrant application in the clinical conditions of utero-placental disease in the absence of aspirin to begin with, to better characterise the platelet changes that are really occurring in the condition. The information that will follow may thus help target individuals in early pregnancy who would otherwise be deemed ‘low-risk’ but who might benefit from aspirin therapy to prevent utero-placental disease. The aim of this thesis is to characterise platelet function in utero-placental disease with different assays of platelet function.
1.7. Screening for utero-placental disease

Modern advances in obstetric care have been heavily invested in developing predictive screening tests for PET and adverse pregnancy outcome. Suggestions of reformed models of antenatal care in favour of concentrating resources to identify high-risk pregnancies in the first trimester have yet to revolutionise standard of care\textsuperscript{53}. This is due, in part to a lack of accurate predictive screening tools for common pregnancy conditions, particularly utero-placental disease\textsuperscript{65,118}. As discussed, hypertensive disorders still remain a leading cause of perinatal and maternal morbidity and mortality. There is evidence to suggest that early (pre-symptomatic) identification of women at risk, followed by intensive surveillance and early intervention, may improve outcomes\textsuperscript{16,119}. Recent evidence from the aforementioned multicentre prospective observational study of fetal growth by The Perinatal Ireland Research Consortium (PORTO) suggests intense monitoring of IUGR improves perinatal morbidity and mortality\textsuperscript{13}.

The challenge remains in early identification of those at risk to redirect appropriate resource allocation in a health service with increasingly limited resources. Ideally developing a screening tool for those at risk of subsequent development of hypertensive and growth disorders could improve maternal and perinatal outcomes by selecting the most appropriate patients for specialist antenatal care. As previously mentioned, markers for identifying pregnancies at risk of developing PET and IUGR have been examined, however no independent clinically useful screening tool has been identified. Biochemical markers such as PAPP-A, placental growth factor (PIGF), VEGF, endoglin, activin-A, inhibin-A, have been heralded as promising markers for PET, however their application is inconclusive in intermediate or later-onset gestational disease\textsuperscript{65,120-124}.
The platelet is a cell that contains multiple growth factors and is involved in a vast array of these individual signalling mechanisms. It is a potential target for extrapolating gross functional information in utero-placental disease that may serve as a general biomarker that represents an end-point of a range of mechanisms listed above.

Even when combining maternal risk factors and characteristics with biomarkers in a multidimensional screening tool, the sensitivity and specificity for the development of adverse maternal complications are far from an acceptable level that can support the widespread generalised clinical introduction of such models\textsuperscript{16,118}. Also, given the differing pathogenesis and phenotypes of utero-placental disease it is not surprising that no single screening tool has emerged. Rather studies should concentrate on elaborating on our basic understanding of pathogenesis such that perhaps newer clinical models and care pathways emerge for specific phenotypes\textsuperscript{125}.

Recalling the first image in this thesis of the 18\textsuperscript{th} century painting, routine antenatal care, by way of identifying raised blood pressure for gestational hypertension, and with the addition of proteinuria for a diagnosis of pre-eclampsia, remains the gold standard ‘screening tool’ for large cohorts of generally well women. The schedule for antenatal hospital visits is therefore highly concentrated in the third trimester, when these conditions are more likely to become clinically apparent (figure 1.5)\textsuperscript{53}. The widespread use of ultrasonography to detect abnormal fetal growth is not recommended for a low-risk population and the identification of IUGR still relies on physical abdominal measurement (SFH). This is largely inaccurate, particularly with the challenges of a pregnant population with an ever increasing BMI.

Current methods for identification of women at risk of utero-placental disease in an index pregnancy solely rely on the presence of maternal medical risk factors or a previous history of adverse pregnancy outcome, an approach that has little use to patients presenting in their first pregnancy. Many high risk patients remain nestled within a large low-risk antenatal population. It is fair to say that in current clinical
practice we are approaching the detection of PET in pregnancy much the same way as we have been doing for a number of centuries: monitoring blood pressure and testing the urine for protein. Unfortunately quite a high proportion of patients remain asymptomatic of disease, and despite the huge resources placed on established antenatal care frameworks, valuable time in managing or preventing associated adverse outcomes is currently being lost by an inability to reliably and promptly identify those at real risk.

Many high-risk patients given aspirin treatment still develop disease. The challenges are that while aspirin appears to prevent disease, we don’t have clear guidance on when exactly to start it in pregnancy. It also not clear exactly how much aspirin we should be using, where ‘low-dose’ constitutes any dose under 300mg and whether we should be adjusting the dose according to gestational age or BMI in pregnancy. Part of the problem is that an accurate measure of aspirin response has yet to be developed in general medicine and current measures of testing aspirin response correlate poorly with each other. Our research group has developed a novel in-vivo dynamic platelet assay that has shown some new aspirin platelet effects. This assay will be tested in this thesis in utero-placental disease.
Figure 1.5 It has been suggested that the pyramid of antenatal care is turned on its head! (reproduced from Nikolaides 2011). The current model of antenatal care (A) consists of intense antenatal hospital visits largely scheduled in the third trimester. A proposed model for antenatal care (B) suggests a multi-parameter screening assessment at booking visit at 12 weeks can help stratify patients into high and low risk and reflect risk stratification.
A comprehensive review of platelet function in pregnancy and pregnancies complicated by utero-placental disease may help identify the gaps in the knowledge and challenge the assumptions of platelet function in pregnancy. The following section is a comprehensive literature review specifically of platelet function studies undertaken in pregnancy to date as a background to the methods of platelet function testing comprising this thesis.
1.8. Platelet function testing: platelet biology, methods of assessment and clinical utility in pregnancy, introduction:

A major function of platelets is primary haemostasis at the site of vessel injury. Platelets adhere to the exposed vessel matrix, activate to recruit additional platelets, and aggregate to each other and to the vessel surface to arrest further bleeding. Thus physiological platelet function can be described as adhesion, activation and aggregation\textsuperscript{126}. Traditional methods of assessing platelet function have focussed on the last phase of platelet function, that is, platelet aggregation\textsuperscript{127,128}. The last few decades have witnessed an evolution in platelet function tests that are now capable of assessing the initial, intermediary and final steps involved in platelet-mediated thrombosis: in other words: platelet adhesion, activation and aggregation.

In the following section I will introduce the role of platelets in haemostasis; the different ways of assessing this function, followed by a literature review of the results of platelet function testing in pregnancy to date. I will review in detail the main platelet function assays that will be tested in this thesis: (i) spontaneous platelet adhesion and aggregation, (ii) agonist-induced aggregation and (iii) shear-induced dynamic platelet function.

1.8.1. Platelet function

When platelets come in contact with exposed sub-endothelial matrix they will (i) attach to the vessel wall (adhesion), (ii) release their granule content (activate) and (iii) clump to each other and eventually form platelet-plug thrombus (aggregation). The primary purpose of this function is to arrest bleeding at the site of vascular injury\textsuperscript{126,129}. Some individuals lack this ability, due to platelet functional defects, and are thus at risk of haemorrhaging\textsuperscript{130}. In one sense, our understanding of the pathway to platelet-plug formation has evolved by understanding the platelet
functions that are deficient in these individuals. Platelet function tests are currently ordered in the clinical setting to identify patients at risk of bleeding. In the past few decades it has become apparent that platelets are involved in diverse physiological and pathological processes, and clinical research groups have invested heavily in developing platelet function assays to predict disease, although these tests still remain largely within clinical research settings. Traditional platelet function tests are still used in the hospital setting.

The first available platelet function tests traditionally focussed on the final phase of primary haemostasis: platelet aggregation. Signals involved in platelet aggregation and activation promote the binding of platelets together and then to the exposed endothelium to form a platelet plug and to prevent further bleeding. There are various ways of assessing platelet aggregation and activation that have been well documented and standardised, and these will be explored later.

Inappropriate or uncontrolled platelet-mediated thrombus formation at the vascular interface can result in ischaemia and infarction. This has encouraged further research into the global function of platelets with newer physiological assays to better understand the real-life platelet behaviour in individuals as opposed to in-vitro and ex-vivo environments that may not be reflective of function in-vivo. Recent experimental data by our research group has focussed on characterising the first phase of platelet haemostasis: platelet adhesion.

The following will summarise the current knowledge of platelet function and the methods of testing each function with specific reference to the assays that will comprise this thesis. Not all platelet function tests measure the same parameter of platelet function. Platelet function tests are based on differing operating principles and most conventional tests focus on the haemostatic function of platelets, as outlined above. The functions that can be tested include platelet adhesion: the measurement of platelet products or the expression of platelet receptors during platelet activation, and the role of platelets in stable clot formation through platelet aggregation. Newer more physiological assays have been developed that can
assess platelet adhesive responses to arterial shear, thus replicating the initial in-vivo platelet behaviour in the circulation.

Since platelet function testing is evolving, the tests used in this thesis are based on platelet adhesion, activation and aggregation. In parallel with a greater understanding of utero-placental disease I used different assays of platelet function to try to comprehensively understand the role of platelets in the condition:

(i) **Adhesion:** I will first assess spontaneous platelet aggregation (SPA). Platelets can adhere and aggregate together when gently stirred. SPA is associated with increased mortality due to recurrent coronary ischaemic events. There are limited reports of SPA in pregnancy. These studies will be explored.

(ii) **Activation and aggregation:** Agonist-induced platelet aggregation will be assessed. This is a measure of the aggregation response of platelets after activation by a number of well-defined agonists. A number of studies have assessed various methods of platelet aggregation in pregnancy with inconsistent results, and these will be described further.

(iii) **Adhesion, activation and aggregation:** Platelet function will be assessed in whole blood at arterial shear-rate using a shear-based vascular platelet assay. This assay can describe the initial platelet adhesion in the vasculature, followed by responses involving platelet activation that ultimately lead to platelet aggregation and stable clot formation. This assay can measure all three major platelet functions. The assay assesses platelet interactions over a well-defined protein-covered surface (von Willebrand Factor, VWF), where interactions are visualised and captured from the first initial interaction on the first frame, through to the final 500th frame, corresponding to a total of 20 seconds of real-time in-vivo platelet function. This assay has never been tested before in pregnancy.
1.8.2. Platelet structure

Platelets are the smallest of blood particles and are found only in mammals. They were originally thought to represent an evolutionary process to allay postpartum haemorrhage\textsuperscript{134}. However both the establishment of a functioning placenta and the usual haemorrhage associated with parturition are not unique to mammals\textsuperscript{135}. It is now thought that the ability of platelets to form thrombus at the site of vessel injury despite high arterial- shear force within the circulatory system represents a later unique mammalian evolutionary process.

With growing interest in the human anatomy and medical research a number of scientists in the late 19\textsuperscript{th} and early 20\textsuperscript{th} century attempted to investigate platelets with early microscopic equipment. The first description of platelets was by George Gulliver, who in 1841 investigated bloods components using the twin lens microscope which permitted improved resolution\textsuperscript{136}. Approximately 40 years later, Max Schultze described platelets as ‘spherules’, particles he noted to be colourless, much smaller than red blood cells and were occasionally clumped together\textsuperscript{137}. The term ‘platelet’ was eventually assigned by James Wright in 1910 while examining them on blood smears, having described what he initially recognised as ‘plates’ in 1906\textsuperscript{138}.

The basic structure of the platelet is in fact credited to the Italian doctor and scientist Giulio Bizzozero, who pioneered the early use of microscopy in medical research\textsuperscript{139}. He was the first scientist to understand the significance of platelets in haemostasis and the relationship between (i) platelet adhesion, (ii) activation and (iii) aggregation and subsequent formation of a fibrin clot, where he summarised “whereas under normal conditions the platelets float isolated in the plasma, when subject to an influence that leads to thrombosis, they adhere to one another to form an accumulation. The blood platelets, free in the blood stream and being hurried along are held up by other platelets that they come into contact with as they become stickier than they are under normal condition”. Here Bizzozero was
describing the traditional model of platelet recruitment and hemostasis. He referred to the platelet as a ‘third blood element’, as distinct from red and white blood cells, and also noted that in a chamber on the slide the platelets initially adhered to one another on a ‘thread’ and over time increased to form layers upon which fibrin was deposited.

The possible predictive role of platelets was even hypothesised by Bizzozero as early as 1882 where he stated ‘it is probable that the blood platelets are involved not only in thrombosis and coagulation but also in other vital reactions of the blood and the blood vessels associated with disease states’. Although there was a burst of interest in platelet biology in the late 18th century, this waned significantly and the platelet was described by one author as the ‘neglected stepchild in the family of blood cells’. It was only in the 1960’s that platelet research became ‘en-vogue’ yet again, with authors interested more in the interaction of platelets within the vasculature and in describing the actual sequence of platelet adhesion and aggregation. It was in this era that platelet activation became apparent, where adenosine di-phosphate (ADP) and collagen were recognised as the major potent platelet pro-aggregants, and even earlier in the fifties, authors had been describing the inhibiting effects of Aspirin on bleeding time.

1.8.3. Platelet production

Platelet turnover is approximately 10 days, with a healthy adult producing an average of $10^{11}$ platelets on a daily basis. A typical platelet count is 150-400 x $10^3/\mu l$ blood for an adult. The average diameter of a platelet is 2.5µm with a volume of 7.1±4.9µm³. Most platelets will circulate in the circulation in the quiescent state and will never interact with the endothelium. Therefore in its lifetime, the platelet will generally assume a discoid ‘resting’ shape, and changes shape upon activation and release of its contents in the wake of vessel injury or when stimulated by an agonist. Platelets are removed by phagocytosis in the spleen and Kupffer cells of the liver.
Platelets are fragments of cytoplasm derived from large nucleated progenitor cells 
megakaryocytes (MK) (50-100µm) produced in the bone marrow. Megakaryocytes 
operate as the ‘factory’, where the processing and assembling of platelets occurs. 
The ‘production line’ comprises of long dendritic MK protrusions that extend into 
sinusoidal vessels, enabling the formation and release of ‘pro-platelets’ and the 
packaging of their various contents\textsuperscript{144,145} (\textbf{figures 1.6,1,7}). The subsequent 
disassembly of the initial large MK cytoplasm into the individual anucleate platelets 
as we recognise them, results from the shear-force of flowing blood predominantly 
within the pulmonary circulation\textsuperscript{146}. It has recently emerged that polypoid 
megakaryocytes have the ability to accumulate large quantities of DNA through 
endomitosis and an invaginated membrane system (IMS), which is thought to 
facilitate the packaging of the large cargo that will eventually be incorporated into 
the granules of individual platelets\textsuperscript{144,147}. 

At any time in a normal healthy person there are both old and new platelets in the 
circulation. Information about the quantity of old and new platelets may be inferred 
through platelet function testing and assessment of the volume of platelets (mean 
platelet volume, MPV). Old and new platelets behave differently: newer or 
immature platelets tend to be larger, and have been shown to be more sensitive to 
agonists than older smaller platelets\textsuperscript{148-150}. Smaller platelets are older and may 
have already undergone platelet shape change and activation. Platelet function 
testing can give a global view of the total population function of platelets.
Figure 1.6 Tubulin staining of a pro-platelet-bearing megakaryocyte (reproduced from Lecine 2000). The pro-platelet tip of the megakaryocyte is presumed to contain granule content that will be present within the platelet.
Platelets are derived from tubules from megakaryocytes produced in bone marrow. Pro-platelets are released from the megakaryocyte body after a retraction and undergo further fragmentation to yield individual platelets (outlined in point 6 on the figure). After commitment to the megakaryocyte lineage, cells undergo cytoplasmic maturation, where pseudopodia are formed and are subsequently remodelled into microtubules that elongate into pro-platelet processes. Swellings along pro-platelet tips are presumed to contain platelet granule content. Pro-platelets are released from the megakaryocyte body after a retraction, undergoing further fragmentation to yield individual platelets.
1.8.3.1. Platelet production in pregnancy

The glycoprotein hormone thrombopoietin (TPO), produced by the kidneys and liver regulates platelet production and is increased in pregnancy\textsuperscript{151-154}. Oestrogen and other hormones may influence megakaryopoiesis, but to a lesser extent\textsuperscript{155}. Thrombocytopenia drives thrombopoiesis. This can result in the production of larger more immature platelets in the circulation. This can be seen as an increased mean platelet volume (MPV) on a full blood count sample. Increased platelet volumes have been associated with diabetes mellitus and myocardial infarction\textsuperscript{148-149}. There have been inconsistent reports of MPV values in pregnancy, though a number of studies have indicated higher MPV levels in PET\textsuperscript{156-158}. This may indicate a response to thrombocytopenia occurring as part of the disease process.

Platelet count can change in pregnancy with a fall in platelet count in apparent healthy pregnancies and with an even greater drop in PET. The mechanism for gestational thrombocytopenia is still largely unknown though it is presumed to be due to haemodilution. Some authors have suggested platelets are activated in pregnancy and further activated in PET, resulting in increased consumption and thrombocytopenia\textsuperscript{26,156}. Assessment of platelet count is a routine investigation in everyday obstetric practice and while it is not a functional measure of platelet behaviour, it is a parameter that is well documented in pregnancy. MPV levels will be assessed in this thesis in normal pregnancy and utero-placental disease and correlated with platelet function results.

1.8.3.2. Gestational thrombocytopenia

A fall in platelet count below 150g/L can complicate between 5-15% of pregnancies\textsuperscript{159-161}. In three quarters of these cases this is an uncomplicated acquired gestational thrombocytopenia, typically identified in the third trimester, and is generally associated with a favourable outcome for mother and infant (figure 1.8). In 5% of cases a reduced platelet count is identified in the first
trimester, and is typically associated with immune thrombocytopenia (ITP), where IgG antibodies to platelet membrane surface are a feature, outcomes are generally favourable\textsuperscript{162}. Even for cases of ITP, over 90% of neonates will not have thrombocytopenia, and less than 1% of cases will be complicated by intracerebral haemorrhage or death\textsuperscript{163}. It is rare that a pregnant patient with ITP would require plasma exchange or a splenectomy (which is usually delayed until the second trimester).

Figure 1.8 Histogram of the platelet count distributions in pregnancy compared to non-pregnant women. Platelet count distributions are different in pregnancy (n=6770) compared with non-pregnant women (n=287) (reproduced from Boehlen 2000)\textsuperscript{159}. This histogram demonstrates there is a large variation of platelet count in pregnant women which is overall much less than non-pregnant women. Platelet counts are reduced in pregnancy (white bars) compared with non-pregnant women (orange bars).
1.8.3.3. Thrombocytopenia in utero-placental disease

The remaining 25% of cases of acquired thrombocytopenia in pregnancy occur in association with PET and HELLP syndrome (haemolysis, elevated liver enzymes and low platelets). A reduction in platelet count may even precede the signs and symptoms of pre-eclampsia. A reduction as low as 30-50% in platelet count may occur in severe PET. The vascular changes of reduced cardiac output and endothelial damage in PET may predispose to increased platelet activation, consumption and increased marrow turnover with clinical and laboratory features of thrombocytopenia and disseminated intravascular coagulation.
1.9. Methods of assessment of platelet function

1.9.1. Tests based on platelet adhesion and methods of assessment

1.9.1.1. Spontaneous platelet adhesion and aggregation

This is the first platelet assay that will be tested in this thesis. It is recognised that platelets have the ability to ‘spontaneously’ adhere and aggregate together when stirred\textsuperscript{167}. There were some studies of spontaneous platelet aggregation (SPA) undertaken a number of decades ago, and it was even proposed as a predictive test for cardiovascular events\textsuperscript{167-172}. SPA could be a marker for disease. Some research groups have described a method of counting the total number of platelets before and after a specified time of ‘gentle stirring’ (usually 10-20 minutes)\textsuperscript{169,170}. The platelet counting system calculates SPA as the number of platelets that have aggregated together by the end of the experiment by subtracting the number of single platelets remaining from the initial total number of platelets. Other studies have described SPA using variations of methods of light transmission aggregation\textsuperscript{167,168}. There is no standardised way of assessing SPA. In this thesis I will assess SPA using a technique of light transmission aggregometry (LTA), where the degree of spontaneous platelet aggregation is calculated as the amount of light transmitted after platelet aggregation. LTA is the gold standard technique of assessing platelet aggregation\textsuperscript{127}. Platelet adhesion and aggregation in the context of novel shear-flow mediated assays will be described last.
Platelet activation and methods of assessment

Platelet activation can be induced by various stimuli and agonists, and via their various receptors platelets subsequently release some or all of their vast dense and α granule content, also known as the platelet secretome, or the totality of secreted platelet proteins or products. The main platelet storage compartments are that of the dense and α-granules. Platelet dense granules are packed with small molecules that contain ADP, adenosine-triphosphate (ATP), serotonin and high levels of calcium, which together promote vasoconstriction and platelet activation, and can contribute to athero-thrombosis. The α-granules are in abundance and contain contents including the proteins VWF, platelet-factor 4 (PF4), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). This cargo is heterogeneous, functioning not only in platelet adhesion and thrombus formation, as novel roles of α-granule releasate in angiogenesis and cancer progression have recently been suggested. As outlined in the introduction, platelet activation is important in the development of a fully functioning placenta, and exaggerated platelet activation is possibly linked to utero-placental disease.

The process of platelet activation is a complex one, involving a plethora of receptors and cell-signalling mechanisms. The binding of various different agonists to the platelet membrane will induce specific changes in the conformation of the platelet receptors described above depending on the initial stimulus. The various steps in platelet receptor activation converge into a common pathway of platelet aggregation and ultimately platelet-plug and thrombus formation. Thus measures of platelet function can include assessments of the change in platelet receptors and/or release of platelet content (platelet activation), or the aggregation response of platelets to various agonists (platelet ‘reactivity’ or aggregation).
1.9.2.1. Methods of assessment of platelet activation:

Activation can be assessed by measuring the expression of markers on the platelet membrane (e.g. P-selectin); measuring the release of dense granule content (ADP); biochemical analysis of specific activation pathways (e.g. TXA₂, a pathway that is a target for aspirin) or by measuring cleaved soluble products of activation (e.g. GPIV)\textsuperscript{173}. The measurement of the expression of surface platelet activation markers or the change in platelet surface receptors can be achieved by flow cytometry. In flow cytometry, platelets are fluorescently labelled with at least two fluorophores; one fluorophore to identify the monoclonal antibody being tested, and a different fluorophore that is the platelet identifier\textsuperscript{174,175}. An agonist is added to the labelled whole blood which is then passed through a flow chamber where a laser beam activates the fluorophobe, intended to target a specific platelet antigen. The amount of light transmitted as a consequence of platelet-platelet interactions and aggregation correlates with the platelet characteristic being tested. Platelet reactivity, changes in platelet-surface antigens, platelet-platelet aggregates, platelet-leucocyte aggregates and markers of platelet activation (P-selectin) can all be tested using flow cytometry. Using this method, platelet disorders (due to a lack of specific glycoprotein platelet receptors) and platelet storage diseases can be diagnosed in addition to heparin-induced thrombocytopenia\textsuperscript{175,176}.

The advantage of flow cytometry for platelet activation is that is requires only a small amount of blood, and uses whole blood for analysis. However, the technique is very complex and expensive to use, it is prone to artefact and it is not standardised\textsuperscript{173}. The relevance of flow cytometry analysis with respect to clinical endpoints is poorly understood. Also the issue with measuring P-selectin or other markers of platelet activation is that is doesn’t give a global view of platelet function. There have been inconsistent reports of platelet activation in pregnancy and utero-placental disease\textsuperscript{44-47,177-192}. Rather the assays tested in this thesis can give information on all three platelet functions of adhesion, activation and aggregation. As outlined above platelet activation does not necessarily imply
platelet thrombosis, and there is also no clear consensus to define platelet activation after adhesion. Thus, a global measure of platelet haemostatic function assessed by this method is not a reliable assessment of individual functions.

1.9.3. Agonist-induced platelet aggregation

Agonist-induced platelet aggregation is the second assay that will be tested in this thesis. Assessment of platelet aggregation by agonist–induction is a better established way of assessing platelet function rather than platelet activation which assesses some but not all platelet activation pathways. The following will introduce the main functions of the platelet with regards to the receptors and agonists involved in stable platelet aggregation.

Upon vascular injury, when the sub-endothelial matrix is exposed, VWF is immobilised from the plasma onto the exposed vasculature. The VWF binds to the platelet receptor GPIb (figure 1.8) resulting in subsequent activation and cell signalling. The platelet receptor GPIIb/IIIa then forms cross links with fibrinogen, which ultimately results in platelet-platelet clumping and aggregation. ADP is subsequently released and this activates P2Y12 which activates IIb/IIIa. ADP and TXA2 are agonists that result in platelet activation and aggregation. This is a general overview of the steps involved in platelet aggregation in relation to specific platelet receptors. Although relatively simplistic and ignoring the 38 other platelet receptors it provides an explanation for platelet aggregation assays. By stimulating a platelet with an agent that is known is result in platelet aggregation and assessing its response or ‘reactivity’, the function of platelets can be determined.
The haemostatic function of platelets involves platelet adhesion, activation and aggregation. As mentioned, the principal agents involved are adenosine diphosphate (ADP) and thromboxane A2 (TXA$_2$), but also collagen, thrombin and to a lesser degree, epinephrine. These are the five agonists that will be tested in this thesis, because they can trigger the downstream platelet aggregation events.

Figure 1.8 Schematic representation of a limited number of platelet receptors involved in the steps of platelet aggregation (platelet adhesion, activation and aggregation) and the anti-platelet agents that can block platelet aggregation (printed with permission, Prof Dermot Kenny). Aspirin inhibits arachidonic acid (AA) mediated thromboxane synthesis; Ticopidine and clopidogrel (Plavix) inhibit the P2Y12 receptor which prevents the release of adenosine diphosphate (ADP) and subsequent platelet activation; Vorapaxar is an anti-thrombin agent which inhibits platelet PAR-1 receptor; Abciximab inhibits Ib/IIa mediated fibrinogen cross-linking of platelets and subsequent platelet aggregation.
Each agonist can examine the response and activation of platelets to that specific stimulus that targets a well-defined platelet receptor and evokes a specific cell signalling mechanism that will ultimately lead to platelet aggregation depending on the dose of the agonist used. By applying multiple different physiological concentrations of agonists, from very small increments to maximal doses, this can give a better meaning of what is happening when platelets are exposed to those sub-maximal concentrations that are ever changing in vivo within the circulation. All the above platelet aggregating agents act synergistically, such that if one of the pathways of aggregation is defective or inhibited, platelet function may be overall greatly impaired\textsuperscript{130}. Previous studies of platelet function in pregnancy have assessed aggregation in response to a limited number of these agonists and at limited concentrations of each, and results have been inconsistent\textsuperscript{197-216}. For this thesis an accurate test of platelet aggregation incorporating all five agonists at incremental and physiological concentrations will be used.

1.9.3.1. Agonists involved in platelet aggregation

The following five agonists will be tested in an aggregometry assay in this thesis:

1.9.3.1.1. ADP activates the P2Y12 receptor and is blocked by Plavix

ADP acts at the P2Y1 and P2Y12 receptor and is blocked by antagonists such as Clopidogrel (Plavix). ADP was recognised as a pivotal platelet agonist in the 1960’s, and its agonistic ability has long been capitalised in platelet function studies\textsuperscript{217}. Over a decade ago, following a greater understanding of the function of the two main G-protein coupled receptors (P2Y12 and P2Y1) involved in ADP-mediated platelet aggregation came the development of specific ADP receptor antagonists which are now widespread in clinical use\textsuperscript{218-220}. ADP platelet responses require initial vascular injury recognition, after which activated platelets release ADP from dense granules which in turn induces a sequence of intra and extracellular events, contributing significantly to the propagation of the platelet
plug. The G-Protein family of platelet receptors, of which there are four (Gq, Gi, G12, Gs), serve to both promote and stabilise platelet activity. Two important receptors for ADP are P2Y12 and P2Y1, with selective actions to enable firm platelet adhesion\textsuperscript{221}. Platelet shape change occurs following binding of the P2Y1 receptor (Gq-coupled) resulting in protein kinase C mediated intra-cellular calcium release from the platelet dense tubular system, compounded by increased intracellular calcium influx. This Gq-coupled ADP complex results conformational change and platelet aggregation. The action of the P2Y12 receptor (Gi-coupled) is to inhibit cyclic adenosine monophosphate (cAMP) production by blocking adenylate cyclase. Adenylate cyclase is an enzyme that normally inhibits platelet aggregation by converting ATP to cAMP. Activation of both Gq and Gi subunits is required for efficient platelet aggregation. Studies in P2Y12-null mice reveal ineffective platelet responses, whereby P2Y1-ADP mediated platelet shape change may occur, however the lack of inhibition of adenylate cyclase due to deficient P2Y12 receptor impairs overall platelet aggregation\textsuperscript{222}.

1.9.3.1.2. AA causes the release of TXA\textsubscript{2} that activates platelets.

The effect of arachidonic acid (AA) is blocked by aspirin. AA is a carboxylic acid (polyunsaturated omega-6-fatty acid) made up of a 20-carbon chain and four cis-double bonds. It is not unique to platelets, present in abundance on the plasma membrane of many cell types including brain and muscle tissue, and is even marketed as an ergogenic supplement\textsuperscript{223}. AA is involved in key inflammatory responses and cell signalling through the release of prostaglandins. It is a powerful mediator of initial platelet activation, but is not required for prolonged platelet propagation\textsuperscript{224}. Therefore studies examining the effect of AA as a single agonist on platelet aggregation should be interpreted with caution, as single agents do not achieve irreversible platelet aggregation independently. This has been one of the limitations of interpreting previous studies of platelet function in pregnancy, and this will be further explored below.
AA is freed from the plasma membrane and cleaved by the inflammatory enzyme phospholipase A\(_2\), ultimately resulting in the formation of TXA\(_2\), and production of phospholipase C, thus contributing to amplification of platelet activation and adhesion respectively. Phospholipase C activation results in increased intracellular calcium influx, achieved by binding of AA to G-protein coupled transmembrane receptors.

The cyclooxygenase enzyme COX-1, contributes in this process by first converting AA to prostaglandin H\(_2\) (PH\(_2\)), which in turn results in the formation of the potent platelet agonist TXA\(_2\). The irreversible action of COX-1 enzyme has been long been capitalised by the anti-platelet therapy Aspirin, prescribed in primary prevention for high risk cardiovascular patients\(^{225}\). However there is substantial inter-individual variability with aspirin and the phenomenon of ‘aspirin resistance’ has emerged\(^{116, 226}\). Recent studies have suggested that this lack of response to aspirin in some individuals is attributed not only to lack of compliance but also to possible residual AA-induced platelet activation in treated patients, perhaps through alternative COX-independent and ADP-dependent pathways\(^{227}\). Other authors have disregarded this potential, citing the significant influence of patient behaviour and non-compliance as central to suboptimal response\(^{228, 229}\).

Aspirin has been recently shown in a meta-analysis to moderately prevent the recurrence of preeclampsia\(^{31, 42, 43}\). Although few studies have examined aspirin response in pregnancy\(^{114, 115}\), the lack of optimal response with this therapy in preventing serious pregnancy complications may be attributed to lack of compliance, suboptimal pharmacy-dynamic or pharmacokinetic effects\(^{230, 231}\).
1.9.3.1.3. Collagen binds the GPIV receptor

Antagonists to collagen are currently in clinical trials. Collagen is a highly thrombogenic platelet agonist and a substantial component of the normal vessel ECM, mediating its effects upon exposure to flowing blood following vascular injury. Collagen types I and III are the major ECM components and type IV contributes to its basement membrane architecture. Within types I and III are GXY motifs, made up of glycine and predominantly proline and hydroxyproline (GPO sequences), which are the platelet pro-aggregants. As previously discussed, the major collagen agonist is the receptor GPIV, and in addition to GPIb, integrin αIIbβ3 (via VWF) and integrin α2β1 effects, these receptors function not only to intensify platelet responses, but to promote their adhesion properties, all in all to contribute significantly to platelet plug formation.

1.9.3.1.4. Epinephrine acts at the α2 receptor.

The platelet response to epinephrine is a useful marker of platelet reactivity. The adrenergic system is a key modulator of cardiovascular and homeostasis of organ function, with its responses heightened in times of stress. Early links between increased epinephrine levels associated with coronary artery disease and ‘fight-or-flight responses’, including surgical and emotional stress, pointed towards the potential pathogenic role of platelets. Studies have confirmed platelet aggregometry responses to epinephrine, with further studies linking adrenergic-platelet responses with the platelet α2a receptor. Epinephrine actions are G-protein coupled, resulting in inhibition of adenylate cyclase and activation of phospholipase C, therefore promoting intracellular calcium influx and rendering the platelet more vulnerable to additional agonist induced activation. However, while other agonists directly activate phospholipase C, epinephrine may only stimulate this enzyme indirectly. It is the direct interaction between the α2a-receptor and the Na+-H+ anti-porter, with the subsequent increase in phospholipase A₂ and AA
that has been proposed as a mediator of secondary wave of epinephrine-associated platelet aggregation\textsuperscript{239,240}.

Considering the diurnal fluctuation in catecholamines, and the potential for considerable unwelcomed in-vivo platelet aggregation in otherwise healthy individuals (e.g. during natural stress of exercise), there appears to be a protective mechanism by which platelets down-regulate responses to epinephrine in a dose and time dependent manner through reduced affinity for $\alpha_2$-receptors\textsuperscript{241}. Thus it appears epinephrine is not a prominent platelet agonist.

Pregnancy places huge demands on the cardiovascular system\textsuperscript{77} and adrenaline-related platelet actions may be useful in determining platelet functional changes in pregnancy. The diurnal variation in adrenaline release will be taken into account in the assessment of platelet reactivity to epinephrine, as all the subjects had blood taken at the same time every morning to limit this effect on platelet function results.

1.9.3.1.5. Thrombin- Receptor Activating Peptide (TRAP) is the active fragment of thrombin that stimulates the PAR receptor

Platelet-mediated thrombin formation is blocked by thrombin antagonists. As previously outlined PAR receptors have been shown to be involved in early placental development in murine models\textsuperscript{100,155}. Thrombin along with collagen is one of the major platelet pro-aggregants, and a key intermediate in the coagulation cascade\textsuperscript{242}. It is a serine protease generated at vascular injury sites and activates a variety of cells types, including platelets, endothelial cells, and leucocytes. As part of the coagulation cascade, activation of factor X (Xa) cleaves pro-thrombin (76,000kDa) to thrombin (32,000kDa), which in turn activates numerous factors and converts soluble fibrinogen into insoluble fibrin and thrombus. Thrombin activates platelets by binding the N terminus of cell membrane proteinase-activated receptors (PAR-1, PAR3, PAR4)\textsuperscript{243}. Unique to these receptors, unlike
other glycoprotein receptors, is that activation in turn leads to the unmasking of the N-terminal which remains attached as a ‘tethered ligand’ where it exerts its platelet agonist potency\textsuperscript{244,245}. PARs are also abundantly expressed on vessel walls, and therefore thrombin may exert an athero-thrombotic and inflammatory role in addition to its primary function in thrombus formation, through platelet and leucocyte recruitment and activation\textsuperscript{246}.

By developing synthetic peptides of thrombin (TRAP, derived from PAR-1 receptor), platelet aggregometry responses as opposed to gross thrombin-mediated aggregation may be assessed. Platelet reactivity to TRAP will be assessed in this thesis. The PAR receptors are G protein-coupled and function to activate platelets (G2) and contribute to platelet shape change (G13), by activating protein kinase C (increasing intracellular Ca\textsuperscript{2+}) and GTPaseRhoA respectively\textsuperscript{247}.

In the past few years a number of studies have emphasised the importance of platelet PAR-thrombin signalling not only in haemostasis, but also in inflammation, cancer and embryonic development\textsuperscript{100,155,248-250}. PAR4 has been implicated in placentation in mouse models\textsuperscript{100}. Most interestingly is the association of the PAR receptors and differential release of angiogenic material from platelet α-granules\textsuperscript{32,251}.

In addition to understanding of the biological role of these receptors, PARs have become a therapeutic target in an effort to inhibit platelet aggregation and thrombosis. Thrombin receptor antagonists (TRA), such as the PAR-1 inhibitors Vorapaxar and Atopaxar have been evaluated following primary coronary intervention, with promising reductions in adverse event rates without an increase in bleeding risk\textsuperscript{252-254}.

Traditional anti-thrombin agents with a long-standing efficacy profile in pregnancy include Warfarin and Heparin. Warfarin inhibits vitamin K dependent carboxylation of prothrombin. Heparin binds anti-thrombin III therefore inactivating thrombin and activated factor X (Xa)\textsuperscript{255}. However, due to a lack of fetal safety data with aforementioned newer class of antithrombotic agents, future studies should focus
on specific antithrombotic agents applied to more homogenous pregnancy groups complicated by placental-mediated conditions in an effort to significantly reduce adverse pregnancy outcome.

These agonists can be tested in methods of platelet aggregation. Some of these agonists have been tested before in pregnancy cohorts, but not all simultaneously. The problem is that a platelet aggregometry test should incorporate all of these agonists, however previous reports in pregnancy have described some but not all agonists and at maximal concentrations, which is not a true physiological assessment of platelet function. The tests that measure platelet aggregation will be described below.

1.9.3.2. Tests of agonist-induced platelet aggregometry

Platelet aggregation can be tested in blood samples of either platelet-rich plasma or whole blood, and variations of both methods have been investigated in pregnancy cohorts to date. A description of the methods of platelet aggregation will be discussed first, beginning with the principle of the assay that will specifically be tested in this thesis, followed by a literature review of the studies in pregnancy.

1.9.3.2.1. Light transmission platelet aggregation (LTA), using platelet-rich plasma (PRP)

Platelet aggregometry by turbidimetric methods was independently described by Born and O'Brien in 1962\textsuperscript{127,256}. It is considered a gold standard assessment of platelet function\textsuperscript{257}. It is an ex-vivo test, which assesses how platelets aggregate in response to soluble agonists. Whole blood is centrifuged to create platelet-rich plasma (PRP), which is then stirred, and differing platelet aggregometry responses
to each individual agonist, and at varying concentrations of each may be assessed by the amount of light transmitted by the aggregates (light-transmission aggregometry)\textsuperscript{258}. There have been a number of studies of versions of LTA in pregnancy and these will be discussed below.

Newer and novel techniques of LTA have evolved to incorporate multiple concentrations of a number of agonists to better represent the actual in-vivo platelet responses to a host of agonists in the circulation. Our research team recently developed a modification of LTA, which has been applied to a number of clinical populations\textsuperscript{33,34,203,229,259}. In a longitudinal study of healthy pregnancy compared with normal controls our research team recently found that platelet aggregation increased as pregnancy advanced, in response to multiple agonists\textsuperscript{203}. This modified version of LTA will be tested in this thesis. Spontaneous platelet aggregation in the absence of agonists will also be assessed using this technique.

1.9.3.2.2. Whole blood aggregometry

Whole blood impedance aggregometry uses the principal of electrical impedance. Agonists are attached to platinum electrodes which cause the platelets to stick to the electrodes. The amount of electrical impedance is calculated as a degree of adhering platelets\textsuperscript{260}. Results from earlier versions of this test were not consistent with those of LTA. Newer models, including the Multiplate platelet function analyser and the VerifyNow point of care test have evolved that are much easier to use and can be applied to a clinical setting, with good correlation with standard aggregometry assays\textsuperscript{173}. Most of the studies of whole blood aggregometry in pregnancy were undertaken decades ago and the more recent models have not been tested in pregnancy to date. The use of whole blood rather than PRP may give a closer reflection of actual platelet responses however this method is still not
physiological. A number of studies have assessed platelet aggregation using electrical impedance and these will be explored further later.

Some new developments in electrical impedance include the development of point of care tests that can be used in the clinical setting. These tests include the Multiplate and the Verifynow assays and are cartridge-based assays. The Multiplate assay is based on the principle of whole blood electrical impedance and has disposable cartridges and ready-to-use agonists. These tests have been shown to correlate well with standard LTA aggregometers, and have the ability to detect patients not responding to aspirin and who are otherwise at risk of major adverse cardiovascular events (MACE), and some studies have shown it can detect patients more likely to bleed post-operatively\textsuperscript{173,261}. The Verify now system is also a point of care (POC) test that assesses whole blood aggregation using a cartridge with beads coated with fibrinogen and agonists. It is a closed system and does not require any blood handling. Its correlation with clinical outcome is debatable.

1.9.4. Shear-induced platelet aggregation

An assay based on shear-induced platelet aggregation will be the final assay tested in this thesis. Recent advances in platelet function testing have taken into consideration the significance of circulatory shear force on the ability of platelets to function in-vivo. Platelets are exposed across a range of circulatory flow conditions, from low shear in venules ($<500\text{s}^{-1}$) to higher shear-force in arterioles ($5000\text{s}^{-1}$), continually surveysing the lining of the vessels (figure 1.10). Shear-force is created as a consequence of the relative parallel motion of blood flow planes within a vessel due to reduced velocity of blood flow at the vessel wall in contrast to the centre, where velocity is highest. Shear rate is expressed in cm/s per cm or inverse second ($s^{-1}$)\textsuperscript{262}. In the resting state, platelets will circulate in a quiescent form and will generally not interact with the vessel wall. Where platelets come into contact with exposed sub-endothelial ECM, they adhere to the site and exert their
effects all the while resisting the dynamics of even the highest shear rates (up to 40,000 s⁻¹ in stenosed arteries)²⁶³.

The concept of shear-flow induced platelet responses was incorporated into platelet function studies as early as the 1970’s²⁶⁴. The ability of VWF, fibrinogen and fibronectin to attract platelets under high-shear rates was demonstrated in the 1980’s²⁶⁵,²⁶⁶. Studies in the decade that followed incorporated improved imaging technology that enabled real-time visualisation of platelet interactions and formed the basis for subsequent shear-flow studies²⁶⁷,²⁶⁸.

The ability of platelets to withstand a system of highest shear, the arterial system, is a function of a range of factors resulting in the preferential binding of platelets to VWF under high flow²⁶⁹. VWF’s large structure (10,000kD) hosts an array of binding sites for collagen and platelets and has the ability to initially increase hydrodynamic drag of platelets from bulk blood flow²⁷⁰. With progressive increases in shear force VWF-GPIb interactions are positively modulated which in turn promotes integrin interactions with collagen and also integrin αIIbβ3-VWF binding, and subsequent stable adhesion²⁶⁷,²⁷¹.
Figure 1.10 Different platelet receptors function in the circulation at different shear rates. At high arterial shear rates, platelets interact with vascular endothelium that has exposed VWF, through platelet GPIb-VWF interactions. These interactions are unstable and platelets can roll along the surface in a stop-start mechanism. The final assay in the thesis, the dynamic platelet function assay (DPFA) will demonstrate this function. At lower venous shear rates, platelet aggregation appears to result in more stable platelet aggregation by platelets receptors GPIV binding to collagen. Thromboembolic disease occurs in the venous system.
Even within the highest shear conditions, it appears platelets may aggregate independently of agonist-induced mechanisms directly through GPIb-VWF interactions, mediated by immobilised and soluble forms of VWF as previously described. Aggregation in lower-shear, typically venous conditions, is thought to be primarily mediated by fibrinogen interaction with integrin αIIbβ3, competing with VWF and fibronectin due to its relatively high plasma molar ratio.

Platelet aggregation under the influence of shear-flow has been assessed by some researchers. Some of the earlier models assessed the effect of shear on gross platelet aggregation and clot formation (PFA-100 analyzer). Our research team recently developed a novel platelet function assay that is a parallel platelet flow chamber, that is capable of assessing the initial GPIb and later downstream platelet-vessel events under the influence of shear-force, and can give information not just on gross platelet aggregation but can detail how platelets roll, tether and eventually stick to a vascular surface within the circulation. In other words, this assay can measure the intimate platelet-vessel behaviours that are believed to greatly affect platelet function.

1.9.4.1. Assessment of shear-induced platelet aggregation

The Platelet-Function Analyser 100 (PFA-100) is an assay that assesses the time taken for whole blood samples under high shear flow to block a hole covered with a thrombogenic surface by forming platelet plug. A limited number of studies have assessed shear-flow mediated platelet aggregation in pregnancy. Blood is collected in citrate and then aspirated under high-shear flow conditions through a microscopic aperture that is coated with collagen. The PFA-100 assesses the ability of platelets to aggregate and form clot under arterial shear force over a highly thrombogenic surface. With the addition of the agonists: epinephrine or ADP, the time to form a platelet plug is measured and is reported as the closure time (CT). This test is useful in diagnosing gross bleeding disorders however it
may miss more subtle platelet function defects. Specific cell signalling mechanisms cannot be assessed in the way that agonist-induced aggregation can describe individual cell signalling mechanisms. The PFA-100 measures only a single parameter of platelet function: time to platelet plug formation, which limits its utility\textsuperscript{275}. The assay has poor sensitivity and specificity in monitoring anti-platelet therapy effects\textsuperscript{276,277}. There are a limited number of studies of the PFA-100 in normal pregnancy, gestational hypertension and PET\textsuperscript{114,115,278-282}.

Depending on whether VWF or collagen is used as the surface, different information on platelet responses can be determined. When a damaged vessel exposes its extracellular matrix, plasma VWF will become bound and will promote the tethering of platelets to the vascular surface under shear-flow by interacting with the platelet receptor GPI\textsubscript{b}269,270. These interactions are initial and reversible, and the net result will be platelet adhesion and activation. Collagen promotes stable platelet-vessel adhesion and aggregation\textsuperscript{234,273}. The initial and perhaps more subtle changes in platelet interactions with VWF are of specific interest because these changes may be associated with adverse cardiovascular events.

1.9.5. Novel methods of shear-induced platelet aggregation: an introduction to the final assay that will be tested in this thesis: dynamic platelet function on VWF surfaces:

Our research group has developed and characterized, using human blood samples, an assay that rapidly and reliably measures the interactions of platelets with VWF under arterial shear\textsuperscript{283,284}. The assay is a microfluidic chip that has VWF uniformly coated over its surface. Microliter samples of whole blood, labelled with fluorescent dye to identify the platelets, are aspirated across the surface at a defined arterial shear rate. Thus the VWF mimics a damaged vascular bed and the assay is capable of describing the interactions of the platelets with the surface, but under the normal arterial flow conditions the platelets would otherwise be exposed
to, as opposed to static in-vitro conditions. This assay will be the final tested in this thesis.

1.10. Literature review of platelet function testing in pregnancy

I will next provide an overview of results of platelet function studies in pregnancy to date that have described methods of (i) platelet adhesion, (ii) activation and (iii) aggregation. I will firstly describe results of spontaneous platelet adhesion and aggregation (SPA). This is the first assay that will be tested in this thesis. I will then provide a brief overview of the various studies that have investigated platelet activation in pregnancy, of which there are many varied methods and results described. I will follow on with a review of platelet aggregation studies in pregnancy. This will serve as a background to the second assay that will be tested; agonist-induced platelet aggregation. I will finally summarise a small number of platelet function studies in pregnancy is that have been based on shear-induced aggregation, as a background to the final assay that I will test in this thesis: the dynamic platelet function assay (DPFA).

1.10.1. Platelet function assay 1: spontaneous platelet adhesion and aggregation

This is a review of the limited previous studies of spontaneous platelet aggregation in pregnancy. Previous reports in pregnancy describe platelet counting techniques, whereas in this thesis I will investigate SPA using a modification of a standardised method of assessing platelet aggregation.
1.10.1.1. Spontaneous platelet adhesion and aggregation in normal pregnancy

There are some limited reports of spontaneous platelet aggregation (SPA) in normal pregnancy and to a lesser degree in complicated pregnancy. Different methods of assessing SPA were described. The interest in SPA in pregnancy arose when it became apparent that heparin led to platelet aggregation and thrombocytopenia, and the term heparin-induced thrombocytopenia (HIT) syndrome was coined. A number of early studies of spontaneous platelet aggregation in pregnancy focussed on the effect of the type of anticoagulant used on platelet results, rather than the effect on clinical outcome. It was established through these studies and similar studies outside of pregnancy that low molecular weight heparin (LMWH) was associated with a lesser degree of HIT, and this is the recommended anticoagulant of choice in pregnancy. A number of studies assessed SPA through platelet counting techniques, where SPA was calculated as the percentage fall in the number of platelets remaining after stirring, where different time-frames were specified in the studies. Methods using whole blood and PRP have been described. One study described whole blood electrical impedance.

Varying results of increased, decreased or no change in SPA in pregnancy were described. One recent study by a Japanese group evaluated SPA in twin versus singleton pregnancies and found significantly increased SPA in twin pregnancies in the second trimester compared with singleton pregnancies. The same research group also published on changes in SPA in 33 women in the first trimester compared with 11 non-pregnant controls using a platelet counting mechanism to determine the percentage fall in single platelets remaining in whole blood after stirring for up to 90 minutes. SPA was reported to be reduced in the first trimester of pregnancy compared with non-pregnant women.

Our research group recently undertook a longitudinal study of spontaneous platelet aggregation in pregnancy using a modification of the standardised test of platelet aggregation using light-transmission, and concluded that spontaneous...
platelet aggregation was also reduced in first trimester compared with non-pregnant controls. We also reported that SPA incrementally increased as pregnancy advanced\textsuperscript{291}. We suggested that spontaneous platelet aggregation could predict adverse pregnancy outcome in the same way that spontaneous platelet aggregation has been linked to adverse cardiovascular outcome.

1.10.1.2. Spontaneous platelet aggregation in hypertension in pregnancy

In one of the aforementioned studies of SPA in pregnancy, a small number of pregnancies complicated by hypertension were examined, and close interpretation of the results indicate that SPA was in fact reduced in pregnancies complicated by hypertension\textsuperscript{286}. There is a dearth of information in the literature regarding SPA in hypertensive disorders in pregnancy and IUGR. Most of the previous studies of SPA in pregnancy were undertaken a number of decades ago and it has not developed beyond this as a useful measure of platelet function. Also, the measures of testing SPA previously described were not standardised. A specific aim of this thesis is to investigate SPA in healthy pregnancies compared with pregnancies complicated by conditions related to utero-placental disease using a gold standard technique of assessing platelet aggregation. The relevance of SPA is that it may reflect platelet behaviour in-vivo.

1.10.2. Platelet activation

1.10.2.1. Platelet activation in normal pregnancy

Platelet activation can be assessed as a measurement of the expression of surface platelet activation markers or the change in platelet surface receptors\textsuperscript{173}. Studies of platelet activation in normal pregnancy to date described platelet activation using either assays based on flow cytometry, or assessments of measurements of platelet activation markers, such as P-selectin or β-thromboglobulin, released after platelet activation\textsuperscript{44,47,177-192}. 
In normal pregnancy, authors have reported inconsistent results of either increased, decreased or no change in platelet activation using flow cytometry methods in healthy pregnancy\textsuperscript{177-179}. Varying combinations of activation markers were described. A well-designed longitudinal study of platelet activation by soluble P-selectin (s-P selectin) levels in pregnancy by Holmes et al showed that levels remained stable throughout pregnancy but significantly higher levels of s-P-selectin levels were found in the second and third trimester compared with non-pregnant controls\textsuperscript{182}. It is worth noting however for this study there was a marked variation in P-selectin levels observed in the controls, and the authors suggested this may be explained by a possible seasonal variation in P-selectin levels, given the time-frame of the study. In the study by Gerbasi et al, increased levels of the platelet activation markers β-thromboglobulin and platelet factor 4 in normal pregnancy compared with non-pregnant controls were found\textsuperscript{180}. Overall results of platelet activation in healthy pregnancy are inconsistent.

1.10.2.2. Platelet activation in Gestational Hypertension and Pre-eclampsia

A limited number of studies examined platelet function in gestational hypertension as a separate entity from pre-eclampsia again with inconsistent results\textsuperscript{47,192}. The limitation is that most of the studies did not specify a group with non-proteinuric hypertension, rather included groups of pre-eclampsia for assessment. There appears to be more lines of evidence that consistently show that platelet activation is increased in pregnancies complicated by pre-eclampsia\textsuperscript{179,183,187-190}. Although two studies in the literature were identified that did not find any difference in PET compared with normal pregnancy controls for some or all of the platelet activation markers tested\textsuperscript{86,184}, and one study found platelet activation to be reduced in PET\textsuperscript{191}.

The difficulty in interpreting these studies is that no two studies examined the same platelet surface markers. Some authors remarked there was a significant variation in expression of different types of activation markers and it was concluded that platelet activation in PET was a feature of a subpopulation of
platelets, and not the total platelet population\textsuperscript{86,179}. The significant individual and seasonal variation in P-selectin levels have been documented for normal pregnancy\textsuperscript{47,182}.

The predictive value of different platelet activation markers for the subsequent development of PET was investigated in a number of longitudinal studies\textsuperscript{44,45,47}. A panel of activation markers were assessed in two studies\textsuperscript{44,45}, whereas a single marker, P-selectin in another study\textsuperscript{47}. For the study by the Dutch group, a total of 7\% of 244 women subsequently developed PET and it worth noting that the pregnancy group on whole included both low and high-risk pregnancies, and included insulin-dependent diabetics\textsuperscript{45}. Diabetes is associated with increased platelet reactivity\textsuperscript{35}. In the Dutch study, of the panel of markers assessed, only a single marker, CD63 expression (lysosomal platelet secretion) was predictive of PET, and when used in combination with assessment of maternal diastolic blood pressure, this increased the likelihood ratio from 1.94 to 9.4 (sensitivity 41\%, specificity 96\%). What is interesting is that for the patients that developed PET, increased expression of platelet activation was not a reproducible finding.

In the study by the Dublin group, of 70 pregnancies, 20 subsequently developed PET and 26 developed GH\textsuperscript{47}. Plasma P-selectin levels were significantly elevated in the first trimester in pregnancies that subsequently went on to develop PET (NPV 99\%). P-selectin was not found to be predictive of GH. According to the study protocol, 400 healthy primigravid patients were originally recruited, and it is unclear as to why such a large proportion of patients were excluded from the final analysis. It is likely a significant proportion of the final 70 patients were already at high risk of developing PET, given that over half of these developed subsequent hypertensive complications. Single biomarkers have not been identified as adequate predictors of PET in several large trials\textsuperscript{65,120-125}. 

100
Assessments of the thromboxane pathway in PET have found evidence for platelet activation, as denoted by increased T\(_{X2}\) and T\(_{X18}\)\(_{2}\). Additional studies have supported increased platelet activation in PET and GH with significantly increased levels of \(\beta\)-thromboglobulin\(^{188,192}\) and CD40\(^{190}\) (a platelet activation marker). These methods are indirect assessments of platelet activation.

1.10.2.3. Platelet activation in IUGR

There is some limited evidence that platelet activation may be occurring in both the mother and fetus in IUGR pregnancies. In a study of 20 mother-fetus pairs with IUGR compared with 15 uncomplicated pairs, platelet activation was assessed by flow-cytometry at delivery\(^{185}\). All cases were delivered by elective caesarean section and paired venous samples were collected in citrate from both the mothers immediately before delivery, and the fetuses (umbilical vein) immediately after delivery and before the third stage was complete. Platelet activation was found to be significantly increased in IUGR fetuses compared with appropriately grown fetuses (\(p=0.03\)), however it is worth pointing out that the complicated pregnancies were delivered at an earlier gestational age than the healthy pregnancies (33.1 compared with 38.5 weeks’ gestation, \(p=0.08\)).

The authors have also disclosed that 7 patients within the IUGR group has consumed aspirin at some time before delivery, however no differences were noted in platelet activation. Another study of 39 healthy pregnant women and their appropriately-grown fetuses undergoing fetal blood sampling at 18-37 weeks' gestation indicated that platelet activation was decreased in both mother and fetus compared with healthy controls, with platelet activation in the fetuses found to be less than that of their mothers\(^{186}\). Platelet activation may be decreased in the fetus compared to the mother but may be increased in IUGR fetuses.

In a study by Wallenburg et al, platelet activation was expressed as level of malonadialdehyde produced (this is the stable metabolite of T\(_{X2}\)) in a number of complicated pregnancies in the third trimester, including a group that were small-
for-gestational age (SGA). TXA₂ production was found to be elevated in SGA pregnancies, and further elevated when SGA occurred with gestational hypertension compared with normotensive pregnancies (6.2±1.4 and 6.6±1.8 compared with 5.3±0.9, p<0.01 respectively). In all groups, a significant correlation was found between MPV and platelet activation, suggesting larger, more activated platelets in IUGR\textsuperscript{187}.

The limitations of the techniques of assessing platelet activation, is that they give information on a \textit{limited} number of specific individual platelet signalling mechanisms, depending on the method tested, rather than an assessment of the actual ‘function’ of the platelets overall. These tests can help determine if platelets are activated, however what happens next is a feature of the platelet’s overall ability to respond to this activation.

### 1.10.3. Platelet function assay 2: agonist-induced platelet aggregation

This is a review of results of similar studies of platelet aggregation in pregnancy that have described methods similar to the methods of the second platelet function assay in this thesis. The assay that will be tested in this thesis describes the \textit{second} and \textit{third} steps of platelet function: the platelet’s overall response (aggregation) to activation.
Table 1.1 Studies of platelet aggregation or reactivity to agonists in healthy pregnancy have yielded inconsistent results.

<table>
<thead>
<tr>
<th>Results of studies of platelet reactivity in normal pregnancy</th>
<th>Increased</th>
<th>Decreased</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aggregometry using platelet-rich plasma (PRP)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whigam(^{197})</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Yamazaki(^{198})</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morrison(^{199})</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis(^{200})</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O’Brien(^{201})</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Sheu(^{202})</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burke(^{203})</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td><strong>Whole blood platelet aggregation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greer(^{204})</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Louden(^{205})</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrol(^{206})</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>
1.10.3.1. Agonist-induced aggregation in normal pregnancy

Specifically addressing platelet aggregation in pregnancy, there is some consensus that platelet aggregation increases as healthy pregnancy advances, although study methods differ somewhat (table 1.1). The general design of these studies is based on the addition of various combinations of platelet agonists to either platelet-rich plasma\textsuperscript{197-203} or whole blood\textsuperscript{204-206}.

1.10.3.1.1. Studies assessing platelet aggregation with PRP

Yamazaki et al compared agonist-induced aggregation in healthy pregnancy with non-pregnant controls in differing phases of the menstrual cycle and found increased platelet reactivity and subsequent aggregation to ADP and adrenaline, but not collagen, in pregnancy\textsuperscript{198}. Platelet aggregation appeared to increase as pregnancy advanced, however results were not found to be significant. A group of 42 non-pregnant females were compared with 20 healthy men and differences were noted in the females depending on menstrual cycle patterns, where menses was associated with a reduction in aggregation. The authors hypothesised that increases in oestrogen observed in pregnancy and the follicular phase of menstruation were triggers for platelet aggregation. Morrison et al found increased platelet reactivity to a single agonist, AA, in a small number of healthy pregnancies (n=14) compared with non-pregnant controls (n=10), with platelet reactivity increasing as healthy pregnancy advanced, and the change in platelet function was apparent as early as 16 weeks’ gestation\textsuperscript{199}.

Lewis et al examined ADP-induced platelet aggregation using a Payton channel aggregometer (a version of light-transmission aggregation), which determines the amount of light transmitted from radiolabelled platelets) and found that platelet reactivity to ADP was markedly increased in late pregnancy compared with earlier pregnancy gestations and non-pregnant groups\textsuperscript{200}. Prostacyclin levels at term were found to be almost double that of early pregnancy samples. Prostacyclin
(PGI$_2$) is a hormone available in abundance in pregnancy and is a potent inhibitor of platelet aggregation. It contributes to vasodilation and counteracts the vasoconstriction associated with thromboxane A2 (TXA$_2$). The authors suggested that that observed increase in prostacyclin in late pregnancy was an ‘adaptive’ response to increased platelet aggregation and thromboxane production. An imbalance in PGI$_2$-TXA$_2$ production, with the balance tipped towards increased TXA$_2$, is well documented in PET$^{23}$.

Sheu et al also examined platelet reactivity in healthy pregnancies compared with non-pregnant participants$^{202}$. A method similar to that described by Lewis et al (Payton lumi-aggregation) was employed, and for this study, rather than use PRP, washed platelets were examined for degree of platelet aggregation. Increased platelet aggregation in response to thrombin and ADP was observed in pregnant participants, alongside increased TXA$_2$ and reduced cAMP. TXA$_2$ mediates platelet aggregation and cAMP inhibits it. The draw-back to this study is a lack of clarity regarding study design and numbers within each group, in addition to a broad range of gestational age at entry to the study.

1.10.3.1.2. Whole blood aggregation

A number of investigators have examined platelet aggregation in whole blood using a platelet counter (thrombo-counter C), which identifies single platelets remaining after stimulation with an agonist as a percentage of the total number of platelets. This is a technique of assessing whole blood aggregation. Louden et assessed platelet reactivity using this technique in a longitudinal study of a small number of pregnancies and found increased reactivity to AA and Adrenaline at 32 weeks’ compared with non-pregnant controls, but no difference was noted for the other agonists tested (ADP, PAF, Collagen, U46619 (TXA$_2^2$))$^{205}$. Additional measures in this study included assessment of serotonin and thromboxane levels. Platelet secretion of serotonin (5HT) was assessed in tandem with platelet reactivity results, and increased levels of 5HT were observed at 32 weeks’ in response to
adrenaline alone. No differences were noted in TXB₂ results, unlike other studies that demonstrated increased levels of this platelet activation marker\textsuperscript{187}.

O’Brien et al also used the method described above of platelet counting with a thrombo-counter-C but PRP was examined instead of whole blood in response to a number of agonists. At odds with the aforementioned studies, normal pregnancy appeared to be associated with a reduction in platelet responsiveness to ADP, epinephrine and AA\textsuperscript{201}. It is worth noting that the normal pregnancy group, though recruited at a mean gestational age of 35 weeks', included some patients with threatened or early labour. This may have influenced the results, as inflammatory states can affect platelet activation. The control group consisted of men and women. Gender differences in platelet function are described\textsuperscript{76}. Greer et al also described the same method using whole blood and failed to detect differences in whole blood aggregation for healthy pregnancy compared with non-pregnant controls, however very small groups for analysis comprised this study\textsuperscript{204}.

Whigam et al compared platelet function in 11 non-pregnant volunteers compared with 11 healthy third trimester pregnancies in a study of platelet function in pre-eclampsia, and found no difference in platelet aggregation as assessed using the thombocounter\textsuperscript{197}. The small study numbers were likely to have contributed to a lack of statistical significance. Carrol et al assessed whole blood aggregation longitudinally in 187 pregnancies from the first through to third trimester using a method of whole blood electrical impedance and did not find any differences in collagen induced platelet aggregation for pregnant women compared with non-pregnant women\textsuperscript{206}.

1.10.3.2. Modification of light-transmission aggregometry: 96 well-plate

This is the specific platelet assay that will be assessed in the second results chapter of agonist-induced platelet aggregation. Our research team recently undertook a longitudinal study of platelet aggregation in normal pregnancy and the results of this study indicated that platelet aggregation was reduced in pregnancy
compared with non-pregnant controls, and within the pregnancy group platelet aggregation increased as pregnancy advanced\textsuperscript{203}. Platelet aggregation was assessed using an advanced modification of the gold standard light transmission aggregometry (LTA) assay.

\textbf{1.10.3.3. Platelet aggregation in Gestational Hypertension}

A small number of studies examined platelet function in gestational hypertension as distinct from pre-eclampsia. There are inconsistent results of no change\textsuperscript{201,211}, increased\textsuperscript{199,204} or decreased\textsuperscript{197,207-209} platelet aggregation in gestational hypertension compared with healthy pregnancy controls. Different agonists were tested and different methods of aggregation were described including methods with PRP\textsuperscript{197,199,201,207,208} and whole blood\textsuperscript{204,209,211}.

O'Brien et al examined PRP platelet aggregation to a range of agonists and did not find any differences in 23 patients with gestational hypertension\textsuperscript{201}. Nissel et al used whole blood impedance methods and failed to demonstrate significant differences in platelet aggregation in response to collagen for GH compared with normotensive pregnancies\textsuperscript{211}. In contrast to these studies, Morrison et al however noted increased aggregation to AA for GH, and this increased reactivity remained a feature at 6 week postnatal review\textsuperscript{199}. Greer et al, using the method of whole blood aggregation found increased to ADP in GH compared with normotensive pregnancies\textsuperscript{204}. Both of these studies tested only a single agonist however.

No differences were found in platelet responses to collagen, AA and ADP for GH in the study by Louden et al, although adrenaline-induced platelet aggregation was significantly less in the GH group compared with normotensive pregnancies\textsuperscript{209}. What is worth nothing is that half of this group were taking aspirin at the time of blood draw and 4 patients went on to develop PET, which may have influenced the significance of the results. Howie et al and Ahmed et al also reported reduced platelet aggregation in response to ADP and ADP and platelet-activating factor (PAF) respectively\textsuperscript{207,208}.
A collaborative group at Trinity College Dublin and St. James’ Hospital investigated platelet reactivity in hypertensive pregnancies further complicated by fetal growth restriction using whole blood aggregation and platelet aggregation was reported as the percentage fall in the number of platelets after the addition of agonist. Hypertension complicated by IUGR compared with normal pregnancy was associated with a 50% reduction in platelet aggregation in response to collagen and ADP (p<0.01). Higher levels of the platelet activation marker, β-thromboglobulin, was a feature of more severe disease, and it was suggested that the reduced platelet aggregation results for hypertension and IUGR was reflective of increased platelet activation. Increased levels of β-thromboglobulin in utero-placental disease have been supported in other studies. A specific aim of this thesis will be to evaluate platelet function changes in IUGR further complicated by hypertension compared with normotensive IUGR.

What might be perhaps relevant in the study of platelet function in pregnancy is the shear effect of arterial blood pressure on platelet behaviour, as a near-physiological or pathological representation of the actual milieu platelets are exposed to during normal and complicated pregnancy respectively. I aim to achieve a global picture of platelet function in pregnancy and utero-placental disease by firstly describing spontaneous platelet effects in PRP (spontaneous platelet aggregation), followed by the response of platelets to multiple agonists (agonist-induced aggregation), and finally, and possibly most clinically relevant and physiological (or pathological), the effect of platelet behaviour under shear-flow conditions (using whole blood in a parallel plate chamber in a vascular platelet assay).

As part of this thesis, platelet function in gestational hypertension will be investigated using physiological assays of the effect of shear-mediated flow on platelet behaviour in conditions that are designed to mimic the circulatory microenvironment in pregnancy. Assays that incorporate whole blood under flow conditions are considered to be more physiological than tests that examine
platelets in either platelet-rich or platelet-poor plasma, though as such these novel assays have yet to be standardised.

1.10.3.4. Platelet aggregation in Pre-eclampsia

The majority of studies of platelet aggregation in PET have cited reduced platelet responses to various agonists. One study concluded no change in whole blood platelet aggregation using a collagen-coated multi-plate assay of electrical impedance in PET compared with healthy pregnancy controls. This appears to be the only study of whole blood electrical impedance in PET in the literature. In light of these findings the authors challenged the ubiquitous use of aspirin in pregnancy; however they did support the concept that it remains largely unknown as to which women are likely to benefit from treatment. One study by an Austrian group found increased platelet aggregation in response to collagen, AA and ADP in a group of 85 women deemed high-risk for the development of utero-placental disease at a time-point before the development of PET, suggesting a predictive role for platelet function testing.

A reduction in platelet aggregation or responsiveness to a variety of agonists in PET has been cited by a number of investigators, however like the studies undertaken in gestational hypertension, these studies were also limited by their study design, definitions used for PET and the differing methods described. Methods of PRP or whole blood aggregation were described (table 1.2). In the study by Peracoli et al, the platelet growth factor TGF-beta was also assessed; this is a marker for platelet activation and was found to be significantly higher in PET compared with normotensive pregnancies. It could be that chronic platelet activation in PET may result in platelet desensitisation and subsequently reduced responsiveness.
1.10.3.5. Platelet aggregation in IUGR

There have been very limited studies of platelet aggregation in intrauterine growth restriction (IUGR)\textsuperscript{215,216}. Two studies have concluded platelet function is reduced in IUGR. Ahmed et al investigated platelet response to the agonist platelet activating factor (PAF) and reported higher concentrations of PAF were required to achieve maximal aggregation in IUGR compared with appropriately grown fetuses\textsuperscript{215}. Only 3 patients were tested in this study. The study by Norris et al has been described above in relation to whole blood aggregation in GH. Platelet reactivity to ADP and collagen was correlated with the platelet activation marker β-thromboglobulin, with findings of increased platelet activation and reduced platelet function as a feature of both normotensive and hypertensive IUGR pregnancies\textsuperscript{216}. When IUGR was further complicated by hypertension, platelets were 50\% less responsive to agonist stimulation in the third trimester compared with uncomplicated pregnancies (p<0.001). Recalling the study by Trudinger et al, where platelet activation assessed with flow cytometry was increased in IUGR fetuses compared with appropriately-grown fetuses, it could be that platelet activation is increased in IUGR however platelet aggregation may be reduced\textsuperscript{185}. In conclusion there are some limited studies of platelet aggregation in IUGR and further larger studies are required.

1.10.4. Platelet function assay 3: shear-induced platelet aggregation

A small number of studies in pregnancy have used assays based on the principle of shear-flow\textsuperscript{278-282}. Two studies assessed aspirin response in pregnancy using a PFA-100 analyser\textsuperscript{114,115}. The following will briefly outline those studies as an introduction to the final assay that will be tested in this thesis; the vascular assay based on shear-flow mediated platelet adhesion, activation and aggregation.
Table 1.2 Studies of platelet aggregation suggest reduced platelet aggregation in PET.

<table>
<thead>
<tr>
<th>PRP assessments of aggregation</th>
<th>Whole blood assessment of platelet aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peracoli\textsuperscript{213}</td>
<td></td>
</tr>
<tr>
<td>O’Brien\textsuperscript{201}</td>
<td></td>
</tr>
<tr>
<td>Whigam\textsuperscript{197}</td>
<td></td>
</tr>
<tr>
<td>Can\textsuperscript{214}</td>
<td></td>
</tr>
<tr>
<td>Felferig-Boehm\textsuperscript{46}</td>
<td></td>
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<tr>
<td>Splawinska\textsuperscript{212}</td>
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<tr>
<td>Norris\textsuperscript{210}</td>
<td></td>
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<tr>
<td>Louden\textsuperscript{209}</td>
<td></td>
</tr>
<tr>
<td>Nissel\textsuperscript{211}</td>
<td></td>
</tr>
</tbody>
</table>
1.10.4.1. Normal pregnancy

The methods of the PFA-100 analyser have already been outlined. In brief, the PFA-100 is a method of assessing the time to platelet clot formation (closure time, CT) when whole blood is aspirated under high shear force over an activated surface. It measures gross clotting time and can be useful in diagnosing bleeding disorders. Using the PFA-100 analyzer, CT has been shown to be shorter in pregnancy compared with the non-pregnant state, which suggests increased coagulation in pregnancy\textsuperscript{278,280}.

A recent study of the effect of shear-flow on platelet-collagen interaction in pregnancy was assessed using a microfluidic assay. Significantly reduced adhesion to collagen and firm platelet-platelet interactions with fibrinogen binding was found in healthy third trimester pregnancies compared with non-pregnant controls\textsuperscript{292}. The authors suggested, in light of the increased thrombotic tendency in pregnancy, this reduced response to collagen might be a ‘normal’ physiological response or an adaptive mechanism in pregnancy to prevent unwanted thrombus formation. The shear-flow assay that will be tested in this thesis describes platelet behaviour on VWF under arterial shear which is more physiologically relevant at high-shear rate than collagen.

1.10.4.2. Pre-eclampsia

In a study comparing PFA-100 and thrombo-elastography (TEG) results for pregnancies complicated by PET to a large number of healthy controls, there was a greater degree of closure time (CT) in PET, which was more pronounced than TEG assessments, suggesting a possible impairment in coagulation and a tendency to bleeding in pregnancy complications\textsuperscript{282}. In the study of assessment of closure time (CT) using the PFA-100, Davies et al found the mean CT was significantly prolonged in severe PET compared with healthy controls, indicating a possible tendency to bleeding for PET\textsuperscript{279}. TEG times, as denoted by the maximum
amplitude (MA) of clot formation were not found to be significantly different based on degree of severity of PET. This finding was also reproduced by Vincelot et al.\textsuperscript{278}. A further study demonstrated prolonged CT in patients with GH\textsuperscript{281}. These studies did not correlate CT results with actual blood loss at delivery or rate of PPH, which would have been a useful correlation for the clinical utility of such tests.

1.10.4.3. Aspirin response in pregnancy

Limited studies of aspirin response in pregnancy were identified using the PFA-100 analyzer\textsuperscript{114,115}. While it was concluded that individualised dosing regimens based on aspirin response in pregnancy were promising, the interpretation of these results is problematic as the PFA-100 is not an accepted measure of aspirin response.

To the best of our knowledge there are no reports of shear-flow induced aggregation in pregnancies complicated by IUGR. While the PFA-100 analyser is a shear-flow based test of platelet function, is measures gross clotting time and under very high shear rates. The assay that will comprise the final assay of this thesis is measuring the initial, intermediary and final stages of platelet function under arterial shear stress, and gives a range of different platelet parameter and outputs that have never been tested before in utero-placental disease.
1.11. Summary of studies of platelet function in healthy pregnancy and pregnancies complicated by utero-placental disease

Physiological pregnancy is associated with changes in haematological indices, including a reduction in platelet count and an apparent increase in MPV. While many different facets of platelet function have been assessed in normal pregnancy, it is fair to say there appears to be some functional difference in platelets in pregnancy compared with the non-pregnant form. Studies conflict and vary significantly in design, and for the large part consist of non-standardised methods of assessing platelet function, thus it is difficult to conclude any clinically relevant information from this available data.

Our understanding of the platelet functional changes in pregnancy and diseased states in pregnancy is limited by the wide variation in results of such studies to date; studies that have assessed various different methods of platelet function. There is also no clear guidance as to the dose of aspirin to give in pregnancy. Platelet function tests have now evolved into more physiological assessments of in-vivo platelet behaviour compared with the static in-vitro assessments previously described. There is a rationale for further platelet function testing in pregnancy.

A reappraisal of platelet function in pregnancy and complicated pregnancies is proposed, not least to better understand the contribution of platelets to the condition, but to also understand why some patients appear to not respond to anti-platelet agents in pregnancy. The key to achieving this will be in the future development of point-of-care tests of platelet function in pregnancy that can be implemented across large prospective studies of platelet function in pregnancy.
1.12. Aims and hypothesis:

The aim of this thesis is to evaluate platelet function using different assays of platelet function in common placental-mediated disorders in pregnancy. The hypothesis is that altered platelet behaviour occurs in conditions mediated by placental disease.

1.11.1 PRIMARY OBJECTIVE:

The primary objective of this PhD is to investigate platelet behaviour in established utero-placental disease using three platelet function assays:

1. Spontaneous platelet aggregation

2. Agonist-induced aggregation

3. Shear-induced dynamic platelet adhesion, activation and aggregation.

1.11.2 SECONDARY OBJECTIVE:

As a secondary objective, the results of multiple platelet parameters will be correlated with clinical outcomes to better describe platelet function changes in pregnancy based on clinical phenotype. A number of sub-analyses will be performed based on severity of disease to determine if platelet function is altered based on clinical presentation on the spectrum of utero-placental disease.

Placental histopathology will be performed and will be correlated with platelet function assay results. To the best of my knowledge this has not been investigated to date.
Chapter 2. **Materials and Methods**
2.1. Study Organisation

My thesis was undertaken at the Rotunda Hospital and the Cardiovascular Biology Research Laboratory, Royal College of Surgeons in Ireland (RCSI), St. Stephen’s Green under the supervision of Professor Fergal Malone and Professor Dermot Kenny.

The Rotunda Hospital is the primary site for the Perinatal Ireland Research Consortium. The recruitment of patients for my PhD thesis was undertaken at the Rotunda Hospital, under the supervision of my lead clinical supervisor, Professor Fergal Malone. The Rotunda Hospital has a birth rate of approximately 9,000 births per annum and this clinical site was well placed for the anticipation of a steady-stream of suitable patients for recruitment to the various platelet function studies that comprised the work of this thesis. The RCSI Department of Obstetrics and Gynecology at the Rotunda Hospital provided the office space, IT systems, and support staff for the study to be undertaken. The day-to-day execution of my thesis was performed with the help of the Perinatal Ireland research manager, Dr Elizabeth Tully who chaired regular departmental research meetings at the Rotunda Hospital. The principal biostatistician for Perinatal Ireland, Patrick Dicker, provided assistance for the statistical analysis of the platelet function studies. Statisticians from the Irish Centre for High-End Computing (ICHEC) provided in-depth tracking analysis assistance and software for the novel dynamic platelet assay.

Professor Dermot Kenny has an extensive background in the area of platelet biology and translational clinical research. I attended and presented at regular departmental laboratory meetings that were held in RCSI with input from a wide range of highly-skilled scientists with experience in platelet biology and the development of industrial point-of-care devices. The principal research site for this study was located at the Cardiovascular Biology Research Laboratory located in the Department of Molecular and Cellular Therapeutics (MCT), RCSI. The
department is an alliance of biochemistry and clinical pharmacology with the focus on understanding the molecular aspects of pathology and its impact in translational medicine. Experiments were predominately undertaken at this primary research site, and a number of pilot experiments were also undertaken at the affiliated research centre in Beaumont Hospital and the Biomedical Diagnostics Institute (BDI) at Dublin City University (DCU) (figure 2.1). The BDI is a Science Foundation Ireland CSET (Centre for Science, Engineering and Technology) committed to the development of cutting-edge research programmes by supporting translational medicine through advances in diagnostic devices and point-of-care solutions (figure 2.2). The BDI mantra is to support these major developments in technology which may impact significantly on diagnosis and tailored management of disease.

The main aim of this thesis was to characterise platelet function in pregnancies complicated by utero-placental disease compared to uncomplicated healthy pregnancies using three different platelet function assays. Following on from the initial work of this thesis, the future aim is the development of point-of-care diagnostic or predictive tools that will assist in the diagnosis and treatment of pregnancy complications. The development of point-of-care platelet and coagulation devices for use within the Irish Maternity health care system may positively impact antenatal care by way of early prediction and management of serious obstetric-related complications.
Figure 2.1 Study design and research collaboration groups

Clinical research site

Perinatal Ireland

Scientific research sites

Molecular and Cellular Therapeutics
Royal College of Surgeons in Ireland,
123 St. Stephen's Green
Dublin 2.
Figure 2.2: An overview of the study design of this thesis including the integration of the clinical and biological platelet function data and analysis with the prospect of future development of point-of-care testing and technologies to assist in high-risk pregnancy screening and management (adapted from the BDI Ireland website).
2.2. Study Design

2.2.1. Recruitment of patients

This programme of work comprised a prospective observational study of platelet function using a range of standardised and novel platelet function assays in pregnancy. Pregnancies complicated by utero-placental disease were compared to normal pregnancy controls. A number of clinical outcomes, as detailed further below, were also recorded and later correlated with platelet function results.

Patients were recruited at the clinical site, the Rotunda Hospital. Patients with singleton pregnancies with utero-placental disease (namely pre-eclampsia (PET), gestational Hypertension (GH), or intrauterine Growth Restriction (IUGR)) detected from 24 weeks’ gestation and beyond were selected and invited to participate in the study. Patients with PET or GH were recruited from the Rotunda Hospital Day Care Unit or as inpatients. The identification of patients with IUGR was facilitated through the Rotunda Hospital Fetal Assessment Unit (FAU). A number of cases of IUGR requiring inpatient surveillance were also recruited as they became available throughout the study time-line.

Cases of prenatally detected IUGR, included pregnancies with an EFW <10th centile; based on sonographic measurements of fetal bi-parietal diameter (BPD), head circumference (HC), abdominal circumference (AC), and femur length (FL) (Hadlock 4). Only accurately dated pregnancies were eligible for inclusion i.e. where a dating ultrasound was performed prior to 14 weeks’ gestation (as estimated by crown rump length, CRL). The presence of abnormal umbilical artery Doppler (UAD) waveform at the time of blood draw was also recorded. Cases of IUGR due to congenital infection and aneuploidy were not included in the study.
To confirm that all IUGR study patients had a true diagnosis of pathological IUGR, patients were followed up at delivery to confirm that birth weight was <10\textsuperscript{th} centile, and in addition, placental histopathology was performed. Thus, the selection of cases of IUGR for the purpose of the platelet function analysis included all participants with a prenatal diagnosis of EFW <10\textsuperscript{th} centile and with subsequent confirmation of birth weight <10\textsuperscript{th} centile, together with histological evidence of utero-placental insufficiency. In those cases where an initial EFW <10\textsuperscript{th} centile was noted at time of antenatal enrolment, but where a birth weight >10\textsuperscript{th} centile was achieved in addition to normal placental histopathology, these were excluded from the IUGR cohort for the purpose of the primary analysis, and were analysed separately (IUGR, birth weight >10\textsuperscript{th} centile).

Consecutive healthy third trimester-matched controls with an appropriately-grown fetus were also recruited for comparison with the test groups. Pregnancy controls were generally recruited as outpatients and some were recruited as inpatients with uncomplicated pregnancies (e.g. awaiting elective caesarean section, unstable lie). All participants were provided a patient information leaflet (appendix 1.1) and all participating patients provided written informed consent (appendix 1.2). Each patient was given the option of retaining a copy of the patient information leaflet.

A specific protocol as outlined below was strictly adhered to (appendix 2.1). Following consent, a single blood sample for platelet function analysis for the various platelet function studies was obtained. All cases were followed up for pregnancy and delivery outcome. Additional obstetric outcomes, such as the subsequent development of hypertensive disorders, were also recorded.
2.2.2. Definitions

For the purpose of this study, the following standard definitions were applied.

2.2.2.1. Utero-placental disease:

Utero-placental disease or placental insufficiency is a term encompassing conditions thought to relate directly to defective placentation\textsuperscript{1-3}. Although the exact pathogenesis is unknown, the precipitating event is thought to be inadequate maternal spiral artery invasion during trophoblastic invasion in early pregnancy\textsuperscript{3}. Both hypertensive disorders in pregnancy, namely PET and GH along-side fetal growth restriction are associated with defective placentation with common features of chronic utero-placental insufficiency evident in the placenta\textsuperscript{1,3}. Although reduced placental perfusion unites these entities, it is usually and most commonly in cases of PET that a maternal syndrome occurs. IUGR is thought to be the ‘fetal’ syndrome of defective placentation. Clinical manifestations of these conditions can appear abruptly and early in the course of the pregnancy\textsuperscript{37}. Occasionally these conditions are superimposed in pregnancy, usually resulting in severe early-onset disease. Placental interrogation will distinguish common and some unique findings for the conditions.

2.2.2.2. Preeclampsia:

Pre-eclampsia is diagnosed as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg in a previously normotensive pregnant woman who is ≥20 weeks’ gestation and has proteinuria >300mg/24 hours\textsuperscript{6}.

2.2.2.3. Gestational hypertension:

Gestational hypertension is defined as new onset hypertension of >140/90 from 20 weeks’ gestation in a previously normotensive pregnancy without proteinuria\textsuperscript{6}.

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2.2.2.4. Intra-uterine growth restriction:

The American Congress of Obstetricians and Gynaecologists (ACOG) guidelines currently define IUGR as a fetus with an estimated weight <10th centile for gestational age. EFW was based on sonographic measurements of fetal bi-parietal diameter (BPD), head circumference (HC), abdominal circumference (AC), and femur length (FL) (Hadlock 4)⁸.

2.2.3. Eligibility criteria

Eligibility criteria were singleton pregnancies from 24+0 weeks’ gestation with a diagnosis of PET, GH or IUGR. Uncomplicated healthy third trimester singleton controls were consecutively selected and were followed up to ensure normal outcome and birth weight at delivery. Only accurately dated pregnancies were eligible for inclusion i.e. where a dating ultrasound was performed prior to 14 weeks’ gestation (as estimated by crown rump length, CRL).

2.2.3.1. Exclusion criteria

The following is a list of exclusion criteria. Criteria were selected on the basis of a theoretical or known effect on platelet function⁶⁸-⁷⁵. Smoking was not an exclusion criterion as it is thought to contribute significantly to placental disease, but smoking status was recorded for later evaluation as a potential confounding factor. Given the findings of one study which suggested Betamethasone administration in pregnancy may affect platelet function²⁹⁴, blood sampling was generally avoided in cases where corticosteroids had been administered for fetal lung maturity within the previous 48 hours. Timing of corticosteroid use was noted and later analysed.
2.2.3.1.1. Maternal characteristics

- Pre-existing maternal medical conditions (e.g. systemic lupus erythematosus, renal disease, thromboembolic disease)
- Known platelet function disorders (Bernard Soulier Syndrome)
- Thrombocytopenia, spherocytosis, Sickle cell anaemia
- History of unexplained recurrent miscarriage or thrombophilia
- Diabetes mellitus (including diet-controlled DM)
- Pre-labour premature rupture of membranes
- Suspected or confirmed maternal sepsis
- Use of non-steroidal anti-inflammatories, aspirin or anticoagulation therapy within 10 days of blood draw
- Administration of l-thyroxine, anti-thyroid medication, selective serotonin reuptake inhibitors (SSRI) (or other antidepressants), or progesterone

2.2.3.1.2. Fetal characteristics

- Multiple gestation
- Known fetal abnormality (structural or genetic)
- Congenital infection (confirmed either pre or postnatally)
2.3. Study procedure

2.3.1. Phlebotomy

For each of the platelet function assays (figure 2.3) described in detail (appendix 2.1), a specific protocol for phlebotomy and blood sample preparation was adhered to. The aim of this procedure was to avoid any inadvertent platelet aggregation and clumping that may hinder platelet function results. I personally performed all of the recruitment and phlebotomy on all recruited subjects. After informed consent, patients were asked to fast from midnight and blood sampling was generally performed in the morning, with platelet analysis occurring within 60 minutes of blood draw. A single blood draw was obtained using a 19-gauge butterfly needle with an un-cuffed technique to prevent platelet clumping. The initial 5ml of blood was discarded and the subsequent 5ml was analysed to confirm a normal platelet count. The un-cuffed technique ensured the free-flow of venous blood and the avoidance of shear stresses that may contribute to platelet aggregation.

After the initial 10ml was drawn from the butterfly needle a further sample of venous blood was obtained for the platelet function testing. For the spontaneous platelet function and light-transmission aggregometry assays, a further 27ml of blood was drawn. For the dynamic platelet function assay only a further 10ml was required. If blood draw proved challenging (whereby the sample did not flow freely or where air aspiration and bubbles occurred) the sample was discarded and repeated. The syringe was gently rocked back and forth to ensure adequate exposure of the sample to the citrate, and the sample was left to rest for approximately 15-30 minutes before preparation for the various platelet function assays.
Figure 2.3 Study Design of the three platelet function assays that will be tested in this thesis in Utero-placental disease compared with healthy pregnant controls
2.3.2. Platelet assay 1: Spontaneous Platelet Aggregation

This first assay described is an assay of spontaneous platelet aggregation (SPA), that is, platelet aggregation without the addition of agonists. As outlined in the introduction, platelets can aggregate together when gently stirred. In brief, SPA was calculated as the amount of light transmitted from platelets that had aggregated together over the specified assay time-frame. A formula was used to calculate the degree of platelet aggregation from the start to the finish.

A summary is presented here, and a more detailed study explanation is outlined in appendix 2.1. After the first 10ml of blood was drawn, a 30ml syringe, pre-treated with 3ml 3.2% sodium citrate (one tenth), was then attached to the needle, and a further 27ml of blood was carefully collected with minimal agitation. The blood was rocked gently to enable adequate mixing of the citrate and the whole blood. The blood sample was then centrifuged for 10 minutes to obtain platelet-rich plasma (PRP) for platelet function testing. The sample was initially transferred in 5ml aliquots into 6 polyethylene test-tubes which were tilted to ensure blood ran from the top of the tube to the base, and therefore each of the test tubes had equivalent measures with minimal sample agitation. Pipettes were used to correct any discrepancies in volumes. The test-tubes were placed in a balanced and reproducible manner inside the centrifuge, where the samples were spun at 150g for 10 minutes. After this, the test-tubes were removed and placed in a holding rack.

Following centrifuging, platelet rich plasma (PRP) was obtained. A further 1ml of the PRP was then spun again to produce platelet-poor-plasma (PPP). SPA was tested using the same 96 well plate used for the agonist-induced aggregation assay, however for measuring SPA only a portion of the plate was required, because this test did not require multiple doses of agonists. PRP and PPP were added to 4 wells each in one column of the plate.
2.3.2.1. Method for calculating spontaneous platelet aggregation

Spontaneous platelet aggregation (SPA) was calculated by converting the raw optical values obtained using a macro excel conversion file. I manually calculated the average SPA values for each patient at two time points: at the start of the assay run (0 minutes, SPA T0) and at the end of the assay run (18 minutes, SPA T18).

The mean SPA was calculated using the following formula:

\[ \{1-(SPA \text{ T18}/ \text{ SPA T0})\} \times 100, \text{ where} \]

SPA Time 0 = average PRP (the average of the values at time 0 for D6, C6, B6, A6) – average PPP (the average of the values for H6, G6, F6, E6).

SPA Time 18 = average PRP (the average of the values at time 18 for D6, C6, B6, A6) – average PPP (the average of the values for H6, G6, F6, E6).

In principle, the mean SPA for each patient was calculated based on the difference in spontaneous platelet aggregation from time 0 to time 18 minutes truly due to platelets (i.e. PRP-PPP). The overall mean SPA for each of the test groups was calculated and was compared to the results for the control group.
2.3.3. Platelet assay 2: Agonist-induced Platelet Aggregation

The second assay tested in this thesis was an assay of agonist-induced platelet aggregation. Platelet aggregation was assessed in response to 8 increasing concentrations of five agonists simultaneously. This was assessed on a plate consisting of 96 wells designed to incorporate the agonists and samples. The same method used to calculate SPA was opted, where the amount of light transmitted from platelets that had aggregated together following agonist-stimulation was obtained and a specified formula was used to calculate the degree of platelet aggregation from the start to the finish.

2.3.3.1. Preparation of the 96-well plates:

The 96 well-plate was used to test each blood sample. The plate consisted of 96 polystyrene wells (12 columns of 8) that accommodated the agonists and the control solution (figure 2.3). The 96 wells within the plate were designed to accommodate the various submaximal concentrations of the 5 test agonists used in this assay: arachidonic acid (AA), collagen (Col), adenosine diphosphate (ADP), epinephrine (Epi), and thrombin receptor-activating protein (TRAP). The 96-well plates were prepared before blood sampling is undertaken, and each well, once fully prepared contained the exact concentration of each of the agonists that were tested. Prior to the addition of each of the agonists, an artificial solution of salts, dextrose and pH buffers (JNL) was added to deionised water to obtain a physiological pH of 7.35 before it was added to each of the wells. This solution best replicates the physiological in-vivo environment for platelets and acted as a buffer solution.
The preparation of agonists and the method used to transfer the various concentrations of agonists by reverse pipetting is outlined in appendix 2.1.

The final incremental concentrations of the agonists added to the plates were 500, 375, 188, 83.8, 46.9, 23.4, 11.8, 5.86 µ/ml for AA; 190, 143, 71.3, 35.6, 17.8, 8.9, 4.45, 2.23 µg/ml for Col; 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µM for ADP and TRAP; and 20, 5, 1.25, 0.313, 0.078, 0.0195, 0.00488, 0.00122 µM for Epi. The agonists were added according to a specific protocol of reverse pipetting (appendix 2.1).

2.3.3.2. Measuring agonist-induced platelet aggregation:

The venous blood sample was initially centrifuged as described already. Starting with an initial sample of at least 8.6ml of PRP, 1ml of PRP was then spun to produce PPP. 180 µl of PRP was then added promptly to all of the wells by reverse pipetting, except for 4 wells, H6-E6, which contained the PPP as a control. The 96-well plate was then placed immediately in the Wallac plate-reader set to a programme at 3-9-15-18 (minutes). A 572 nm filter measured the amount of light absorbed as the plate was rotated constantly at 1000 r.p.m. through a 0.1 mm orbit in between measurements. The total time for full sample testing took approximately 25 minutes, after which the 96-well plate was then read using a Victor 3 Multilabel plate reader (Perkin Elmer, Wellesley, MA). This assay therefore comprised a modification of standard light transmission aggregometry, where the light absorbance values were normalised based on the PRP and PPP control absorbance values, representing 0 and 100% aggregation. In order words, the calculation was based on the difference in aggregation of the PRP minus the aggregation caused by PPP (i.e. aggregation that may be due to other blood components).
JNL buffer and agonists: AA, Col, ADP, Epi, TRAP

Platelet rich plasma (PRP)

96-well plate

Victor 3TH Multilabel plate reader

Optical Density readout @ 0.3, 9, 15, 18 mins

% Platelet Aggregation

Figure 2.4 Method for testing agonist-induced platelet aggregation (Platelet Assay 2). Buffer solution (JNL) and various concentrations of agonists: arachidonic acid (AA), collagen (Col), adenosine di-phosphate (ADP), epinephrine (Epi) and thrombin-receptor activating peptide (TRAP) are first added to the 96-well plate by reverse pipetting. After centrifuging the blood sample, platelet-rich plasma (PRP) is then added to the 96-well plate containing the buffer and agonists. The plate is placed in the Victor Multilabel plate reader set at 37 degrees and spun at orbital rotation. After 20 minutes an optical density read-out is obtained at the times specified. These raw values are the amount of light absorbed by platelet aggregates and are converted using a conversion file and a pre-set equation to the percentage platelet aggregation.
Each sample was labelled and filed appropriately (e.g. for IUGR sample 1, the raw platelet data output was stored as ‘IU01’ to enable compatibility with Wallac transforming data system). All data was stored as raw values in light absorption units, which were then converted into % platelet aggregation using the equation:

\[
\% \text{aggregation} = 100 \times \frac{(\text{PRP} - \text{Well})}{(\text{PRP} - \text{PPP})}
\]

The log values of the agonists were then used to calculate dose-response curves using GraphPad Prism software (GraphPad Prism©, San Diego, CA).

The T1/2 (or half-maximal effective concentration (EC\text{50}) values were further generated from the dose-response curves. The EC\text{50} is the concentration of an agent which induces a response or reaction at the halfway point between baseline and maximum response after a specified time-frame. It is a useful method of analysing dose-response in relation to time for a given agent and is used regularly to assess pharmacological response (figure 2.4).

![Dose-response curve](image)

**Figure 2.5** The EC\text{50} represents the concentration of agonist required to provoke a response halfway between the baseline and maximum platelet aggregation responses (reproduced from the GraphPad Prism© website)\textsuperscript{285}. In this figure the data is normalised without subtracting the baseline of 20%, therefore the EC\text{50} in this example is the concentration of agonist that evokes a response of approximately 60% (halfway between 20% and 100%).
2.3.4. Platelet assay 3: Dynamic Platelet Function Assay

This is the final assay that will be tested in this thesis. The rationale for this assay is to mimic actual in-vivo platelet interactions in the arterial circulation. Platelets tether to VWF via the platelet GPIb receptor, which induces signalling and platelet activation that leads to activation of the platelet GPIIb/IIIa receptor and subsequent adhesion on VWF. These initial ‘up-stream’ platelet behaviours ultimately result in subsequent ‘down-stream’ platelet aggregation. The full operational protocol for the dynamic platelet assay is described in detail in appendix 2.2. A novel parallel plate flow chamber coated with purified human VWF is used along with a sophisticated custom-designed platelet tracking software. A camera captures and quantifies the platelet-VWF interactions, under physiological arterial shear-flow and is a better physiological representation of the actual in-vivo platelet effects. Platelets can be visualised initially tethering to the VWF surface, rolling along the surface or sticking to it. Some platelets roll along the surface; some platelets are very adhesive and can be seen sticking firmly to the surface; some platelets can roll along the surface in a ‘stop-start’ way. Using this system, platelets translocating across VWF under the influence of arterial-shear can be captured in real-time and a comprehensive analysis and tracking of the platelets is achieved.

The novel platelet tracking algorithm accurately identified individual platelets in each frame (figure 2.5). Platelet movement from frame to frame was then tracked and recorded giving multiple parameters of platelet translocation behaviour that related to the biological activity of platelets in vivo (table 2.1). The assay determined the numbers of platelets that interacted with VWF (platelet tracks), the number of platelets that translocated on the surface (platelet translocation), the velocity of platelet translocation, the number of platelets that stably adhered to the surface (static platelets), the number of platelets that interacted but did not adhere to the surface (unstable platelets), and finally the percentage of platelets that remained on the VWF surface at the final frame (percentage of platelet surface coverage). These are the six platelet parameters defined using the DPFA.
Figure 2.6: Schematic representation of the dynamic platelet function assay (DPFA) with custom designed platelet tracking software (reproduced from Lincoln 2010). (A) Whole blood is perfused over the von Willebrand factor (VWF) surface at arterial shear after the sample has been fluorescently labelled, and platelet interactions with the VWF are captured using an Andor camera. (B) Images of platelet interactions with VWF are captured at 30 frames a second for a total of 500 frames. (C) Each platelet is identified on each frame and the platelet is tracked from one frame to the next. (D) The movement of the platelet on VWF is represented as platelet tracks. These platelets tracks are used to generate multiple parameters of platelet behaviour.
A list of experimental reagents and experimental equipment for the DPFA is available in appendix 2.2.

2.3.4.1. Key outputs/platelet parameters of DPFA

The key outputs we get from our analysis and their definitions are summarised in the table below with further reference in the appendix 2.2.

**Table 2.1 Definitions of the various platelet parameters assessed in the dynamic platelet function assay**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet tracks</td>
<td>The number of platelets that interact with VWF</td>
</tr>
<tr>
<td>Platelet translocation</td>
<td>The number of platelets that travel &gt; 1.5 times the average radius of the platelets</td>
</tr>
<tr>
<td>Velocity</td>
<td>Speed a translocating platelet moves in the direction of flow (µM/sec)</td>
</tr>
<tr>
<td>Static platelets</td>
<td>The number of platelets that travel &lt;1.5 times the average radius of the platelet</td>
</tr>
<tr>
<td>Unstable platelet interactions</td>
<td>Where a platelet interacts with VWF for a period of &gt;10 frames but &lt;490 frames.</td>
</tr>
<tr>
<td>% platelet surface coverage (end)</td>
<td>The percentage platelet surface coverage on the final frame (frame 500)</td>
</tr>
</tbody>
</table>
2.3.5. Definitions of platelet parameters tested with the DPFA

1. **Platelet tracks**

   The total numbers of platelets that interact with VWF over a period of 500 frames.

2. **Number of translocating platelets**

   This is the number of platelets that are translocating over 500 frames; our preliminary data suggests that this parameter is different between normal healthy volunteers and patients with cardiovascular disease on dual antiplatelet therapy.

3. **Velocity.**

   This is the average velocity of all the platelets in the direction of flow over 500 frames. This value tends to be relatively uniform as it is a function of the pump used in the assay in that the platelets translocate at the maximum flow rate set by the pump.

4. **The number of stably adhered platelets.**

   A stably adhered platelet is defined as a platelet that has not moved more than 1.5 times its diameter over the image acquisition time of 500 frames. This number is generated over 500 frames.

5. **Unstable platelet interactions**

   Platelets that interact with VWF for a period that is greater than 10 frames but less than 490 frames.

6. **Percentage surface coverage**

   At frame 500 this is defined as the percentage of the visible surface covered by adhered platelets in the imaged area relative to the background. This is a relatively standard measurement used by many groups.
2.4. Data collection

The data collection sheet and details recorded for case subjects at enrolment are outlined in Appendix 1.3. The details recorded included standard maternal demographic details, previous obstetric history, and details pertaining to the current pregnancy including gestational age at diagnosis of PET, GH or IUGR, and any additional complications. The timing of administration of corticosteroids was also recorded, and patients were not enrolled in the study where corticosteroids had been administered within the previous 48 hours. In addition, platelet count, haematocrit and blood group was recorded at the time of blood draw. Samples were not included in the final analysis if the platelet count was <100x10⁹/L. All pregnancies were followed up and delivery details obtained included birth weight, mode of delivery and estimated blood loss. Placental histopathology was generally performed in most cases of utero-placental insufficiency.

All participants were assigned a unique study number that was coded for analysis. The key for the code and the data collection sheets remain with the lead researcher in a secure location in a locked cupboard at the RCSI unit, the Rotunda Hospital. The database is password protected. All data will be maintained for 5 years after the final publication as per ethical requirements and will be destroyed after this time by the lead researcher.
2.5. Statistical analysis

2.5.1. Demographic data

Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5).

2.5.2. Platelet function analysis

The specific statistical analysis applied to each of the platelet assays are detailed in each of the results section. Platelet aggregometry data were analysed using specific modelling approaches. The analysis for the final platelet assay, the dynamic platelet assay required specialised software and with an inbuilt tracking analysis and algorithm for platelet racking.
2.6. Ethical approval

Ethical approval was obtained from the Rotunda Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Participants were provided with written information prior to informed consent and blood draw, and each patient was given the option of retaining a copy of the patient information leaflet.

2.7. Funding

Funding was provided by the RCSI CLAT scheme and the Rotunda Foundation.

Figure 2.7 The Rotunda Foundation logo (reproduced from the Rotunda Hospital website).
Chapter 3.  **Results of Spontaneous Platelet Aggregation**
3.1. Concise summary

Platelets can adhere and aggregate together spontaneously when gently stirred. This phenomenon is known as spontaneous platelet aggregation (SPA) and has been identified as a feature in some individuals at risk of cardiovascular disease. In this study I found the degree of SPA to be significantly greater in healthy normal pregnancies compared to pregnancies complicated by utero-placental disease. Thus SPA may be a marker for disease.
3.2. Introduction

As outlined in the introduction to the work of this thesis, pregnancy complications may be associated with changes in platelet behaviour. There are a number of different ways of assessing platelet function, and many varied techniques have been described in the literature in pregnancy cohorts to date. As already described, measures of platelet function can include an assessment of the release of platelet granule content or the change in platelet membrane proteins (platelet activation), or the response of platelets to a stimulus or agonist (platelet ‘reactivity’ or platelet aggregation).

In our blood, platelets generally circulate as separate particles. When obtaining a whole blood sample using the standard method of anticoagulation, citrate, this property of the platelet should be maintained and platelets should not aggregate. Platelets can ‘spontaneously’ adhere and aggregate together when gently stirred. Spontaneous platelet aggregation is a feature that does not appear to require an initial stimulus or agonist, but may represent a platelet aggregation effect. The clinical importance of this finding is that it has been associated with increased mortality in cardiovascular patients. A limited number of studies have assessed spontaneous platelet aggregation in pregnancy.

Spontaneous platelet aggregation was first described by O’Brien et al in 1966 where it was noted that platelet rich plasma (PRP) could demonstrate some degree of platelet aggregation simply after stirring the sample at 37°C in citrate. Spontaneous platelet aggregation was assessed using an aggregometer, where the optical density of the platelet aggregates was obtained by light-transmission aggregometry. An adaption of the method described in 1966 was adopted in a further study by Friedlander 1971 who investigated spontaneous platelet aggregation in 244 healthy volunteers and patients at risk of cardiovascular disease. Optical density read-outs of platelet aggregation were assessed at zero and ten minutes, where spontaneous platelet aggregation was considered to
have occurred if there was a change in the optical density of 20 units or more after ten minutes. A number of platelet agonists were also tested to determine platelet reactivity. Spontaneous platelet aggregation was identified in 8% of all patients at risk of major cardiovascular adverse outcomes (MACE), and this was a reproducible finding in a total of 80% of these participants. Another study by Trip et al published some decades later investigated SPA in 149 survivors of infarction and found that the SPA-positive group (which was determined in this study as SPA occurring within 10 minutes) had a relative risk of death of 5.4 (95%CI, 2.2-13.4) after 5 years\textsuperscript{172}.

In the study by Friedlander et al factors that may have affected or explained spontaneous platelet aggregation results were also examined, and no correlation was found between age, gender, type of anticoagulation used or platelet count and platelet aggregation results\textsuperscript{168}. Although one study did find that age, haematocrit level and platelet count can significantly affect SPA results\textsuperscript{170}. Each of these variables can change in pregnancy, and it may well be that spontaneous platelet aggregation is either affected by, or reflects changes in haematological indices in healthy pregnancy, with further changes in pregnancy-related complications. Different studies have assessed SPA at different time intervals, but most studies have used a cut-off of about 20 minutes. When samples were subjected to longer time intervals (2 hours) this was actually found to result in platelet disaggregation\textsuperscript{168}.

Spontaneous platelet aggregation was found to be associated clinically in two case reports of peripheral vascular disease associated with thrombocytopenia\textsuperscript{296,297}. When the patients in question were treated with antiplatelet agent aspirin, the peripheral ischaemia was noted to be reversible\textsuperscript{297}. In patients with metastatic cancer and diabetes, spontaneous platelet aggregation has also been shown to be significantly increased\textsuperscript{33,298}. Pregnancy is a diabetogenic state, and is also associated with significant changes in the cardiovascular system, and it is possible that this may affect spontaneous platelet aggregation, in the same way that these conditions appear to result in platelet
functional changes in conditions outside of pregnancy. A familial occurrence of SPA has also been described\textsuperscript{299}. A familial link is a feature of pre-eclampsia where the relative risk of developing the condition in pregnancy is 3 fold where a first degree family member has previously been affected\textsuperscript{6}.

In two early longitudinal studies in pregnancy, spontaneous platelet aggregation was found to be increased compared with non-pregnant participants\textsuperscript{285,286}. The research interest of these early studies was the effect of the type of anticoagulant used, rather than a pregnancy-effect per say, on platelet aggregation results, given earlier reports showing that SPA was dependent on the type of anticoagulant used\textsuperscript{300,301}. One of the first such studies in pregnancy identified in the literature was by a group in Queens Medical Centre, University Hospital, Nottingham in 1985\textsuperscript{286}. The group demonstrated a marked increase in spontaneous platelet aggregation in whole blood in a longitudinal study in 46 pregnancies compared with 10 non-pregnant women. Spontaneous platelet aggregation was found to be increased in normal pregnancy (n=14), pregnancy with essential hypertension (n=11) and gestational hypertension (n=21), returning to normal within 6 weeks postpartum. SPA was examined in whole blood containing either heparin or citrate. Samples obtained in heparin were found to be associated with significantly increased SPA compared with those of citrate. The mechanism proposed for this increased platelet aggregation in pregnancy was the effect of heparin rather than citrate on aggregation results.

A serious side-effect of heparin is heparin-induced thrombocytopenia syndrome (HITS). Heparin is a highly charged sulphate molecule and can bind to charged epitopes on the platelet membrane, resulting in platelet dense granule release and aggregation\textsuperscript{300}. Unfractionated heparin has been found to enhance the rate at which ADP is liberated from red blood cells and platelets\textsuperscript{302}. Pregnancy is also associated with ADP leucocyte-platelet interactions\textsuperscript{303,304} and a possible ‘dual-effect’ may explain the results of increased spontaneous platelet aggregation when heparin is used pregnancy. Low-molecular weight heparin (LMWH) is associated with a lesser degree of heparin-platelet binding and HITS syndrome,
and has a well-established safety profile in pregnancy, and is therefore currently the anticoagulant of choice for pregnant patients at risk of thromboembolic disease. It is possible the mechanism behind the thrombocytopenia of HITS is related to heparin-induced ADP potentiation of platelet aggregation and subsequent thrombocytopenia\textsuperscript{304}.

Regarding the theme of the potential influence of the anticoagulant agent on platelet aggregation results, the second study in pregnancy by Fox et al investigated spontaneous platelet aggregation longitudinally in healthy pregnancies compared with non-pregnant volunteers, comparing results with both citrate and hirudin (the most potent natural inhibitor of thrombin which is obtained from medicinal leeches)\textsuperscript{285}. Spontaneous platelet aggregation was again found to be increased in normal pregnancy compared with non-pregnant controls regardless of whether citrate or hirudin was used. Platelet reactivity to adrenaline was found to be enhanced in pregnancy. The authors concluded that platelet reactivity was increased in pregnancy and this could impact on the complications of pregnancy related to long-term cardiovascular disease.

In a study by Hof et al, whole blood impedance and platelet counting mechanisms were used to compare SPA of 120 healthy pregnancy women to 22 healthy non-pregnant women and found no differences in SPA between the two groups\textsuperscript{287}. It is worth noting however that the non-pregnant group were taking oral contraceptives at the time of the study. A hormonal influence on platelet function has been described\textsuperscript{198,203}.

In a further study by Ajayi et al, the effect of LMWH on both spontaneous platelet aggregation and ADP-platelet reactivity was investigated in third trimester pregnancies\textsuperscript{288}. No difference was found in heparin-induced SPA in pregnancy compared with non-pregnant controls for LMWH, however when unfractionated heparin was used as the anticoagulant, like the findings of previous studies, this was found to be associated with significantly increased platelet aggregation (p<0.01). An ADP inhibitor, apyrase grade II (ADP scavenger) was found to inhibit
the effects of heparin-induced platelet aggregation and HIT in pregnancy, lending further weight to the suggestion that heparin-platelet interactions are enhanced in pregnancy.

A recent study of spontaneous platelet aggregation in first trimester of 33 pregnancies compared with 11 non-pregnant females was described by a Japanese research group, where SPA was found to be reduced in the first trimester of pregnancy. It is unclear from this study how the healthy pregnant women were selected or what their clinical outcomes were, although there were no differences in age or BMI between the two groups289.

The same group also examined SPA in singleton versus twin pregnancies290. The research question was whether gestational thrombocytopenia, which is observed more frequently in multiple versus singleton pregnancies, could be related to degree of platelet reactivity and pregnancy outcome. Spontaneous platelet aggregation in this study was found to increase over time during sample processing, and was found to be proportional to increasing gestation in a total of 59 singleton pregnancies and 17 sets of twins (chorionicity was not specified in the study). Platelet aggregates were found to be significantly increased in the 2nd trimester of twin pregnancies compared with gestation-matched singleton pregnancies. The suggestion was made that based on the increased placental mass in twin pregnancies this may be the main ‘location’ for the increased platelet reactivity observed in twins for this study. Placenta volumetric assessment or histopathological correlations with platelet aggregation were not available in this publication. This study lends further weight to a potential relationship between platelet activation and aggregation and gestational thrombocytopenia. For this thesis I will examine platelet weight and placental histopathological results in relation to different platelet functions in the final results chapter.

Our research group recently undertook a longitudinal study of spontaneous platelet aggregation in healthy pregnancy and found that platelet aggregation was reduced in first trimester compared with non-pregnant controls291. Spontaneous
aggregation was then found to incrementally increase as pregnancy advanced. The preliminary results suggested that spontaneous platelet aggregation could predict adverse pregnancy outcome in the same way that spontaneous platelet aggregation has been linked to adverse cardiovascular outcome. The literature review of spontaneous platelet aggregation in pregnancy is limited to a few studies largely undertaken a couple of decades ago, and it is difficult to clean a consensus given the differing types of anticoagulation used during sample preparation. Nonetheless an interesting concept of the relationship between platelet function and pregnancy, with indirect evidence to suggest a role for platelet function in adverse pregnancy outcome, has emerged.

Given the dearth of information in the literature regarding spontaneous platelet aggregation in complicated pregnancies, as the next logical step, I sought to investigate spontaneous platelet aggregation in healthy pregnancies compared with pregnancies complicated by utero-placental disease. The hypothesis is that given the similarities between pregnancies complicated by conditions related to placental ischaemia and cardiovascular disease, alongside the association between spontaneous platelet aggregation and generalised pathologies, that SPA would be different in healthy pregnancy compared with pregnancies complicated by utero-placental disease. Also, haematological changes in pregnancy, including a reduction in platelet count and haematocrit may further affect spontaneous platelet aggregation, and that this phenomenon may in part explain a mechanism for a worsening gestational thrombocytopenia in pre-eclampsia. Finally, given the limited information regarding the effect of confounding variables on platelet aggregation in pregnancy, a specific aim of this study was to investigate the effect a number of maternal variables on the spontaneous platelet aggregation results.
3.3. Study Design

The general study design is outlined in the materials and methods chapter. Over the course of two years (2012-2014) a total of 150 pregnant patients were recruited to participate in a study of spontaneous platelet aggregation in pregnancy at the Rotunda Hospital. Platelet function in third trimester pregnancies complicated by utero-placental disease was compared to those of gestation-matched normal pregnant controls.

3.4. Statistical analysis for Spontaneous platelet aggregation

The study was powered (80%) to detect an 8% change in spontaneous platelet aggregation for pregnancies complicated by utero-placental disease. Comparisons between spontaneous platelet aggregation assessments for the three complicated pregnancy groups were made individually with the third trimester healthy pregnancy results using Mann Whitney two tailed t tests (SPSS Version 22® and Graph Pad Prism Version 6.0®). Categorical data was analysed using Chi-square and correlations between spontaneous platelet aggregation results and maternal variables were analysed using Pearson’s correlation.
3.5. Results: Spontaneous Platelet Aggregation in pregnancies complicated by utero-placental disease compared with normal pregnancies

3.5.1. Clinical results

A total number of 150 patients were recruited for this study. In 31 cases these did not fulfil the strict inclusion criteria, resulting in the final numbers for analysis of 34 healthy pregnancy third trimester controls compared with 44 patients with prenatally detected IUGR, 14 patients with GH and 27 patients with PET (figure 3.1). Demographic data are represented at the end of the chapter in table 3.1.
Figure 3.1 Study Design of the patients recruited for the study of spontaneous platelet aggregation (SPA) and the total numbers excluded from the final analysis.
3.5.2. **Spontaneous platelet aggregation is reduced in utero-placental disease compared with normal pregnancy controls**

The mean values (± standard error of the mean, SEM) for spontaneous platelet aggregation (SPA) results in healthy pregnancy compared with utero-placental disease are represented in figure 3.2. The mean value of SPA for healthy third trimester pregnancies was 6.7±1.4% SEM (95% CI 3.7-9.6). This was found to be increased compared to the three pregnancy groups complicated by utero-placental disease with significance obtained for the IUGR group: GH 2.7 ±1.5% SEM (95% CI -0.5 to 5.99); PET 2.7 ± 1.2% SEM (95% CI 0.21 to 5.12) and IUGR 1.8 ±0.8% SEM (95% CI -0.5 to 2.8).

Results of the mean spontaneous platelet aggregation for pregnancies complicated by utero-placental disease were compared individually with the results of the healthy third trimester group. As the data were not normally distributed, comparisons for continuous data were made using Mann-Whitney two-tailed analysis. Overall, spontaneous platelet aggregation appeared to be reduced in each of the complicated pregnancies compared with healthy pregnancy controls. Reductions in SPA for GH and PET were similar but were not found to be significant (p=0.18, p=0.11 respectively). However results were highly significant for a reduction in SPA in IUGR pregnancies compared with normal pregnancy controls (p=0.002).
Spontaneous platelet aggregation is reduced in Utero-placental Disease compared with healthy third trimester pregnancies.

Figure 3.2 Spontaneous platelet aggregation in healthy third trimester pregnancies (n=34), is increased compared to pregnancies complicated by utero-placental disease (gestational hypertension (GH, n=14, p=0.18), pre-eclampsia (PET, n=27, p=0.11) or intrauterine growth restriction (IUGR, n=44, p=0.002). Comparisons between the groups were made using Mann Whitney two-tailed t independent test for non-normal data.
Spontaneous platelet aggregation is reduced in Gestational Hypertension compared with normotensive third trimester pregnancies.

Figure 3.3 Spontaneous platelet aggregation in gestational hypertension (GH, n=14) is reduced compared with healthy normotensive third trimester pregnancies (n=34), (2.7 ±1.5% SEM (95% CI -0.5 to 5.99) versus 6.7±1.4% SEM (95% CI 3.7-9.6) respectively, p=0.18, n/s). Comparisons between the two groups were made using Mann Whitney two-tailed t independent test for non-normal data.
Spontaneous platelet aggregation is reduced in Pre-eclampsia compared with healthy third trimester pregnancies

Figure 3.4. Spontaneous platelet aggregation in pre-eclampsia (PET, n=27) is reduced compared with healthy normotensive third trimester pregnancies (n=34) (2.7 ± 1.2% SEM, (95% CI 0.21 to 5.12) versus 6.7±1.4% SEM (95% CI 3.7-9.6) respectively, p=0.11, n/s). Comparisons between the two groups were made using Mann Whitney two-tailed t independent test for non-normal data.
Spontaneous platelet aggregation is significantly reduced in Intra-uterine Growth restriction compared with normotensive third trimester pregnancies.

Figure 3.5. Spontaneous platelet aggregation in fetal growth restriction (IUGR, n=44) is significantly reduced compared with healthy normotensive third trimester pregnancies (n=34), (1.8 ±0.8% SEM (95% CI -0.5 to 2.8) versus 6.7±1.4% SEM (95% CI 3.7-9.6) respectively, p=0.002). Comparisons between the two groups were made using Mann Whitney two-tailed t independent test for non-normal data.
Spontaneous platelet aggregation in healthy pregnancy is increased compared with utero-placental disease. SPA appears to be reduced in complicated pregnancies. The reason for this is unclear. I next hypothesised whether maternal haematological and demographic variables may have impacted on the results of platelet aggregation. I performed a number of correlations of clinical results with spontaneous platelet function assay results (table 3.2). I was specifically interested in determining if any maternal factors (e.g. smoking) could explain the significant results obtained for platelet function in pregnancies with IUGR.

Referring to the demographic data available at the end of this chapter, no differences were noted for ethnicity or parity, or any corticosteroid use (table 3.1). Interestingly, although only a total of 16 patients were taking antihypertensive medications at the time of blood draw, no differences were noted between cases of GH/PET that were treated medically and cases that were managed expectantly.

In keeping with the literature, I did find a trend towards a positive correlation between increasing BMI and SPA, although this was not found to be significant ($r=0.13$, 95% CI -0.05-0.3), $p=0.15$) (figure 3.7). When analysing the differences in the means between all smokers (n=16) and non-smokers (n=103), significantly less mean spontaneous platelet aggregation was noted for those who were smoking at the time of blood draw compared with non-smokers (-0.87 compared with 4.09, $p=0.007$, figure 3.8), though the small number of smokers overall must be noted.
Table 3.1 The effect of maternal characteristics on Spontaneous Platelet Aggregation (SPA). Pearson’s correlation (r, with p value) was used for comparisons between continuous data sets (r values >0.7 indicated positive correlations, r<0.7 indicated negative correlations). Mann Whitney independent sample’s two-tailed t-test was used for differences in mean SPA between grouping maternal variables. There appears to be a positive correlation between increasing BMI and SPA* (n/s) and a negative correlation between MPV and SPA** (r=−0.2, p=0.04). Mean SPA appears to be significantly less in smokers¥ (n=16) compared with non-smokers (n=103), p=0.007.

<table>
<thead>
<tr>
<th>Maternal characteristic (n=119)</th>
<th>Average/Percentage of the whole sample</th>
<th>Spontaneous Platelet Aggregation Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>30 (17-44)</td>
<td>r=−0.19 (CI -0.2-0.7), p=0.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.5 (16-45)</td>
<td>r=0.13(95% CI -0.05-0.3), p=0.15*</td>
</tr>
<tr>
<td>Ethnicity (Caucasian)</td>
<td>101 (85%)</td>
<td>p=0.59</td>
</tr>
<tr>
<td>Smoker</td>
<td>16(13%)</td>
<td>p=0.007¥</td>
</tr>
<tr>
<td>Steroids</td>
<td>43(36%)</td>
<td>p=0.52</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>53(45%)</td>
<td>p=0.48</td>
</tr>
<tr>
<td>Multiparous</td>
<td>56(55%)</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>31(26%)</td>
<td>p=0.76</td>
</tr>
<tr>
<td>Phlebotomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet count</td>
<td>239 (107-406)</td>
<td>r=−0.04 (95% CI -0.14-0.22) , p=0.69</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.35(0.29-0.43)</td>
<td>r=−0.004(95% CI -0.18-0.18), p=0.96</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>9.7(4.3-14.8)</td>
<td>r=−0.2 (95% CI -0.35- -0.009), p=0.04**</td>
</tr>
</tbody>
</table>
There is a positive correlation between BMI and Spontaneous Platelet Aggregation (n/s, n=119).

Figure 3.6 There is a non-significant trend towards increasing BMI and spontaneous platelet aggregation ($r=0.13$, $p=0.15$, $n=119$). Pearson’s correlation was used for correlations between two groups of continuous data.
Smoking is associated with significantly reduced Spontaneous Platelet Aggregation.

Figure 3.7 Smokers (n=16) have significantly less spontaneous platelet aggregation than non-smokers (n=103), p=0.007, Mann Whitney two-tailed t test was used for comparing non-normal SPA data between the two groups.
Regarding maternal haematological indices obtained at the time of platelet function testing, I next assessed whether platelet count, haematocrit (hct) and mean platelet volume (MPV) were independently associated with spontaneous platelet aggregation. Pearson’s correlation was used to examine if there were positive or negative linear associations between groups of continuous variables.

Although, as expected the platelet count was significantly less in pregnancies complicated by PET compared with normotensive pregnancies (211± 65 versus 250± 45 respectively, p=0.007), overall platelet count values did not appear to be predictive of spontaneous platelet aggregation results for the group as a whole (n=119), (r=0.05 (05% CI -0.144-0.215), p=0.69) (figure 3.8). A correlation was not identified either between hct and spontaneous platelet aggregation (figure 3.9).
There is no correlation between Platelet Count and Spontaneous Platelet Aggregation (n=119)

![Graph showing no correlation between platelet count and spontaneous platelet aggregation](image)

Figure 3.8 There is no correlation between platelet count and spontaneous platelet aggregation (r=0.04 p=0.69, n=119). Pearson’s correlation was used to compare continuous data.
There is no correlation between Haematocrit and Spontaneous Platelet Aggregation (n=119)

Figure 3.9 There is no correlation between haematocrit (hct) and spontaneous platelet aggregation ($r=0.004$, $p=0.96$, $n=119$). Pearson’s correlation was used to determine an association between two sets of continuous data.
Although a lack of an association was found between platelet count, hct and spontaneous platelet aggregation, the volume of platelets appeared to correlate better with aggregation results. Platelet volumetric analysis in the context of spontaneous platelet aggregation revealed that mean platelet volume (MPV) was negatively associated with platelet aggregation ($r=-0.2$ 95% CI $-0.35$-$0.009$, $p=0.04$) (**figure 3.10**). That is, higher MPV is associated with lowered spontaneous platelet aggregation. The mean MPV for the group on whole was 9.7 (range 4.3-1.8), broken down by MPV for healthy pregnancy 8.4± 1.7, GH 11.05±2.2 (p=0.004); PET 10.37±2.0 (p=0.001), and IUGR 9.9±1.9 (p=0.006). Although significantly higher MPV values were found in pregnancies complicated by differing phenotypes of utero-placental disease compared with healthy pregnancies, overall significance in SPA was only noted between the IUGR and healthy pregnancy groups.

This result implies that platelets with a higher mean platelet volume (in other words, ‘newer’, larger or more immature platelets) in the maternal circulation of pregnancies complicated by fetal growth restriction are associated with reduced platelet aggregation, and that MPV does not appear to significantly affect results in hypertensive disorders. The lack of significance between higher MPV values in GH and PET compared with healthy pregnancy and spontaneous platelet aggregation results imply that MPV alone is not responsible for the observed platelet dysfunction.
There is a negative correlation between Mean Platelet Volume and Spontaneous Platelet Aggregation (r=-0.2, p=0.04, n=119)

Figure 3.10. There is a negative correlation between MPV and spontaneous platelet aggregation (SPA). MPV has been shown to be increased in pregnancies complicated by utero-placental disease; a negative correlation was found between increasing MPV and spontaneous platelet aggregation (SPA) (r=-0.2 p=0.04, n=119). Although overall, higher mean MPV values were found in pregnancies complicated by gestational hypertension (GH) and pre-eclampsia (PET), this did not appear to correlate with SPA for these groups. Pearson’s correlation was used to determine correlations between two sets of continuous data.
Although previous investigators have demonstrated an association between age and haematocrit level\textsuperscript{170} and spontaneous platelet aggregation, this study could not concur with these findings. I did however find that increasing BMI had a slight, though non-significant, positive association with spontaneous platelet aggregation. Given that patients in the GH and PET groups compared with healthy pregnancies had higher BMI’s (p=0.01 and p=0.03 respectively), this may have explained, in part, the lack of significance in the observed reduction in spontaneous platelet aggregation in these groups. In other words, a subpopulation of platelets in obese patients with hypertensive disease may have a tendency to increased platelet aggregation. Indeed there were some outliers with increasing SPA values for the hypertensive groups.

In summary, the main compounding variable affecting spontaneous platelet aggregation could be smoking status. Although previous investigators have found that smoking was not associated with significant differences in SPA\textsuperscript{172}, for this study it was noted that for the pregnancy study group as a whole (n=119), smoking was associated with significant reductions in spontaneous platelet aggregation. The small number of smokers in this study (n=16) needs to be taken into account when extrapolating statistical conclusions. It may be that smoking in pregnancy is associated with pregnancy-specific changes in platelet aggregation responses? What is worth noting is that although the total number of patients that smoked within our study population was only 16, ten of these comprised the IUGR group. Smoking is significantly associated with IUGR.

To further investigate the significance of smoking status within the IUGR group as a whole (44), a sub-group analysis was subsequently performed. When compared with non-smokers (n=33), patients who admitted to smoking in the context of a pregnancy complicated by fetal growth restriction (n=10) surprisingly did not appear to have any significant differences in mean spontaneous platelet aggregation results (2.2 (95% CI -0.63-3.8) compared with -0.3 (95% CI -2.8-2.2) respectively, p=0.22). It can be deduced that the significant results of reduced spontaneous platelet aggregation for pregnancies with IUGR is not explained by
smoking alone, and that perhaps some other plasma factor is contributing to the platelet dysfunction in IUGR.

In conclusion, I have assessed platelet aggregation using a simple assay of spontaneous platelet aggregation and have found that aggregation is increased in healthy pregnancy and is reduced in pregnancies complicated by utero-placental disease. There does not appear to be a clear correlation with maternal demographic or clinical features apart from smoking status. SPA may be a useful marker for identifying utero-placental disease.
Table 3.2 Patient demographic data for Platelet Function Assay 1: Spontaneous Platelet Aggregation (SPA), demonstrating some differences between the healthy and complicated pregnancy groups. *IUGR estimated fetal weight <10th centile. † Normal Control: pregnancy controls consisted of women in the third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. ¥ Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ‡ Mode of delivery (MOD): p value quoted for any difference in delivery between groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control † Healthy 3rd trimester (n=34)</th>
<th>IUGR* (n=44)</th>
<th>p-value</th>
<th>Gestational Hypertension (n=14)</th>
<th>p-value</th>
<th>Pre-eclampsia (n=27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>32 ± 4</td>
<td>28 ± 6</td>
<td>&lt;0.001</td>
<td>31±6</td>
<td>0.39</td>
<td>31±74</td>
<td>0.26</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 4</td>
<td>25 ± 6</td>
<td>0.30</td>
<td>27±4</td>
<td>0.01</td>
<td>26±36</td>
<td>0.03</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>32 (94%)</td>
<td>35(80%)</td>
<td>0.06</td>
<td>14(100%)</td>
<td>0.49</td>
<td>20(74%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Smoker</td>
<td>0</td>
<td>10 (23%)</td>
<td>0.002</td>
<td>3(21%)</td>
<td>0.03</td>
<td>3(11%)</td>
<td>0.03¥</td>
</tr>
<tr>
<td>Steroids</td>
<td>0</td>
<td>31(70%)</td>
<td>&lt;0.001</td>
<td>3(21%)¥</td>
<td>0.21</td>
<td>9(33%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>11(34%)</td>
<td>20(46%)</td>
<td>0.241</td>
<td>9(64%)</td>
<td>0.041</td>
<td>13(48%)</td>
<td>0.210</td>
</tr>
<tr>
<td>Multiparous</td>
<td>23(76%)</td>
<td>24(56%)</td>
<td></td>
<td>5(36%)</td>
<td></td>
<td>14(52%)</td>
<td></td>
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<td>History</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Utero-placental disease</td>
<td>0%</td>
<td>12(27%)</td>
<td>&lt;0.001</td>
<td>1(7%)</td>
<td>__</td>
<td>9(33%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chronic HTN</td>
<td>0%</td>
<td>1(2%)</td>
<td>__</td>
<td>1(7%)</td>
<td>__</td>
<td>3(11%)</td>
<td>0.08¥</td>
</tr>
<tr>
<td>Parameter</td>
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<td>IUGR* (n=44)</td>
<td>p-value</td>
<td>Gestational Hypertension (n=14)</td>
<td>p-value</td>
<td>Pre-eclampsia (n=27)</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------------</td>
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<td>---------</td>
<td>---------------------------------</td>
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</tr>
<tr>
<td>Phlebotomy:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>32±4/7</td>
<td>33±4</td>
<td>0.18</td>
<td>37±1/7</td>
<td>&lt;0.001</td>
<td>35±4</td>
<td>0.005</td>
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<td>Platelet count</td>
<td>250 ± 45</td>
<td>244 ± 60</td>
<td>0.59</td>
<td>249±45</td>
<td>0.95</td>
<td>211 ± 65</td>
<td>0.007</td>
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<td>Haematocrit (hct)</td>
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<td>0.36±0.02</td>
<td>0.75</td>
<td>0.35±0.04</td>
<td>0.23</td>
<td>0.35±0.03</td>
<td>0.110</td>
</tr>
<tr>
<td>Mean platelet volume (MPV)</td>
<td>8.4±1.7</td>
<td>11.05±2.2</td>
<td>0.004</td>
<td>10.37±2.0</td>
<td>0.001</td>
<td>9.9±1.9</td>
<td>0.006</td>
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<td>Obstetric Outcome:</td>
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</tr>
<tr>
<td>Gestation at delivery (weeks)</td>
<td>40±1</td>
<td>36 ± 3</td>
<td>&lt;0.001</td>
<td>38±3</td>
<td>0.02</td>
<td>36 ± 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mode of delivery ‡</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>30(88%)</td>
<td>15 (34%)</td>
<td></td>
<td></td>
<td></td>
<td>11(41%)</td>
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<tr>
<td>Elective caesarean section</td>
<td>2(6%)</td>
<td>16(37%)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>4(15%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Emergency caesarean section</td>
<td>2(6%)</td>
<td>13(39%)</td>
<td></td>
<td></td>
<td></td>
<td>2(14%)</td>
<td></td>
</tr>
<tr>
<td>Birth-weight (kg)</td>
<td>3.7 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>&lt;0.001</td>
<td>3.02±.7</td>
<td>0.06</td>
<td>2.7 ± 0.9</td>
<td>&lt;0.001</td>
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</table>
3.6. Discussion

The results of this section of my thesis demonstrate that spontaneous platelet aggregation (SPA) is significantly reduced in pregnancies complicated by utero-placental disease compared with uncomplicated gestation-matched pregnancies. The most significant reductions in SPA were observed in the IUGR group.

I found a positive though non-significant correlation between increasing BMI and spontaneous platelet aggregation. The hypertensive groups (GH and PET) consisted of women with significantly higher BMI’s than their normotensive counterparts therefore it may be that BMI was a factor in increasing spontaneous platelet aggregation towards that of the healthy pregnancies. Smoking was found to be associated with a significant reduction in spontaneous platelet aggregation. The IUGR group had a high proportion of smokers within the group. A further analysis within the IUGR group did not conclude that smoking could explain the statistically significant reductions in SPA observed for this group. Other factors known to affect platelet aggregation (age, platelet count, hct) did not appear to influence the results of this study of spontaneous platelet aggregation in pregnancy and utero-placental disease.

Mean platelet volume appears to be increased in utero-placental disease\textsuperscript{156-158}. I found a negative correlation between increasing MPV and spontaneous platelet aggregation. For each of the complicated pregnancies compared with healthy pregnancy, MPV appeared to be increased, and this may have, in part, explained the overall reduction in spontaneous platelet aggregation for pregnancies complicated by utero-placental disease. Results from this study indicate that a reduction in spontaneous platelet aggregation appears to be a real feature of pregnancies complicated by utero-placental disease. This finding may indicate the presence of pregnancy-specific plasma factor that may inhibit spontaneous platelet aggregation in these conditions.
Overall the lack of a clear association with clinical parameters known to be associated with utero-placental disease and spontaneous platelet aggregation for this study implies (1) that we cannot clinically predict the patients who will have platelet dysfunction related to utero-placental disease, and (2) there isn’t any confounding variable that could have explained our results of reduced spontaneous platelet aggregation in utero-placental disease, and specifically the reductions in SPA observed in IUGR. Differences in platelet function appear to be a real feature of differing phenotypes of utero-placental disease. Given the strict criteria adopted for platelet function analysis, we feel confident that results are true.

As mentioned, our research group previously investigated spontaneous platelet aggregation in a well-designed longitudinal study in normal pregnancy compared with non-pregnant controls\textsuperscript{291}. Spontaneous platelet aggregation was found to be reduced overall compared to a non-pregnant control group. Spontaneous platelet aggregation rose incrementally across the second and third trimesters. Within this study, a pilot study of spontaneous platelet aggregation in pregnancies complicated by utero-placental disease compared with healthy pregnancies was conducted. Due to the small pilot numbers, patients with gestational hypertension were combined with patients with pre-eclampsia. In these preliminary findings, spontaneous platelet aggregation was also found to be reduced in complicated pregnancies compared with healthy pregnancies. It was observed that the reduction in spontaneous platelet aggregation for complicated pregnancies was similar to the findings of platelet aggregation in the first trimester of healthy pregnancies. These findings may suggest that platelet function in established utero-placental disease could mimic the behaviour of normal first trimester platelet behaviour. Whether this feature is a compensatory effect or represents an angiogenic attempt at revascularisation of the ischaemic placenta in utero-placental disease remains to be determined?
SPA was also found to be reduced in the first trimester of pregnancy compared with non-pregnant females in another recent study of SPA in pregnancy. In other studies spontaneous platelet aggregation was found to be increased in normal pregnancy compared with non-pregnant controls. One study did not find any difference in SPA for healthy pregnancies compared with non-pregnant women. A total of only 10 pregnant volunteers and 8 non-pregnant controls however were tested in one of these studies. In contrast to the study of SPA in pregnancy by our research group, where spontaneous platelet aggregation incrementally increased with advancing gestational age, in the study by Fox et al there were no significant differences in SPA noted according to gestational age. Perhaps a limitation of the study by Fox et al was the small sample number, in addition to a considerable lack of clinical data regarding maternal demographic data and pregnancy outcome.

Another study by Burgess et al also concluded increased spontaneous platelet aggregation in normal pregnancy; the findings were most significant when samples were anticoagulated with heparin compared with citrate. A small number of pregnancies complicated by hypertension (grouped as either gestational hypertension or pre-eclampsia) were also examined. Spontaneous platelet aggregation was reported to be increased both in normal pregnancies (evident at 16 weeks’ gestation) and pregnancies complicated by essential hypertension (evident by 24 weeks’ gestation), but not gestational hypertension. However, when examining the results of Burgess et al more closely, the main results reported appeared to be presented for the samples that were collected in heparinised blood. In citrated blood however the changes in SPA were reported to be much less marked.

For our study of spontaneous platelet aggregation in utero-placental disease we used citrate as the anticoagulant, which is the standard method of anticoagulation. When manually reviewing the graphs of Burgess et al, it actually appears that SPA in citrate at 32 weeks’ gestation for pregnancies with essential hypertension was in fact reduced compared to normotensive pregnancies assayed at the same time-
point. What is interesting is that for our study, pregnancies complicated by either gestational hypertension and pre-eclampsia were recruited at a similar time-point to this study, and therefore some similarities between our study and the study by Burgess et al can be gleaned.

The clinical relevance of the finding of this study of reduced or ‘absent’ spontaneous platelet aggregation in pregnancies complicated by utero-placental disease could be, at first, counter-intuitive to the clinician. What is well established is that low-dose aspirin can prevent the development of utero-placental disease when administered to high-risk patients and when commenced less than 16 weeks’ gestation\textsuperscript{31,42,43}. There is now direct evidence that platelet function may be involved in the development of adverse outcomes directly related to defective placentation\textsuperscript{50-52}. Given that aspirin is a potent anti-platelet agent, it could be stipulated that in cases where aspirin is not administered, for whatever reason, one might deduce that platelet function could be \textit{adversely increased} in pregnancies complicated by PET or IUGR, and that the function of aspirin is to prevent this aberrant platelet activity. It is worth reiterating again that platelet activation and aggregation are two separate measures of platelet function and that platelet activation does not automatically imply platelet aggregation. Platelets may become activated and release their cargo but may not necessarily aggregate.

What is not well established is the longitudinal profile of platelet function in pregnancies destined to develop complications directly related to adverse placentation? It may well be that platelet function is altered early in pregnancies that subsequently go on to develop later disease in pregnancy, a theory supported by the observation that the optimal benefit of aspirin for PET prevention is when it is administered from the first trimester.

What remains to be determined is the biological mechanism behind the findings of reduced or ‘absent’ spontaneous platelet aggregation in pregnancies complicated by utero-placental disease compared with healthy pregnancy controls. Whether this altered platelet function is representative of a pregnancy-specific mechanism
occurring in tandem with the clinical spectrum of utero-placental disease, or a failure of normal cell-signalling pathways in platelet activation and aggregation in response to the ischaemic event occurring in the placenta, remains to be established. Though suggested, a causal relationship between SPA and adverse pregnancy outcome cannot be concluded from this current study. Future larger prospective studies of SPA in pregnancies destined to develop complications may uncover insights into this aspect of platelet function as a novel predictive tool.

The strengths of this study included the use of a standardised technique to assess spontaneous platelet aggregation by a modification of light-transmission aggregometry, a technique which, to the best of our knowledge has not been assessed in pregnancy complications to date. The aforementioned studies in pregnancy reported spontaneous platelet aggregation as the percentage of platelet aggregates, a technique which is not standardised. A strict study protocol was adhered to which included specific inclusion and exclusion criteria to control for factors that can affect spontaneous platelet aggregation results (including circadian rhythm and fasting). This study comprised of well-defined study groups. All pregnancies were followed up for pregnancy outcome to ensure the groups were accurately defined for analysis, and as a result a significant number of participants were excluded. Patients with gestational hypertension and pre-eclampsia were identified separately, whereas other studies have combined these into one ‘hypertensive’ group or reported little clinical information on pregnancy outcome. While patients with gestational hypertension are at higher risk of subsequently developing PET, they are recognised as separate clinical entities.

It should be pointed out that smoking, which has been shown in some studies to be associated with increased platelet reactivity, was not an exclusion criterion for this study as it contributes significantly to utero-placental disease, specifically of the IUGR-type and a specific aim was to investigate the impact of smoking in pregnancy on platelet function. Rather than exclude smokers unanimously, smoking status was recorded at the time of recruitment and later analysed for effect on platelet function results. The most significant reduction in spontaneous
platelet aggregation for our study occurred in the IUGR group, the group that had the highest proportion of smokers. However when controlling for smoking status in the spontaneous platelet function analysis, no differences were noted for smokers versus non-smokers. In the large study of SPA in 149 myocardial infarction survivors, approximately 20% of patients were smokers, and the authors also did not find any difference in SPA for smokers versus non-smokers\(^\text{172}\). While smoking has been established to affect platelet reactivity, it may well be that the utero-placental insult is the over-riding event contributing to the reduction in platelet function for IUGR rather than any confounding maternal variable effect.

A weakness of this study is the lack of longitudinal data for complicated pregnancies from first through to third trimester. For such a prospective study to be adequately powered, this would require a large pregnancy population. Given the specialised technique of testing platelet aggregation required to conduct this assay, including the requirement for a strict protocol for blood sampling and handling; study procedure; resources and equipment, and skilled operators, such a study using this method would not be currently feasible. The significant differences in spontaneous platelet aggregation demonstrated between normal and pathological pregnancies in this study are worth exploring further. To this end, if developed, a bedside point-of-care test of spontaneous platelet aggregation could be incorporated into a multi-biomarker screening tool for adverse pregnancy outcome within a larger prospective study. In support of a predictive role for platelet function testing in a large and heterogeneous pregnancy population is the fact that maternal characteristics such as BMI and increasing age did not appear to impact platelet function results, and therefore this or similar methods of platelet testing in pregnancy may have wide clinical applicability.

As previously mentioned, spontaneous platelet aggregation has been proposed as a useful biomarker in predicting patients who will have a further ischaemic event after a recent myocardial infarction\(^\text{168,172}\). Spontaneous platelet aggregation is a novel measure of platelet function. While it is attractive in terms of a single assessment of platelet function, it can only assess one facet of platelet function
and its usefulness has not yet translated into clinical practice. The accepted method of assessing platelet function is by determining how platelets react and aggregate to known platelet agonists (‘platelet reactivity’) rather than assessments of spontaneous platelet aggregation, in the absence of such agonists.

Nonetheless, following on from these findings of significantly reduced spontaneous platelet aggregation in complicated pregnancies I sought to investigate platelet function using a standardised method of platelet function testing as the next logical step. In the succeeding chapter, platelet aggregation was assessed in response to a number of agonists using the same technique that was used to assess spontaneous platelet aggregation: a modification of light-transmission aggregometry. This is a more comprehensive assay than that of SPA. This method is considered the gold standard test of platelet aggregation as it incorporates multiple agonists at submaximal concentrations. Each of the agonists can stimulate the platelet via different platelet surface receptors, subsequently resulting in aggregation. The result is five different measures of specific cell-signalling mechanisms of platelet function.
Chapter 4. Results of Agonist-induced Platelet Aggregation
4.1. Concise summary

In the first results chapter I found that spontaneous platelet aggregation (SPA), that is platelet adhesion and aggregation in the absence of agonists, was reduced in pregnancies that were complicated by hypertension (gestational hypertension (GH) or pre-eclampsia (PET)) and intra-uterine growth restriction (IUGR) compared with healthy gestation-matched pregnancies. In this chapter I investigated a more complex measure of platelet function; agonist-induced platelet aggregation. I found platelet aggregation to be significantly reduced in response to some or all agonists, depending on the clinical subtype of utero-placental disease (GH, PET or IUGR) in question, with the greatest significance in reduction in platelet aggregation observed for IUGR.
4.2. Introduction

Research groups have attempted to define platelet function in pregnancy and pregnancy complications over the past number of years. Various methods have been described\textsuperscript{44,45,47,177-192} and most studies focussed on platelet aggregation, describing differing methods\textsuperscript{46,197-209}. Some studies described methods similar to that of this study, where optical responses of platelet-rich plasma (PRP) to agonists were described, but as previously mentioned a limited number of agonists were generally used in most of these studies. Some studies described platelet aggregation in whole blood, using a method of either counting platelet aggregates after the addition of agonists ('Thrombocounter') or by whole blood electrical impedance, where platelets attach and aggregate to electrodes after stimulation. Other studies described platelet activation, and a limited number of studies examined shear-flow mediated platelet aggregation.

These lines of evidence suggest that platelet function changes throughout pregnancy, albeit with inconsistent results reported\textsuperscript{46,197-209}. Most of the evidence regarding platelet function results in healthy pregnancy is nestled within studies of platelet function in PET, whereby groups of healthy pregnancy and non-pregnant participants were also analysed. A number of studies have examined platelet function longitudinally in healthy pregnancy\textsuperscript{45,47,177,178,182,200,203,204,206}. Results of platelet function testing in gestational hypertension and pre-eclampsia described various techniques, with some inconsistencies and cross-over with the definitions used for the conditions, and some studies had very small sample sizes of less than 10 for analysis\textsuperscript{179,183-192,197,214}. There is a considerable lack of information regarding platelet function in IUGR\textsuperscript{185,215,216}. As detailed in the introduction, platelet function appears to be altered in utero-placental disease, but results have been unclear and inconsistent to date.
As already outlined, most platelet function tests are based generally on the overall principle of the haemostatic function of platelets: platelet adhesion, activation and aggregation. The final step in this pathway, platelet aggregation is the function on which most of these assays are based. To test agonist-induced platelet aggregation, platelets are exposed to known stimuli ex vivo. The agonists that are commonly used are those that are known to evoke platelet activation. Following exposure to the agonist, platelets form aggregates with each other and these aggregates create optical responses, such that a measure of the change in light absorbed or transmitted corresponds to the degree of platelet aggregation. In this test, platelet-to-platelet aggregation in response to various concentrations of agonists is measured in platelet-rich plasma (PRP). This is known as light-transmission aggregometry (LTA) and is the method that will be tested in this study and has been described in a number of clinical studies by our research group.

The standardised method of LTA requires multiple submaximal concentrations of agonists, beginning from very low concentrations to higher concentrations. Platelet aggregation in response to agonists in humans is characterised by a sigmoidal relationship. Computer programs will generate a sigmoidal curve based on platelet aggregation between the primary aggregation response rate and the log dose of the aggregating agent used. However this requires multiple increasing doses of agonists to generate such a response. By estimating the shape of the curve, an individual’s platelet aggregometry response to that agonist can be calculated. The method of assessing platelet aggregation in response to the five agonists that will be tested in this study has already been validated in 100 healthy individuals by our research group. This will be outlined in the context of the results of aggregation curves generated in this study in pregnancy.

Our research group previously investigated platelet aggregation using this specific technique in healthy pregnancies from the first through to the third trimesters. The dose-response curves for pregnancy overall were less than those of healthy non-pregnant controls, and the shape of the curves increased from the first
through to the third trimester, albeit still remaining less than the curves for non-pregnant women. We concluded that platelet aggregation was reduced in pregnancy but increased as pregnancy advanced, possibly in preparation for the haemodynamic changes at delivery where increased haemostatic platelet function is required.

In this study I assessed platelet function in response to multiple agonists. Five different agonists were assessed including ADP, Collagen, AA, TRAP and epinephrine. Why these agonists? The haemostatic function of platelets involves platelet adhesion, activation and aggregation. The principal agents involved are adenosine diphosphate (ADP), collagen (Col), thromboxane A₂ (TXA₂ via AA), and thrombin. Epinephrine is a weaker agonist. Platelets have receptors for each of these aggregating agents. These agonists are released during the process of platelet adhesion and activation, and trigger aggregation. All the above platelet aggregating agents act synergistically, such that if one of the pathways of aggregation is defective or inhibited, platelet function may be overall greatly impaired. These agonists target different receptors and different pathways and they are physiologically relevant.

By assessing these agonists simultaneously, information regarding pathways that are targets for antiplatelet therapies can be assessed: AA assesses the ‘aspirin’ pathway; ADP assesses the ‘clopidogrel pathway’ (via P2Y12 receptor); collagen assesses the ‘collagen pathway’ (assesses stable platelet aggregation via glycoprotein VI; a target for newer antiplatelet agents); TRAP assesses the ‘PAR pathway’ (anti-thrombin agents are currently in clinical use) and finally epinephrine assesses the ‘adrenaline pathway’ (a pathway that is likely altered in pregnancy alongside the associated cardiovascular changes). By understanding these platelet pathways more comprehensively there may be an opportunity to investigate alternative antiplatelet therapies for utero-placental disease prevention, because it is clear that aspirin has its limitations. Trials are already underway of anti-thrombin agents for PET prevention.
A more comprehensive evaluation of platelet function in pregnancy and utero-placental disease using a range of these physiological agonists and at physiological concentrations can provide a summary of individual platelet cell-signalling mechanisms, and an overall global picture of platelet function. This is the aim of this chapter. I sought to comprehensively investigate platelet function using the method of LTA in pregnancies complicated by utero-placental disease. The hypothesis is that platelet function is altered in established utero-placental disease. A more comprehensive and accurate assessment of platelet function in pregnancy may help identify those platelet changes that may also be occurring early in disease in pregnancy, and thus bring us one step closer to developing a screening aid, in addition to targeted platelet therapies for utero-placental disease.
4.3. **Study Design**

As outlined in the main materials and methods chapter, pregnant patients were recruited at the Rotunda Hospital to participate in this study of agonist-induced platelet aggregation in pregnancy. This study was a prospective observational study of platelet aggregation in pregnancy using a modification of a standardised method of platelet function testing: light-transmission aggregometry (LTA). Platelet function results of pregnancies complicated by utero-placental disease were compared to those of normal trimester-matched pregnant controls. A number of clinical outcomes were also recorded, and were later correlated with platelet function results.

The recruitment, inclusion and exclusion criteria have been described in detail in the materials and methods chapter.

4.3.1. **Methods of Platelet function testing**

4.3.1.1. **Phlebotomy**

A specific protocol for blood handling and aggregometry analysis was strictly adhered to ([appendix 2.1](#)). Following consent, a single blood sample was obtained for platelet function analysis in a standardised way. The large number of agonists at differing concentrations of each agonist tested in this second platelet function assay necessitated a substantial volume of venous blood draw. After informed consent, patients were asked to fast from midnight and blood sampling was performed before 8am. A single blood draw was obtained using a 19-gauge butterfly needle with an un-cuffed technique to prevent platelet clumping, with the initial 5ml analysed to confirm a normal platelet count. A 30ml syringe, pre-treated with 3.2% sodium citrate, was then attached to the needle and 27ml of blood was carefully collected with minimal agitation. Owing to the large volume of blood
required for platelet functional testing, in a number of cases blood draws were challenging, and in cases where a suboptimal volume was obtained or it was highly likely that inadvertent platelet aggregation was likely to have occurred secondary to the collection, these samples were excluded from the analysis.

4.3.1.2. Platelet aggregometry testing

All samples were analysed within 60 minutes of blood draw. The sample of blood was firstly centrifuged for 10 minutes, following which platelet rich plasma (PRP) was obtained and dispensed into a plate that contained 96 individual wells (isoplate with clear flat-bottomed wells; Perkin Elmer, Wellesley, MA). This plate is designed to accommodate 8 different concentrations of the five agonists including: arachidonic acid (AA)(500, 375, 188, 83.8, 46.9, 23.4, 11.8, 5.86 g/mL); collagen (Col) (190, 143, 71.3, 35.6, 17.8, 8.9, 4.45,2.23 g/mL); adenosine diphosphate (ADP) and thrombin receptor-activating peptide (TRAP) (20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 mol/L), and epinephrine (Epi) (20, 5, 1.25, 0.313, 0.078, 0.0195, 0.00488, and 0.00122 mol/L).

In brief, platelet function was assessed using a novel modification of light transmission aggregometry which assesses platelet aggregation in response to the given range of concentrations of agonists, where the percentage of light absorbance from baseline corresponds to the degree of platelet aggregation. The percentage aggregation response for each concentration of each agonist was calculated and plotted against the log values of the concentrations of agonists using Graphprism software (Graphprism®, San Diego, CA). The T1/2 (or half-maximal effective concentration (EC\textsubscript{50}) values were further generated from the dose-response curves. Our group has described this assay in detail previously\textsuperscript{33-35,203,259}.35,203,259
4.4. Statistical analysis

The study was powered (80%) to detect a 5% change in platelet aggregation for pregnancies complicated by utero-placental disease. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample independent t-test for continuous data. Continuous data were checked for non-normality and for outliers. Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). Data management and statistical analysis were performed using SAS Version 9.3®, GraphPad PRISM Version 6® and SPSS Version 22®.

4.4.1. Advanced statistical analysis for agonist-induced platelet aggregation

The degree of platelet aggregation in response to the increasing concentrations of each of the 5 agonists tested was assessed as dose-response curves. This is the standardised way of assessing platelet aggregation to these agonists.

[1] Platelet Aggregation Profile Model. ANOVA-type models with group and dose as factors, plus interaction term. These allowed a free-varying dose-response relationship. Group comparisons were therefore not dependent upon any assumed growth curve model. The likelihood-ratio test (for group main effect plus group-by-dose interaction effect) was used to determine any difference in platelet aggregation profiles between groups. This method determines the best fit model for the dose response curves and the differences between the groups.

Additional complex platelet aggregometry data were analysed using modelling approaches created by statistician Mr. Patrick Dicker (Perinatal Ireland and RCSI) to further investigate the platelet effects describing the dose-response sigmoidal
curves. The results of this analysis will be outlined in the chapter presented after the main results of agonist-induced aggregation.

[2] Platelet Agonist Potency Model. Assuming a range of 0-100% for platelet aggregation, two-parameter sigmoidal growth-curve models with EC\textsubscript{50} and hill parameters were fitted. Comparisons between groups were made with the addition of an EC\textsubscript{50} group difference parameter:

\[
\text{Platelet aggregation} = \frac{100}{1 + 10^{[(\text{EC}_{50} + \text{group effect}) - \text{agonist dose}] \times \text{hill}}
\]
4.5. Results

4.5.1. Clinical

A total of 150 patients with a singleton pregnancy and with a diagnosis of either gestational hypertension (GH), pre-eclampsia (PET) or IUGR (EFW<10th centile) were recruited for agonist-induced platelet aggregation testing from 24 weeks’ gestation and beyond at the clinical research site. Platelet function results were compared to the next available healthy singleton third trimester pregnancies (who were subsequently confirmed to have a normal pregnancy outcome). After excluding a number of patients that did not fulfill the inclusion criteria (n=29), the remaining patients for analysis included 36 normal pregnancy controls, 14 patients with GH, 27 patients with PET and 44 patients with prenatally detected IUGR. (figure 4.1). Pregnancies were followed up for pregnancy outcome to ensure the participants retained their original diagnosis.

As outlined in the methods, the IUGR group was further grouped and analysed based on the actual birth weight and placenta histopathology results (figure 4.2). Overall demographic and clinical data are available in the next chapter, which is the second part of the results of the sub-analysis of this study.
Figure 4.1 Study Design of the patients recruited for the study of agonist-induced platelet aggregation and the total numbers excluded from the final analysis.
Figure 4.2 Study design of the IUGR group.

*IUGR <10th centile: based on prenatal EFW <10th centile and subsequent birth weight <10th centile with evidence of utero-placental insufficiency

**IUGR >10th centile: based on prenatal EFW <10th centile with subsequent birth weight >10th centile without evidence of utero-placental insufficiency

***SLE (systemic lupus erythematosus)
4.5.2. Platelet function analysis in utero-placental disease

Does response curves of platelet aggregation in response to incremental concentrations of each of the five agonists were generated and plotted. This is the standardised method of assessing agonist-induced platelet aggregation. In healthy individuals, patterns of platelet aggregation responses have been established in 100 individuals. The curves show a sigmoidal pattern of platelet aggregation response to increasing doses of the agonists (figure 4.3). Our group has previously demonstrated that in healthy pregnancy, dose response curves are different compared with non-pregnant women\textsuperscript{203}.

In this study, I found that these curves were further significantly different in pregnancies complicated by utero-placental disease compared with normal pregnancy controls (figures 4.4-4.6). Platelet aggregation in GH, PET and IUGR was significantly reduced compared with normal pregnancy controls. For GH platelet aggregation was reduced in response to three agonists: AA, COL, and TRAP. For PET, platelet aggregation was reduced in response to four agonists: AA, COL, TRAP and EPI. For IUGR, platelet aggregation was reduced in response to all five agonists: AA, COL, TRAP, Epi and ADP.
Figure 4.3 Platelet aggregation in response to a number of agonists in healthy non-pregnant donors follows a sigmoidal curve (reproduced from Peace 2009 with permission from Professor Dermot Kenny)\textsuperscript{307}. The platelet aggregation response in 100 healthy donors follows a sigmoidal curve to each of the given agonists arachidonic acid (AA), Collagen, ADP, EPI and TRAP. The X axis represents the log of the incremental concentrations of each of the agonists, and the Y axis is the % platelet aggregation response.
Figure 4.4 In gestational hypertension (GH, n=14) the platelet aggregation response to AA, TRAP and Collagen (graphs to the left) is significantly reduced compared with normotensive pregnant controls (n=36). Dose-responses of platelet aggregation are similar to Epi and ADP (graphs to the right) for GH compared with healthy pregnant controls. Platelet aggregation was significantly less in response to all concentrations of AA, TRAP and Col in GH. *TRAP (thrombin receptor-activating peptide), **ADP (adenosine diphosphate).
Figure 4.5 In pre-eclampsia (PET, n=27), the platelet aggregation response to AA, TRAP, Collagen and epinephrine is significantly reduced compared with normotensive pregnant controls (n=36). Dose-responses of platelet aggregation to ADP are similar for PET compared with healthy pregnant controls. *TRAP (thrombin receptor-activating peptide), **ADP (adenosine diphosphate).
Figure 4.6 In Intra-Uterine growth restriction (IUGR, n=33) the platelet aggregation response to all five agonists: AA, TRAP, Collagen, epinephrine and ADP is significantly reduced compared with normotensive pregnant controls (n=36). This indicates a global platelet hypofunction in IUGR. *TRAP (thrombin receptor-activating peptide), **ADP (adenosine diphosphate).
4.5.3. **Explanation of dose-response curves of platelet aggregation in utero-placental disease**

4.5.3.1. **Platelet aggregation in Gestational Hypertension is reduced in response to AA, TRAP and Col compared with normotensive pregnancy controls.**

When pregnancy is complicated by hypertension, the ability of platelets to respond to a number of physiological agonists and thus aggregate was found to be overall less than when a pregnancy remains normotensive. Platelet aggregation profile models and dose-response curves generated for AA \( (p<0.0095) \), TRAP \( (<0.001) \), Col \( (<0.0115) \) demonstrated statistically different agonist responses in GH compared with healthy third trimester controls (figure 4.4). This pattern of reduced platelet reactivity to agonists was not replicated in response to epinephrine \( (p=0.129) \) or ADP \( (0.1051) \). The reason for this is unclear. It could be that platelet function is impaired overall in GH, but that specific responses to epinephrine and ADP are increasing towards normal pregnancy values in compensation for this platelet dysfunction. Platelet-epinephrine responses are likely affected by the presence of increased circulatory shears in hypertension. ADP is a potent platelet stimulus and represents stable platelet aggregation. A sub-population of platelets may demonstrate a tendency to 'increased' sensitivity to certain agonists, the concentrations of which may be altered depending on the clinical phenotype.

Overall, these findings demonstrate a significant reduction in platelet aggregation in pregnancies complicated by GH compared to normotensive third trimester pregnancies.
4.5.3.2. **Platelet aggregation in Pre-eclampsia is reduced in response to AA, TRAP, Col and Epi compared with normotensive pregnancy controls.**

When pregnancy is complicated by pre-eclampsia, the ability of platelets to respond to a range of platelet agonists is overall much less than when a pregnancy remains normotensive. Results of reduced platelet responsiveness in PET were even more significant than the reductions in platelet responses to each of the agonists than for GH. As with GH, platelet aggregation profile models and dose-response curves generated specifically for agonists AA (p<0.03), TRAP (<0.0001), Col (<0.001) in PET demonstrated statistically reduced responses and subsequently reduced aggregation compared with healthy third trimester controls (figure 4.6). Unlike GH however, for the PET group, significantly reduced platelet responses were also found in response to epinephrine (p <0.001).

A similar result of no difference in platelet aggregation in response to ADP was obtained in PET and GH, where platelet aggregation was not found to be significantly different compared with normotensive pregnancy controls (p=0.49). The pattern of no change in platelet reactivity to ADP was found for both GH and PET. It could be that platelet function is impaired overall in hypertension and PET, but that specific responses to ADP are increasing towards normal pregnancy values in PET. A possible reason for the differences in the dose-response curves of platelet aggregation for GH and PET will be explored later.
4.5.3.3. Platelet aggregation in IUGR is reduced in response to all 5 agonists: AA, TRAP, Col, Epi and ADP compared with normotensive pregnancy controls

For platelet function analysis for the IUGR birth weight <10\textsuperscript{th} centile group (n=33), platelet aggregation profile models generated for each agonist demonstrated statistically significantly reduced platelet aggregation in response to all agonists compared with normal pregnancy controls (figure 4.6). These findings imply a global ‘hypo-reactivity’ of platelets across all five agonists and at all concentrations in growth restricted pregnancies compared to pregnancies with an appropriately-grown fetus. When analysed separately, the IUGR birth weight >10\textsuperscript{th} centile group also demonstrated a similar effect, with significantly reduced or altered platelet function compared to normal pregnancy controls.

Platelet function appears to be different based on clinical presentation on the spectrum of utero-placental disease. Platelet function appears to differentiate between GH to PET to IUGR, where platelet aggregation appears to be significantly reduced in response to 3, 4 and 5 agonists respectively.

To explore the differences obtained for platelet aggregation results in GH, PET and IUGR I examined these findings in more detail. I next performed a number of more advanced statistical analyses with the assistance of Mr. Patrick Dicker, statistician, RCSI, to determine any differences in platelet responses to each of the agonists that may have explained the different appearances of the growth curves based on clinical condition.

One way of examining the appearance of the curves more closely is to determine the EC\textsubscript{50}. The EC\textsubscript{50} is the concentration of an agonist which induces a response or reaction at the halfway point between baseline and maximum response after a specified time-frame. It is a useful method of analysing dose-response in relation to time for a given agent and is used regularly to assess pharmacological response (figure 4.7).

![Graph](image)

Figure 4.7 The EC\textsubscript{50} represents the concentration of agonist required to provoke a response halfway between the baseline and maximum platelet aggregation responses (reproduced from the GraphPad Prism© website)\textsuperscript{295}. The EC\textsubscript{50} will be greater if the platelet responses to that agonist are reduced (i.e. a higher concentration of the agonist will be required to obtain a similar result of platelet aggregation between two groups)\textsuperscript{295}.
4.6.1. Advanced platelet aggregometry analysis with calculations of the EC\textsubscript{50} (tables 4.1 and 4.2)

4.6.1.1. Gestational Hypertension (table 4.1)

The overall mean platelet aggregation for AA, TRAP and Collagen was significantly reduced in GH compared with normotensive pregnancy. The EC\textsubscript{50} (as a measure of platelet agonist potency), was increased for the agonists AA, TRAP and Collagen (i.e. a higher concentration of the given agonist was required in GH to result in the same degree of platelet aggregation at the half-way mark of that of normotensive controls (who required less agonist accordingly and were therefore more responsive). Although no differences were found in the dose-response curves for Epinephrine and ADP, the EC\textsubscript{50} was found to be lower in GH compared to normal pregnancy controls, indicating increased platelet responses to these two agonists in GH.

4.6.1.2. Pre-eclampsia (table 4.1)

The overall mean platelet aggregation for agonists AA, TRAP, Collagen and ADP was significantly reduced in PET compared with normotensive pregnancy, and the EC\textsubscript{50} (the dose of agonist required to reach 50\% platelet aggregation, as a measure of platelet agonist potency), was increased accordingly for these agonists (i.e. for PET a significantly higher concentration of the agonist was required to reach the same degree of platelet aggregation of healthy pregnancy controls), implying the platelets were not as responsive to these given agonists in PET. However the response to ADP was increased in PET, where less EC\textsubscript{50} was required to generate a similar response in normal pregnancy controls.
Table 4.1 Results of the EC$_{50}$ for AA, TRAP, Collagen, epinephrine and ADP for normal pregnancy controls is compared to results of gestational hypertension (GH) and pre-eclampsia (PET) in the third trimester. * A significantly different EC$_{50}$ compared to healthy pregnancy controls.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Controls (n=36)</th>
<th>GH (n=14)</th>
<th>P value</th>
<th>PET (n=27)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid (AA)</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>-0.7</td>
<td>0.59</td>
<td>-0.6</td>
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<tr>
<td>Thrombin-receptor activating peptide (TRAP)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>EC$_{50}$</td>
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<td>1.9</td>
<td>&lt;0.0001*</td>
<td>1.9</td>
<td>&lt;0.0001*</td>
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<tr>
<td>EC$_{50}$</td>
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<td>3.3</td>
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<td>1.5</td>
<td>0.05*</td>
<td>1.6</td>
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</table>
4.6.1.3. **Intrauterine growth restriction (table 4.2)**

The IUGR group as a whole was further analysed based on final birth weight <10th or >10th centile and results of the EC50 are represented in a separate table (table 4.2). The overall mean platelet aggregation for each agonist was significantly reduced in IUGR. The EC50 (as a measure of platelet agonist potency) for each agonist in IUGR (for both groups, IUGR birth weight <10th centile and IUGR birth weight >10th centile) was correspondingly significantly greater compared to normal controls. Again, this implies that higher concentrations of each agonist in cases of IUGR were required to produce a response halfway from baseline to maximum aggregation to produce a similar response to that of controls. This implies a global platelet hyporesponsiveness in IUGR.

The dose-response curves and advanced platelet function analyses correlate and explain the appearance of the curves, demonstrating a significant deterioration in a reduction in platelet reactivity in pregnancies complicated by GH, PET and IUGR compared to normotensive third trimester pregnancies. To further investigate the different findings of platelet function obtained for GH, PET and IUGR, I next investigated the effect of a range of maternal demographic and clinical variables on platelet function results. This will be outlined in the succeeding chapter of the sub-group analyses of the agonist-induced aggregation results.
Table 4.2 Results of the EC\textsubscript{50} for AA, TRAP, Collagen, epinephrine and ADP for normal pregnancy controls is compared to the results for the IUGR group (based on birth weight <10\textsuperscript{th} centile and birth weight >10\textsuperscript{th} centile). * A significantly different EC\textsubscript{50} compared to healthy pregnancy controls.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Controls (n=36)</th>
<th>IUGR &lt;10\textsuperscript{th} centile (n=33)</th>
<th>(P) value</th>
<th>IUGR &gt;10\textsuperscript{th} centile (n=11)</th>
<th>(P) value</th>
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<td>Thrombin-receptor activating peptide (TRAP)</td>
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<td></td>
<td></td>
</tr>
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<td>1.98</td>
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<td>2.13</td>
<td>&lt;0.001*</td>
</tr>
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<td>Collagen</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EC\textsubscript{50}</td>
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<td>&lt;0.0001*</td>
<td>-0.99</td>
<td>0.005*</td>
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<td>1.98</td>
<td>0.02*</td>
<td>1.65</td>
<td>0.006*</td>
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</tbody>
</table>
4.7. Conclusion

Platelet aggregation, using a modification of light-transmission aggregometry, appears to be reduced in utero-placental disease compared with healthy pregnancy controls. Platelet aggregation in GH is reduced in response to 3 agonists (AA, TRAP and Collagen). Platelet aggregation in PET is reduced in response to 4 agonists (AA, TRAP, Collagen and Epinephrine). Platelet aggregation in IUGR is significantly reduced in response to all 5 agonists tested (AA, TRAP, Collagen, Epinephrine and ADP). To explore these findings further I performed a number of comprehensive sub-group analyses to determine the effect of maternal demographic and clinical variables on the agonist-induced platelet function aggregation results.
Chapter 5. **Sub-group analysis of Agonist-induced Platelet Aggregation based on demographic and clinical variables**
5.1. Concise summary

The main findings of significantly reduced platelet function in response to 3 agonists for GH, 4 agonists for PET and all 5 agonists in IUGR were further explored through sub-group analyses to determine any confounding variables on platelet function results. Agonist-induced platelet aggregation did not appear to be affected by maternal demographic and clinical variables. Different agonist responses were identified for a range of different clinical sub-types based on severity of disease. The most significant finding was a reduction in platelet responses to all 5 agonists when IUGR was further complicated by hypertension compared to cases of IUGR that remained normotensive. This suggests platelet dysfunction may be a marker of severe utero-placental disease.
5.2. Introduction:

Platelet function has been shown in some studies to be affected by variables such as age, BMI and medication use\textsuperscript{62,73,170,294,306-309}. I investigated some of these specific variables in pregnancy in relation to agonist-induced platelet aggregation results. Because similar results were obtained for platelet aggregation in response to the agonist ADP for GH and PET, and because a significant proportion of patients with either gestational hypertension or pre-eclampsia were treated with antihypertensive medications, I specifically determined if there was an effect of antihypertensive use on platelet aggregation results.

The overall clinical and demographic data that complement these analyses are available at the end of this chapter (tables 5.3-5.5).

5.3. Effect of potential confounding maternal variables on platelet function results

I determined the effect of maternal variables on platelet function results for the hypertensive groups combined (GH and PET) and the IUGR groups separately. I combined the hypertensive groups because of the small numbers of variables for analysis. For the hypertensive group, no differences in platelet function were noted for age, BMI, ethnicity, parity, smokers, or any corticosteroid use for fetal lung maturity (p>0.05). Regarding blood pressure measurement prior to platelet function testing, the average systolic and diastolic blood pressure readings for GH and PET were 138±16/86±7 and 143±16/88±8 respectively. No differences were noted in platelet function based on BP ≥140/90 prior to platelet function testing. Increasing BMI, older age categories, smoking and hypertension have been shown to affect platelet function outside of pregnancy\textsuperscript{62,73,170,306}. Also, a single
study suggested corticosteroid use in pregnancy can also affect platelet function\textsuperscript{294}. It is worth pointing out that in light of this particular study, I generally avoided platelet function testing for at least 48 hours in cases where corticosteroids had been administered, and for the pregnancy group as a whole, steroids were generally administered on average a week prior to recruitment to the study. The lack of effect of these factors on platelet function results in pregnancy in this study indicates that for hypertension in pregnancy, platelet functional changes appear to be real and may be a reflective of the utero-placental condition, rather than any confounding maternal factors.

Specifically for the PET group there did not appear to be a correlation with the level of proteinuria (average 833g, range 300-3390g) or the subsequent development of abnormal liver function tests (n=6) with platelet function results (p>0.05). A small number of the PET patients subsequently developed fulminating PET (n=7). There were no cases of eclampsia. Though numbers were small for analysis, there were no differences in platelet function for cases of fulminating PET compared to milder cases of PET (p>0.05).

When evaluating the effects of maternal characteristics on platelet function in IUGR pregnancies there were no differences in total platelet aggregation for smokers (n=9), pregnancies with a BMI >30 (n=3), or concurrent antihypertensive medication use (n=3) (p>0.05).
5.3.1. Antihypertensive medication use

The beta blocker labetalol is the usual first line therapy for hypertension in pregnancy, and the majority of the cases in this study were treated with this antihypertensive medication\textsuperscript{6}. Although principally a beta blocker, labetalol also has alpha blocking properties. Platelets have an alpha receptor\textsuperscript{310}. Thus I hypothesised that the alpha blocking properties of labetalol might affect platelet aggregation results in utero-placental disease. A total of 64% and 66% of the GH and PET groups were treated with antihypertensive medications respectively, of which labetalol was the predominant medication used (refer to figure 5.1, tables 5.3, 5.4 at the end of this chapter). When comparing platelet aggregation results for the GH and PET groups combined, and for those treated with antihypertensives (n=27) compared to those without (n=14), no differences were noted in platelet aggregation results in response to any of the five agonists used, and specifically for ADP (p=0.31) and epinephrine (n=0.39).

In conclusion, the use of antihypertensive medication, specifically labetalol, does not appear to affect platelet function in pregnancy when this is tested using standardised platelet function techniques. For the IUGR group, 3 patients were treated with labetalol, and no differences were noted in platelet aggregation (p>0.05). In the final chapter of dynamic platelet function in pregnancy, I explore this concept further, and assay the effect of labetalol on dynamic platelet-VWF interactions because I hypothesised there was possibly a subtle effect of antihypertensive medications on platelet haemostatic function that may not be apparent using gross platelet functional assays. This will be explored further.

To summarise, overall there appears to be a reduced response of platelets to a range of physiological agonists in conditions affected by utero-placental disease. Platelet function appears to differentiate between gestational hypertension, pre-eclampsia and intra-uterine growth restriction. The results of the differing patterns obtained for GH, PET and IUGR could not be explained by maternal demographic
variables or antihypertensive use. This suggests that the reduction in platelet aggregation of these clinical entities on the spectrum of utero-placental disease is not explained by maternal demographic or clinical confounding variables. Maternal platelet function appears to be altered in utero-placental disease and is different based on the clinical presentation.

Figure 5.1 Study design of sub-group analysis of the platelet aggregation results for patients with hypertension in pregnancy (combining the groups gestational hypertension (GH) & pre-eclampsia (PET)) treated with antihypertensive medications or conservatively managed).
5.4. Sub-group analysis of platelet aggregation based on severity of utero-placental disease

Both IUGR and hypertension can occur together in pregnancy, and when both features are superimposed they represent cases on the most severe end of the spectrum of utero-placental disease\(^{311}\). As part of this study design, all cases were followed up for clinical outcome. While every effort was made to avoid recruiting cases where it was apparent these clinical entities were superimposed at the time of recruitment, after following up all cases for delivery outcome, it was apparent that some cases of hypertension (GH and PET) had likely been complicated by IUGR, and also a small number of cases of IUGR were complicated further by superimposed hypertension.

To recap, overall, platelet function was reduced in

(i) GH in response to 3 agonists (AA, TRAP, Col),

(ii) PET in response to 4 agonists (AA, TRAP, Col, Epi) and

(iii) IUGR in response to all 5 agonists (AA, TRAP, Col, Epi, ADP).

I next performed a further comparison of the platelet aggregation results within these three groups for cases that were subsequently complicated by additional features of utero-placental disease, to ascertain if severity of disease correlated with deterioration in platelet function, and whether this could explain the highly significant global reduction in platelet aggregation in response to five agonists in IUGR.
Firstly examining the hypertensive group (GH and PET), I performed two sub-group analyses: (i) cases that were further complicated by IUGR compared to cases that were not affected by fetal growth restriction and (ii) cases of hypertension that were diagnosed early (<34 weeks’ gestation) versus later-onset disease (>34 weeks’ gestation) (figure 5.2).

5.4.1. Hypertension sub-analysis (table 5.1)

5.4.1.1. GH and PET superimposed with IUGR

I combined the GH and PET group for this sub-group analysis. In the hypertensive group (GH and PET), a total of 30% (12/41) were found to have an infant with a birth weight <10th centile for gestational age, and therefore it could be inferred these cases had been superimposed on IUGR, although it may not have been apparent at the time of recruitment. I determined if there were any differences in platelet function based on cases that were likely superimposed with IUGR compared to cases that were not (table 5.1).

Platelet responses specifically to the agonist ADP were significantly reduced when GH/PET was further complicated by IUGR (n=12) compared to cases that had an appropriately grown fetus (n=27) (p=0.02). Recalling that in general for the GH and PET groups, platelet responses to ADP were not significantly different compared with normal pregnancy controls, it appears that if these conditions are further complicated by fetal growth restriction, platelet function is impaired in response to this specific agonist.

5.4.1.2. Early-versus late-onset utero-placental disease

Next, I compared cases of GH/PET that were diagnosed early (early-onset, EO, < 34 weeks’ gestation) compared with later-onset disease (LO, >34 weeks’ gestation), (table 5.1). For GH/PET when compared with LO disease diagnosed
>34 weeks’ gestation (n=27), EO GH/PET diagnosed <34 weeks’ gestation (n=12) was associated with reduced platelet aggregation to TRAP (p=0.01).

Figure 5.2 Study Design of the Study design of sub-group analysis of the platelet aggregation results for patients with hypertension in pregnancy (combining the groups gestational hypertension (GH) & pre-eclampsia (PET)) based on (i) the presence of IUGR at delivery compared with AGA and (ii) early-versus-late-onset disease

*AGA (appropriate for gestational age)
Table 5.1 Subgroup-analysis of the hypertension group (GH/PET) showing platelet aggregation was reduced in response to: (i) ADP for the hypertension group combined (GH/PET) that were further complicated by IUGR compared to cases that were not and (ii) TRAP for cases of early-onset hypertension compared to later-onset disease. * A significantly different dose-response profile (p<0.05). Significance remained after Bonferroni adjustment for multiple group comparisons.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Hypertension with IUGR (n=12) versus Hypertension with AGA (n=29)</th>
<th>Early (n=12) versus late onset hypertension (n=29)</th>
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</thead>
<tbody>
<tr>
<td>Arachidonic acid (AA)</td>
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<td>0.88</td>
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<td>Dose-response profile</td>
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</tr>
<tr>
<td>Thrombin-receptor activating peptide (TRAP)</td>
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<td>0.01*</td>
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<td>Dose-response profile</td>
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<td>Collagen</td>
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<tr>
<td>Dose-response profile</td>
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<td></td>
</tr>
</tbody>
</table>
5.5. IUGR sub-analysis (table 5.2)

Because of the significant results of a global reduction in platelet function obtained for the IUGR group independently, to further investigate whether differences in platelet aggregation correlated with severity of IUGR, a final subgroup analysis was performed. The recent PORTO study demonstrated that cases of IUGR with abnormal UA Doppler waveform or cases that were <3rd centile were most at risk of adverse perinatal outcome. I divided the high-risk IUGR group (n=33) into subgroups of pregnancies complicated by (i) abnormal umbilical artery (UA) Doppler (this consisted of AEDF) (n=13); or (ii) EFW <3rd centile (n=15). I also analysed (iii) differences in platelet function based on early versus late-onset presentation (iv) and IUGR with the subsequent development of hypertension (figure 5.3, table 5.2).

5.5.1. Abnormal UA Doppler

Within the abnormal UA Doppler group compared to cases with normal UA Doppler waveforms, there was significantly reduced platelet reactivity in response to the agonists ADP and TRAP (p=0.01 and <0.001 respectively). What is interesting is that the agonist ADP was the single agonist that resulted in reduced platelet function when GH/PET was further superimposed by IUGR. IUGR complicated by abnormal UA Doppler or hypertensive disease are both severe conditions in the fetus and mother respectively. This finding may indicate that platelet reactivity to this agonist, ADP, is significantly reduced for the more severe clinical entities of utero-placental disease. Different platelet responses to different agonists may distinguish between clinical presentations of utero-placental disease.
5.5.2. EFW <3rd centile

There were no differences found in platelet aggregometry profiles in IUGR cases stratified according to EFW<3rd centile when compared with IUGR >3rd centile.

5.5.3. Early-versus late onset IUGR

Cases of early-onset IUGR < 34 weeks’ were also associated with a significantly reduced platelet responses to TRAP, but also collagen (p<0.0001 and 0.0019 respectively) Table 4.91. It appears that different clinical entities on the spectrum of utero-placental disease might result in different and selective platelet functional responses based on gestational age at presentation.

5.5.4. IUGR superimposed with hypertension

In over a third of the IUGR group, hypertension (GH/PET) further complicated the pregnancy (12/33). It is worth noting that at the time of enrolment, a total of 3 cases of IUGR out of the final 33 cases were complicated by hypertension, and these 3 patients were taking antihypertensive medications at the time of recruitment. The remaining 9 cases subsequently developed superimposed GH/PET. I have already determined that antihypertensive medication in general does not appear to affect platelet aggregation using this assay.

For this sub-group analysis, the most prominent finding was that of statistically significantly reduced platelet aggregation in response to all agonists when IUGR occurred in the setting of subsequent development of GH/PET compared to normotensive IUGR, which is not surprising as this represents a severe end of the spectrum of utero-placental disease. This is a compelling finding and may explain
the highly significant global reduction in platelet function for the IUGR group as a whole.

**Figure 5.3 Study Design of sub-group analyses of the platelet aggregation results the IUGR group as a whole.**
Table 5.2 Subgroup-analysis of the IUGR group showing platelet aggregation is reduced in response to: (i) TRAP for abnormal UA Doppler (UAD) compared to cases with normal UAD; (ii) TRAP and Collagen for early-onset IUGR compared to later-onset disease and (iii) all five agonists for IUGR complicated by hypertension compared to normotensive IUGR. * A significantly different dose-response profile (p<0.05). Significance remained after Bonferroni adjustment for multiple group comparisons.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Abnormal UAD (n=13) versus normal UAD (n=20)</th>
<th>Early (n=14) versus late onset IUGR (n=19)</th>
<th>IUGR with hypertension (n=12) versus normotensive IUGR (n=21)</th>
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</thead>
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<tr>
<td>Arachidonic acid (AA)</td>
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<td>Dose-response profile</td>
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<td>Dose-response profile</td>
<td>0.01*</td>
<td>0.05</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>
5.6. Summary of sub-group analyses of platelet function based on clinical severity

Different platelet responses to agonists appear to be a feature of differing presentations of the clinical spectrum of utero-placental disease.

Firstly for the hypertension group (GH/PET), the subsequent development of IUGR is associated with reduced platelet responses to ADP. In early versus later onset GH/PET, platelet responses are reduced in response to TRAP. For the IUGR group, the presence of abnormal UA Doppler affects both of these agonists with reductions again in platelet responses to the same. Also, when IUGR presents early at < 34 weeks’ gestation, responses to TRAP and Collagen are also reduced. Furthermore, IUGR subsequently complicated by hypertension results in significantly reduced platelet responses to all five agonists. Findings remained significant for the IUGR group after Bonferroni adjustment for multiple comparisons.

It appears that for cases where GH/PET occurs first in pregnancy and IUGR later, platelet responses appear to be reduced only in response to the agonist ADP compared to GH/PET alone. Whereas when IUGR presents first in pregnancy and is later superimposed by GH/PET, platelet function appears to be globally reduced in response to all agonists and at all concentrations. This is a novel finding and may indicate degrees of severity of utero-placental disease based on whether a fetal or maternal condition presents first, suggesting that when IUGR appears first in a pregnancy and is later complicated by hypertension, the condition may be more severe. These findings may also suggest that for utero-placental disease, platelet function may be a marker for severity of disease or the development of further pregnancy complications. Additionally, reduced selective responses to TRAP and collagen, based on sub-type of disease, may suggest these agonists are also be selectively involved in platelet functional changes on the spectrum of utero-placental disease, and may be useful targets for antiplatelet therapy.
5.7. Demographic data of patients

5.7.1. Clinical results for Gestational Hypertension:

Patients with GH had a higher BMI compared with their normotensive counterparts (27±4 versus 24±4, p=0.001) (table 5.3). A total of 4 patients with GH had a BMI >30 and all patients with hypertension were Caucasian. Cases of GH were recruited at a later gestational age than healthy pregnancy controls. This was to limit the chances of recruiting patients with hypertension in pregnancy that subsequently developed PET. The numbers recruited were thus slightly lower than anticipated, however they represent a group of ‘true’ pregnancy-related hypertension not due to ‘undiected’ pre-eclampsia. As expected more patients with gestational hypertension were treated medically (n=9) than conservatively managed, although we were afforded the opportunity of recruiting a small number of patients presenting with gestational hypertension before antihypertensive medication was prescribed (n=5) so that we could later compare platelet function results for those treated compared to those not on medications. The antihypertensive medications used consisted of labetalol (n=6) and labetalol combined with nifedipine (n=3).

Although for GH there were no differences in platelet counts, platelet volumes (MPV) were significantly higher in the hypertensive group compared with normal pregnancy controls. In terms of delivery outcomes, patients with GH were delivered at a slightly earlier gestational age (GA), although this did not correspond with a difference in birth weight compared with controls who were delivered at a later GA. Almost half of cases with GH were delivered by caesarean section compared with only 12% of the healthy pregnancy cohort (p=0.07). This specific result will be explored in the final chapter in relation to placental changes in hypertension in pregnancy, platelet function analysis and mode of delivery.
5.7.2. Clinical results in Pre-eclampsia:

Regarding maternal demographics for the PET group, there was a slightly higher BMI in this group compared with normal controls (26 versus 24, p=0.03) (table 5.4). When compared with 94% of controls, only 74% of cases of PET were of Caucasian ethnicity. Non-Caucasian ethnicity is an independent risk factor for developing PET. A total of 66% of patients with PET were treated medically with anti-hypertensives, and results of platelet aggregation were compared for those not taking medications at the time of recruitment. The antihypertensive medications prescribed for the most part were labetalol (n=13), labetalol combined with nifedipine (n=2) and alpha-methyldopa (n=3).

As expected, the platelet counts were significantly lower in cases complicated by PET compared with normal controls, and MPV values were higher in PET. Other groups have reported on this finding. Cases of PET were delivered at an earlier GA, and with a corresponding lower birth weight compared with controls. What is interesting is that although in contemporary practice, it is often expected that cases of PET will labour effectively, for our study almost half of all cases of PET were delivered by emergency caesarean section (44%) compared with only 6% of the healthy pregnancy cohort (p<0.001).

5.7.3. Clinical results in Intrauterine growth restriction

Patients with IUGR were more likely to have had a previous history of PET (p=0.034) or IUGR (p=0.001) compared to normal pregnancy controls (table 5.5). Within the IUGR group birth weight <10th centile, there were significantly more smokers (n=9, 27%) compared to normal pregnancy controls (p=0.001). As discussed previously smokers were not excluded at enrolment as smoking contributes significantly to the development of IUGR, however its potential confounding effects on platelet aggregometry results were analysed.
Within the IUGR birth weight >10\textsuperscript{th} centile group, mothers were significantly younger (p=0.001) and not surprisingly 36% represented a non-Caucasian ethnic group (p=0.02). Again, this group likely consisted of cases of constitutional cases of IUGR, and non-Caucasian ethnicities are associated with smaller birth weights than their Caucasian counterparts. There were no significant differences in BMI and GA at blood draw for this group compared with normal pregnancy controls, however the platelet count was noted to be lower than the control group, albeit within a normal range. The IUGR birth weight >10\textsuperscript{th} centile group were also delivered earlier, resulting in an overall lower birth weight compared with appropriately grown pregnancies (p<0.001).

The mean gestational age at enrolment to the study for the IUGR group was 34 weeks’ (±3 days) and the mean gestational age at delivery was 36 weeks’ (±3 days), resulting in a significantly lower birth weight compared with controls (p<0.001). Congenital infection as a cause for IUGR was out-ruled in all cases. Antenatal corticosteroids for fetal lung maturation were administered in 79% and 45% of the IUGR birth weight <10\textsuperscript{th} centile and IUGR birth weight >10\textsuperscript{th} centile groups respectively. In cases where antenatal corticosteroids were administered, platelet function testing was generally delayed for greater than 48 hours from the time of corticosteroid administration to minimise any potential effect on platelet function, and in most cases testing was undertaken on average a week from the administration of corticosteroids. None of the women had taken aspirin or were administered low-molecular weight heparin at any point in the pregnancy.
Table 5.3 Overall patient demographic data for Platelet Function Assay 2: agonist-induced platelet aggregation demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by gestational hypertension. †Normal Control: pregnancy controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers."p-value=<0.05. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery (MOD).

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Normotensive 3rd trimester (n=36)</th>
<th>Gestational Hypertension (n=14)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>32 ± 4</td>
<td>31±6</td>
<td>0.39</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 4</td>
<td>27±4</td>
<td>0.01*</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>32 (94%)</td>
<td>14(100%)</td>
<td>0.49</td>
</tr>
<tr>
<td>Smoker</td>
<td>0</td>
<td>3(21%)¥</td>
<td>0.03*</td>
</tr>
<tr>
<td>Steroids</td>
<td>0</td>
<td>3(21%)¥</td>
<td>0.21</td>
</tr>
<tr>
<td>Parity</td>
<td>Nulliparous</td>
<td>11(32%)</td>
<td>0.04*</td>
</tr>
<tr>
<td></td>
<td>Multiparous</td>
<td>23(68%)</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>0</td>
<td>9(64%)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

| Phlebotomy:             |                                          |                                 |         |
| Gestational age(weeks)  | 32±4/7                                   | 37±1/7                          | <0.001* |
| Platelet count          | 250 ± 45                                 | 249±45                          | 0.95    |
| Haematocrit             | 0.36±0.02                                | 0.35±0.04                       | 0.23    |
| Mean platelet volume    | 8.4±1.7                                  | 10.37±2.0                       | 0.001*  |

| Obstetric Outcome:      |                                          |                                 |         |
| Gestation at delivery (weeks) | 40±1                                  | 38±3                            | 0.02*   |

| Mode of delivery†‡      |                                          |                                 |         |
| Vaginal delivery        | 30(85%)                                  | 8(57%)                          |         |
| Elective caesarean section | 2(6%)                                  | 4(29%)                          |         |
| Emergency caesarean section | 2(6%)                                  | 2(14%)                          | 0.07**  |
| Birth-weight (g)        | 3700 ±500                                | 3017±710                        | 0.06    |
Table 5.4 Overall patient demographic data for Platelet Function Assay 2: agonist-induced platelet aggregation demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by pre-eclampsia.

†Normal Control: pregnancy controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. *p-value=<0.05. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery (MOD).

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Normotensive 3rd trimester (n=36)</th>
<th>Pre-eclampsia (n=27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>32 ± 4</td>
<td>31 ±74</td>
<td>0.26</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 4</td>
<td>26 ±36</td>
<td>0.03*</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>32 (94%)</td>
<td>20 (74%)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Smoker</td>
<td>0</td>
<td>3 (11%)¥</td>
<td>0.03*</td>
</tr>
<tr>
<td>Steroids</td>
<td>0</td>
<td>9 (33%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Parity</td>
<td>Nulliparous</td>
<td>11 (32%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multiparous</td>
<td>13 (48%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.210</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>0</td>
<td>18 (66%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Phlebotomy:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age(weeks)</td>
<td>32±4/7</td>
<td>35±4</td>
<td>0.005*</td>
</tr>
<tr>
<td>Platelet count</td>
<td>250 ± 45</td>
<td>211 ± 65</td>
<td>0.007*</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.36±0.02</td>
<td>0.35±0.03</td>
<td>0.110</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>8.4±1.7</td>
<td>9.9±1.9</td>
<td>0.006*</td>
</tr>
<tr>
<td>Obstetric outcome:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation at delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(weeks)</td>
<td>40±1</td>
<td>36 ±5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mode of delivery†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>30 (85%)</td>
<td>11 (41%)</td>
<td></td>
</tr>
<tr>
<td>Elective caesarean section</td>
<td>2 (6%)</td>
<td>4 (15%)</td>
<td></td>
</tr>
<tr>
<td>Emergency caesarean section</td>
<td>2 (6%)</td>
<td>12 (44%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Birth-weight (g)</td>
<td>3700 ± 500</td>
<td>2700 ± 900</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
Table 5.5 Overall patient demographic data for Platelet Function Assay 2: agonist-induced platelet aggregation demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by intra-uterine growth restriction:†Normal Control: pregnancy controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. *IUGR birth weight >10th centile: cases where an initial estimated fetal weight <10th centile was noted at the time of antenatal enrolment, but with an actual birth weight of >10th centile were analysed separately (n=11). † p-value comparisons are firstly for IUGR <10th centile versus controls, and secondly for IUGR >10th centile versus controls. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery (MOD).

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Appropriately-grown, 3rd trimester (n=36)</th>
<th>IUGR birth weight &lt;10th centile (n=33)</th>
<th>p-value†</th>
<th>IUGR birth weight &gt;10th centile* (n=11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>32 ± 4</td>
<td>29±6</td>
<td>0.06*</td>
<td>26±4</td>
<td>0.001*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 4</td>
<td>25±6</td>
<td>0.52</td>
<td>27±6</td>
<td>0.14</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>32 (94%)</td>
<td>28(85%)</td>
<td>0.24</td>
<td>7(64%)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Smoker</td>
<td>0</td>
<td>9(27%)</td>
<td>0.001</td>
<td>1(9%)†</td>
<td>0.001*</td>
</tr>
<tr>
<td>Steroids</td>
<td>0</td>
<td>26(79%)</td>
<td>&lt;0.001*</td>
<td>5(45%)†</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Parity</td>
<td>Nulliparous</td>
<td>11(32%)</td>
<td>0.81</td>
<td>6(55%)</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Multiparous</td>
<td>23(68%)</td>
<td></td>
<td>5(45%)</td>
<td></td>
</tr>
<tr>
<td>Phlebotomy:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age(weeks)</td>
<td>32±4/7</td>
<td>35±4</td>
<td>0.009*</td>
<td>32±4</td>
<td>0.54</td>
</tr>
<tr>
<td>Platelet count</td>
<td>250 ± 45</td>
<td>252±62</td>
<td>0.98</td>
<td>206±35</td>
<td>0.004*</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.36±0.02</td>
<td>0.36±0.03</td>
<td>0.98</td>
<td>0.34±0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>8.4±1.7</td>
<td>9.6±2.2</td>
<td>0.009*</td>
<td>10.45±2.6</td>
<td>0.03*</td>
</tr>
<tr>
<td>Obstetric outcome:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA delivery (weeks)</td>
<td>40±1</td>
<td>36 ± 3</td>
<td>&lt;0.001*</td>
<td>39±1</td>
<td>0.002*</td>
</tr>
<tr>
<td>Mode of delivery†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>30(85%)</td>
<td>10(30%)</td>
<td></td>
<td>5(46%)</td>
<td></td>
</tr>
<tr>
<td>Elective caesarean section</td>
<td>2(6%)</td>
<td>11(33%)</td>
<td></td>
<td>5(46%)</td>
<td></td>
</tr>
<tr>
<td>Emergency caesarean section</td>
<td>2(6%)</td>
<td>12(37%)</td>
<td>&lt;0.001*</td>
<td>1(8%)</td>
<td>0.75</td>
</tr>
<tr>
<td>Birth-weight (g)</td>
<td>3700±500</td>
<td>2000±600</td>
<td>&lt;0.001*</td>
<td>3000±500</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>
5.8. Conclusion

5.8.1. Main findings

The main finding of this study of agonist-induced platelet aggregation is significantly reduced platelet function in GH, PET and IUGR compared with normal pregnancy controls. For GH platelet aggregation is reduced in response to AA, COL, and TRAP. For PET, platelet aggregation is reduced in response to AA, COL, TRAP and additionally Epinephrine (Epi). For IUGR, platelet aggregation is reduced in response to all five agonists: AA, COL, TRAP, Epi and ADP. Where platelet function in GH and PET appear to be affected by 3 and 4 agonists respectively, for IUGR there appears to be a highly statistically significant global ‘hypo-reactivity’ of platelets in response to all five agonists and at all concentrations of each agonist. Similar findings were recorded in pregnancies where the fetus was considered constitutionally small. Platelet aggregation was represented as dose-response curves to each of the 5 agonists AA, TRAP, Col, Epi and ADP.

In healthy individuals, a sigmoidal curve is generated for agonist-platelet aggregation results. In pregnancy, these curves show a pattern of a reduction in response to each agonist, and this reduction appears to be further reduced in pregnancy based on severity of utero-placental disease. Advanced analyses of these curves using statistical models did not explain the differences according to disease, where the EC<sub>50</sub> and the total platelet aggregation correlated with the dose response curves. Results of platelet aggregation in response to multiple concentrations of these agonists indicate significantly reduced responses in GH, PET and IUGR compared with healthy pregnancy controls. Platelet function results did not appear to be affected by a range of maternal demographic and clinical data.
5.8.2. Platelet aggregation in normal pregnancy

As previously mentioned, results specifically of platelet aggregation in normal pregnancy and pregnancies complicated by utero-placental disease available in the literature are inconsistent. Nonetheless some similarities can be gleaned with this study. In previous studies of platelet aggregation in normal pregnancy, some of the methods described were similar to this study, in that they described platelet aggregation to agonists using versions of light transmission aggregometry, and usually with PRP\textsuperscript{197-203}. Some studies described using washed platelets\textsuperscript{202}, and some used whole blood aggregation with platelet aggregate counting mechanisms and electrical impedance\textsuperscript{206}.

For this study, healthy pregnancy subjects were recruited in the third trimester. The problem with interpreting previous studies of platelet aggregation in healthy pregnancy is numerous-fold: studies describe varying gestational ages at entry; some studies were not longitudinal in nature, and not all studies compared results in pregnancy to a control group of non-pregnant subjects. No two studies of platelet aggregation in pregnancy described the same platelet function methods. The issues with some of the study designs of these studies were that some studies included men\textsuperscript{198} as control subjects, and this may have affected platelet function results, and our group recently found that there are differences in platelet function based on age and gender\textsuperscript{73}. One study included patients in early or threatened pre-term labour\textsuperscript{201}; infection and inflammation can affect platelet function. Other studies consisted of either very small study numbers for analysis (in some cases less than ten patients\textsuperscript{197,204}), or included a broad range of gestational ages for entry\textsuperscript{202}. 

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Overall, compared to non-pregnant controls, platelet aggregation has been found to be either increased\textsuperscript{198-200}, decreased\textsuperscript{203}, or unchanged\textsuperscript{197,201,204,206} in pregnancy. There appears to be some consensus from limited longitudinal studies in pregnancy that platelet reactivity to agonists appears to increase as gestation advances\textsuperscript{200,201,203}.

Our research team recently investigated platelet aggregation in response to multiple agonists in a longitudinal study in normal pregnancy from the first to the third trimesters compared to non-pregnant controls using the modification of the gold standard light transmission aggregometry (LTA) that I used in this study\textsuperscript{203}. Compared with normal pregnancy, platelet reactivity was found to be reduced in non-pregnant women. Findings were found to be most significant for a reduction in platelet response to collagen in pregnancy compared to non-pregnant women. Following on from this, as pregnancy advanced from the first through to the third trimester, platelet aggregation was found to incrementally increase, though it still remained less than non-pregnant controls across each gestation. The strengths of this study compared to previous studies of platelet aggregation in pregnancy were that a very specific protocol for platelet handing was adopted, specific inclusion and exclusion criteria were adhered to, multiple agonists at minimal to sub-maximal concentrations were used and dose–response curves were generated. Previous studies reported near-maximal or limited concentrations of a limited number of agonists and did not describe a dose-response to multiple concentrations. All of the agonists used in this assay (AA, ADP, COL, TRAP and EPI), act synergistically to result in platelet aggregation. Therefore for a global assessment of platelet function, all of these agonists should be tested simultaneously, and this assay is capable of doing this.
5.8.3. Platelet aggregation in Gestational Hypertension

For this study I found that in GH, platelet aggregation was significantly *reduced* in response to AA, COL, and TRAP, but I did not find any differences in platelet aggregation in response to Epi and ADP. The results were not apparently affected by antihypertensive medication use or maternal demographic variables. There are some previous studies of platelet aggregation in gestational hypertension in the literature, as distinct from studies of proteinuric hypertension\textsuperscript{197,199,204,207-209,211}. In two studies, no significant differences in platelet aggregation were found in gestational hypertension compared with normotensive pregnancy\textsuperscript{201,211}. Studies described either platelet aggregation tested with PRP, whole blood (platelet counting) or whole blood electrical impedance. Two of the studies found that platelet aggregation was increased in GH compared with normal pregnancy, albeit only 1 agonist was tested\textsuperscript{199,204}. As discussed with the studies undertaken previously in healthy pregnancy, these studies in gestational hypertension were limited by the use of either a single agonist; small study group numbers for analysis\textsuperscript{204,209}, and in one study half of the patients were taking aspirin at the time of blood draw which is likely to have significantly affected platelet function results\textsuperscript{209}. A number of studies have found platelet aggregation in GH to be reduced in response to a range of different agonists\textsuperscript{197,207-209}. The results of this study indicate that platelet reactivity in response to 3 different agonists and at a range of concentrations of each agonist are reduced in GH.

5.8.4. Platelet aggregation in PET

For PET, as distinct from gestational hypertension, I found that platelet aggregation in response to AA, COL, TRAP and also Epi was even more significantly reduced than the findings in gestational hypertension compared with normotensive pregnancies. Most of the studies of platelet aggregation in pregnancy have concentrated efforts on PET, although studies have been
complicated with the same issues of variation in methods and design that comprise most previous studies of platelet function in pregnancy. There appears to be some consensus in the literature that platelet reactivity to some agonists is reduced in PET \cite{197,201,209,212,214}, and this study supports this finding. Different methods of whole blood aggregation (including percentage fall in platelets using a ‘thrombocounter’ \cite{209,212} and whole blood electrical impedance \cite{214}) and methods using PRP and variations of photometric assessments \cite{197,201} have been described.

Following studies of the pathway of platelet haemostasis in PET, as outlined, there appears to be evidence for (i) increased platelet adhesion and spontaneous platelet aggregation in healthy pregnancy \cite{285,286,288} (ii) there is some evidence for enhanced platelet activation in PET \cite{179,183,187-190,192}, and (iii) a reduction in platelet aggregation or responsiveness to a variety of agonists has been cited by a number of investigators \cite{197,201,209-213}. Significance was reached for some or all agonists used. In the study by Peracoli et al, the platelet growth factor TGF-beta was also assessed; this is a marker for platelet activation and was found to be significantly higher in PET compared with normotensive pregnancies \cite{213}. Nissel et al described whole blood aggregation with filtrometry with reduced aggregation for PET compared with normal pregnancy \cite{211}.

A research group in Baltimore, USA, concluded no change in platelet aggregation in PET, however a limitation of this study was the fact that only collagen was assessed using whole blood electrical impedance \cite{214}. A similar technique of whole blood electrical impedance was also described by an Austrian group, but with conflicting results. The latter group assessed ADP, AA and collagen as opposed to collagen alone. This group found significantly increased whole blood platelet aggregation to collagen (p<0.0001) and a non-significant increase in aggregation to ADP and AA in 10 patients at 25 weeks’ gestation who went on to develop PET \cite{46}. A study by Norris et al of whole blood platelet aggregation found that for severe PET compared with moderate PET, platelet aggregation was found to be significantly less (ADP, collagen, platelet activating factor (PAF)) \cite{210}. The authors hypothesised that the severity of disease corresponded with a reduction in platelet
function and that this was reflected by an ‘inability to produce new platelets to keep pace with the enhanced platelet destruction and consumption’, leading to a decline in platelet count and subsequent reduced platelet function.

While there appears to be some consensus regarding reduced platelet function in PET, no two studies are comparable, as the method used to assess platelet aggregation was different and not standardised (i.e. dose-response curves were not reported) such that it is difficult to interpret the findings. This study of dose-response platelet aggregation, which uses a more accurate assessment of platelet aggregation, however does appear to agree with this hypothesis, but with the most convincing evidence to date.

To further investigate the differences in platelet function based on severity of disease, further subgroup analyses revealed some compelling findings. When hypertension was further complicated by IUGR, platelet responses to ADP were found to be reduced compared with appropriately-grown pregnancies. Also compared with later-onset disease, early-onset hypertension was associated with reduced platelet responses to TRAP. Recalling one prior study of whole blood aggregation that investigated platelet function in hypertensive pregnancies complicated with IUGR compared to appropriately grown pregnancies, significantly less platelet aggregation in response to ADP was found for this study\textsuperscript{216}. Different agonists appear to be affected based on differing disease sub-type. This may support a rationale for the investigation of additional antiplatelet therapies for utero-placental disease prevention (i.e. clopidogrel for the ADP pathway; thrombin antagonists for the TRAP pathway). Larger randomised controlled trials will be required to determine the safety profiles of these medications in pregnancy.
5.8.5. Platelet aggregation in Intrauterine growth restriction

For the IUGR group, I found platelet aggregation to be significantly reduced in response to all five agonists: AA, COL, TRAP, Epi and ADP. There appears to be global ‘hypo-reactivity’ of platelets in response to all agonists and at all concentrations of each agonist in growth restricted pregnancies. Similar findings were recorded in pregnancies where the fetus was considered constitutionally small.

There are very limited studies specifically of platelet aggregation in IUGR available the literature for comparison. They conclude that platelet aggregation is reduced in IUGR\textsuperscript{215,216}. Ahmed at al investigated platelet response to the agonist platelet activating factor (PAF) in IUGR, and reported higher concentrations of PAF were required to achieve maximal aggregation compared with appropriately grown fetuses\textsuperscript{215}. However, a limitation of this study was the fact that only a single agonist was used, and only 3 patients were tested in this study.

The aim of the study by Norris et al, was to investigate if GH was complicated by IUGR as opposed to normally-grown pregnancies, did this result in a further deterioration in platelet function? When hypertension occurred in tandem with IUGR, platelets were 50% less responsive to agonist stimulation in the third trimester compared with uncomplicated pregnancies (p<0.001)\textsuperscript{216}. Platelet reactivity to ADP and collagen was correlated with the platelet activation marker β-thromboglobulin, with findings of increased platelet activation and reduced platelet function as a feature of both normotensive and hypertensive IUGR pregnancies. This indicated that a more severe clinical entity for both mother and fetus is associated with significantly less platelet aggregation in platelet function. For this study, I also found that when IUGR is further complicated by hypertension, platelet aggregation is most significantly reduced compared to normotensive IUGR. For this study I further categorised all conditions in utero-placental disease, based on
severity of disease or gestational age at diagnosis. It is not clear if other researchers did this.

There is some limited evidence to suggest platelet activation is different in pregnancies complicated by IUGR for both mother and fetus: platelet activation appears to be increased and consequently platelet reactivity may be reduced\(^\text{185}\). There is some consistency in the reports of MPV in established IUGR, where mean platelet volume has been shown to be increased in pregnancies that go on to develop IUGR, though MPV has not been established as a useful predictor of disease\(^\text{157,312,313}\).

Further sub-group analysis in this study found that platelet aggregometry results in patients with IUGR and abnormal UA Doppler waveform (\(n=13\)) to ADP and TRAP were significantly reduced in comparison to those with normal UA Doppler. Doppler changes have been correlated with platelet function and similar agonists in some prior studies. ADP-induced platelet aggregation was found to be increased in patients with abnormal uterine artery Doppler who subsequently developed IUGR\(^\text{312}\). Assessment of uterine artery Doppler waveforms was not included in my study, and therefore a comparison with this study cannot be achieved but it suggests that platelet responses to ADP may be altered in severe utero-placental disease, or that platelet function could be a useful predictor of disease. A limitation of the aforementioned study was that near-maximal concentrations of the agonist ADP was used (0.5, 1, 2, 5µM), compared to the multiple submaximal concentrations of agonists I used in this current study for ADP (20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µM).

A further study of platelet function in mother-fetus pairs with abnormal UA Doppler found no difference in maternal platelet aggregation, however in response to limited concentrations of a single agonist, thrombin\(^\text{185}\). Also, within this study 7 patients treated with aspirin were included in the overall analysis. For this study differences in response to TRAP (a synthetic thrombin) were however noted for cases with abnormal UA Doppler in IUGR.
To further investigate the differences in platelet function based on severity of disease, further subgroup analyses revealed some compelling findings. It appears that when IUGR is complicated by superimposed GH/PET, platelet function appears to be further globally impaired, indicating that platelet function may already be deteriorating in a pregnancy complicated by IUGR that will later develop hypertension. This may suggest a strong indication for aspirin for IUGR. This may also suggest that platelet function could be a useful predictive tool for the further deterioration of established utero-placental disease.

In conclusion there are some limited studies of platelet aggregation in IUGR, and although study designs and methods of assessing platelet aggregation differ between these studies, the results of this study appear to also conclude that platelet function is reduced in IUGR. I have used a standardised platelet assay to accurately describe platelet function in utero-placental disease. The studies of platelet function in pregnancy to date have used either single agonists, or limited concentrations of agonists, whereas in this study incremental concentrations of five different agonists, which represent a more physiological assessment, were tested.

For this study the study groups, particularly for IUGR, comprised of a well-defined cohort, with analysis occurring after delivery outcomes and placental histopathology results were available. This data collection afforded me the opportunity to confirm the antenatal diagnosis and to investigate platelet behaviour on not only ‘pathologically small’ but also on infants that demonstrated increasing in-utero growth trajectories with a normal delivery outcome, which has not been previously reported to my knowledge to date. Subgroup analysis based on severity of disease gave more comprehensive information regarding deterioration in platelet function based on worsening clinical condition for the mother and fetus.

IUGR with superimposed PET often occurs prior to 37 weeks' gestation and contributes significantly to iatrogenic preterm delivery and perinatal morbidity\textsuperscript{37,311}. These results suggest that by interrogating platelet function in IUGR, the
pregnancies most at risk for adverse maternal and fetal outcome may be identified early, perhaps necessitating more intense surveillance, or delivery where appropriate. The concept is a little like the ‘chicken and the egg’, where it is difficult to predict which comes first (i.e. the fetal condition, IUGR or the maternal condition, GH/PET)? What would be useful in clinical practice would be a test that could predict at an early stage those cases that will likely be further complicated and thus warrant closer supervision in pregnancy, or appropriate timing of corticosteroid administration prior to delivery. This is always a challenge in obstetrics and has considerable implications for neonatal care, because these severe cases tend to be delivered at an earlier gestational age, placing a huge resource burden on neonatal services. If we had a test that could help us predict severe disease early, then this would revolutionise the way we care for these patients, and would help us focus our already limited resources. Further longitudinal studies of platelet function are required to determine the utility of platelet function testing as a predictor for utero-placental disease.

A weakness of this study could be the lack of longitudinal data particularly from the first trimester. Given the natural evolution of utero-placental disease I missed an opportunity to study platelet changes during a subclinical period before established disease. Information on platelet function early in complicated pregnancies might afford the opportunity for an accurate predictive test of adverse pregnancy outcome. However the method of blood sampling for this study and the large blood volume required (30ml) for testing would make serial sampling impractical within the setting of a large prospective study in pregnancy throughout all trimesters.

In the next chapter of platelet function in utero-placental disease, I will investigate platelet function using a near-physiological assay that is capable of describing the more subtle platelet behaviours occurring in the actual maternal circulatory conditions. This assay uses only 150µml of blood and is therefore one step closer to a point-of-care test of platelet function that may become a useful marker in pregnancy. Also the assay uses whole blood rather that platelet-rich plasma, and
is therefore more physiological. In unpublished data from our research group this assay has also been tested and validated in a large cohort of non-pregnant healthy people and those at risk of major adverse cardiovascular events (MACE), with interesting results indicating a possible correlation with clinical outcome. This assay has never been tested before in pregnancy. Investigations using this final assay may help to further investigate the differences in agonist-induced platelet aggregation depending on the spectrum of clinical presentation.
Chapter 6. **Results of the Dynamic Platelet Function Assay**
6.1. Concise summary

In the arterial circulation following plaque rupture or endothelial damage platelets initially tether, adhere and roll on the exposed vascular surface by interacting with von Willebrand factor (VWF), and will eventually aggregate to form platelet-mediated thrombus. In this final chapter I studied platelet function using a novel microfluidic platelet vascular assay that measures these initial platelet interactions in whole blood under arterial shear flow, on a surface that is designed to mimic a damaged vascular bed. In utero-placental disease, significant differences in platelet parameters on VWF were identified compared to healthy third trimester pregnancies. Differences in platelet behaviours were found across the three conditions of gestational hypertension (GH), pre-eclampsia (PET) and intrauterine growth restriction (IUGR).
6.2. Introduction

Recalling the sequence of events of the haemostatic function of platelets from platelet adhesion to activation to aggregation, most available assays are unable to describe the initial platelet adhesive properties. The interaction of platelets with the vascular surface in the arterial system is mediated by the exposed extracellular matrix and von Willebrand Factor (VWF) (which is immobilised to the exposed endothelium from plasma)\textsuperscript{194}. Platelets in the circulation recognise the damaged vascular nidus and VWF and will tether to the surface (adhesion), ‘roll’ or translocate over the surface (mediated by platelet activation), and will finally ‘stick’ to the surface (aggregation) by interacting with VWF. These interactions are multiple and complex and generally require just seconds before the final result of platelet-plug formation is obtained. These platelet functions are mediated by arterial shear-flow, which propels platelets across the vascular surface\textsuperscript{262,263,265,266,269}.

To date, our research group has developed and characterised, using human blood samples, an assay that rapidly and reliably measures the interactions of platelets with VWF under arterial shear\textsuperscript{283,284}. The assay is a microfluidic chip that has VWF uniformly coated over its surface. Microliter samples of whole blood, labelled with fluorescent dye to identify the platelets, are aspirated across the surface at a defined arterial shear rate. Thus the VWF mimics a damaged vascular bed and the assay is capable of describing the interactions of the platelets with the surface, but under the normal arterial flow conditions the platelets would otherwise be exposed to, as opposed to static in-vitro conditions. This assay is called the dynamic platelet function assay (DPFA), and has been described in more detail in the materials and methods chapter.

As outlined in the introduction to this chapter, in the arterial circulation, platelets initially tether to VWF via the Glycoprotein (GP) GPIb receptor\textsuperscript{193,194}. Final arrest of the platelet and platelet-platelet aggregation is mediated by GPIIb/IIIa\textsuperscript{195}. The first
step is highly reversible, and in unpublished data from our research group on over 500 non-pregnant donors we have shown that platelets in some individuals tether rapidly to the VWF and remain ‘stuck’ or static; some platelets continue to tether or ‘roll’ along the surface, and some platelets do not interact with the VWF surface at all. These initial interactions can contribute to the process of arterial platelet thrombus formation, where for some individuals this may lead to inadvertent arterial micro-thrombus formation however for others this may place the patient at risk of bleeding. Since GPIb binding to VWF is the initiating process for arterial thrombosis, and platelet arrest via GPIIb/IIIa is the final element, the DPFA mimics the fundamental dynamic behavior of platelet thrombosis in-vivo, in effect from beginning to end.

Several new parameters of platelet function are thus measured using the DPFA as platelets tether, adhere and translocate or roll across VWF. These interactions are captured in real-time from frame to frame over a total of 500 frames (corresponding to approximately 20 seconds) such that not only the end-result of platelet thrombus coverage on the final frame is achieved, but also all of the platelet interactions that led to that point. Individual platelets can actually be visualised mobilising across the chip under the experimental conditions that are set to recreate arterial flow rates, and because the sample has lipophilic dye added to it before the experiment, platelets are easily identified. Using a novel parallel plate flow chamber coated with purified human VWF and custom-designed platelet tracking software, this system can accurately measure multiple platelet behaviours on VWF.

The dynamic platelet parameters have been characterised and defined in detail in previous publications\textsuperscript{73,283,284}. In unpublished data from our research group significant differences in platelet function using the DPFA in patients with stable and unstable coronary artery disease were found that may indicate patients who are possibly at risk of future major adverse cardiovascular events (MACE). I recently published with our research group on age-related changes in platelet function using the DPFA, where platelet parameters were found to be more
profound in women than men\textsuperscript{73}. We hypothesised that given the flux of oestrogen levels in women in later life this may result in changes in platelet function, and this assay may identify women at risk of MACE. The contribution of shear mechanical force in promoting this platelet-vessel adhesion with WVF is of specific interest in this study in pregnant patients because platelet function changes in the maternal arterial system extending into the maternal spiral arterioles of the placenta may link utero-placental disease and cardiovascular disease.

I also collaborated recently on a study using the DPFA in preterm infants (Cowman et al 2016). Compared with term infants (n=13) preterm infants < 32 weeks’ gestation (n=15) demonstrated significantly differing patterns of platelet translocation on VWF\textsuperscript{314}. These subtle changes in platelet parameters may either contribute to an increased bleeding tendency in this vulnerable group, or may indicate a compensatory mechanism to counteract the increased bleeding risk. Because a huge weight of the pre-eclampsia and intrauterine growth restriction burden is attributed to prematurity, there is a compelling rationale to understand the effect of platelet function in utero-placental disease. Prevention of disease in the neonate begins with prevention of disease in the mother. The focus of this chapter is to understand the differences in dynamic platelet function in mothers that may be related to utero-placental disease that ultimately affects the neonate.

Results of the different platelet function assays previously investigated in pregnancy to date have already been described in detail in this thesis. Most of the assays used were not capable of replicating the flow and shear environment that platelets are exposed to in-vivo. An inherent challenge of platelet function testing in pregnancy is that of the significant cardiovascular and hormonal changes that should be taken in account when assessing platelet function in pregnancy\textsuperscript{77}. Cardiac output is increased by 30% by the second trimester, with further haemodynamic changes in pregnancies complicated by gestational hypertension and PET, placing a huge physiological strain on maternal endothelium. The vascular and haemodynamic changes in normal and complicated pregnancy are
likely to affect platelet function in-vivo. The literature on platelet function in pregnancy to date describes static, in-vitro conditions that are not physiological.

There are limited studies in the literature regarding assays broadly based on shear-flow platelet function in pregnancy. A type of shear-based assay called the Platelet-Function Analyser 100 (PFA-100 analyser) has previously been investigated in pregnancy\textsuperscript{114,115,278-282}. This platelet assay assesses the effect of shear flow on gross platelet aggregation and clot formation. The assay can measure the time taken for whole blood samples under high shear flow to block a hole pre-treated with a thrombogenic surface, e.g. collagen. Thus, under shear flow, a platelet plug will form in the collagen filled hole, and the time it takes for this to occur is measured and described as the ‘closure time’, CT, such that a longer CT is associated with bleeding tendencies and a shorter CT is associated with thrombosis.

Using the PFA-100 analyzer, CT has been shown to be less in pregnancy compared with the non-pregnant state, which suggests a possible increased coagulation in pregnancy (i.e. it takes less time for platelet-plug to form in pregnancy using this assay)\textsuperscript{280}. In pregnancies complicated by PET compared with healthy pregnancy controls, the magnitude of CT was found to be greater in PET in a number of further studies, suggesting a possible impairment in coagulation and a tendency to bleeding in PET\textsuperscript{279,281,282}. One study demonstrated prolonged CT in patients with GH\textsuperscript{278}. These studies did not correlate CT results with actual blood loss at delivery or rate of PPH, which would have been a useful correlation to investigate the clinical utility of such tests. In the final results chapter in this thesis I will investigate if there is a correlation between platelet parameters assessed using the DPFA and blood loss at delivery.

While the PFA-100 analyser is a type of shear-flow based test of platelet function, it only measures a single output, gross platelet-mediated clotting time, under very high shear rates, and it does not represent the true physiological or pathological conditions platelets are exposed to in-vivo. Collagen is the surface generally used
in the PFA-100 system. Collagen is highly thrombogenic, and when used in shear-flow assays, it can give information about stable platelet-platelet aggregation. This interaction is the final step in platelet aggregation\textsuperscript{234,273}. The PFA-100 does not provide information on specific platelet behaviours, rather it measures the general ability of platelets to form clot. The process of platelet adhesion, activation and aggregation and thrombus formation is not a static condition. In order to replicate these functions in the most physiological way possible, devices that mimic in-vivo flow conditions and are capable of capturing these specific interactions are required. The DPFA is capable of achieving this.

A recent study using a more advanced shear-flow assay than the PFA-100 was evaluated in pregnancy\textsuperscript{292}. This study described a platelet function assay that is probably the most comparable to the DPFA, the assay that will be tested in this thesis. In this study by Valera et al, collagen was used to coat the surface of a microfluidic chip, as opposed to just the aperture of the PFA-100 analyser (in the DPFA VWF is used to coat the surface of the chip). Anticoagulated blood pre-treated with dye to identify platelets was perfused over the collagen surface for a total of 2 minutes at high-shear rate. Platelet thrombus formation was actually visualised at the end of the 2 minutes by confocal microscopy, and was expressed as a percentage of the total area covered for normal pregnant participants compared with non-pregnant controls. Significantly less platelet-collagen thrombus formation was found in pregnancy compared with non-pregnant controls (p<0.001).

This study appears to be the only available study in the literature of a microfluidic-type shear-based assay in pregnancy. The study indicates that under high shear-stress, platelet binding to collagen is reduced in pregnancy. Again, though, like the PFA-100 analyser, the results using this particular assay describe the final steps in platelet aggregation, not the initial steps. To the best of my knowledge there are no previous studies of shear-flow mediated platelet behaviour on VWF in pregnancy and utero-placental disease. Compared with the aforementioned study that used collagen as the surface, and reported on the final platelet-mediated
thrombus after 2 minutes, the DPFA measures all of the individual platelet-VWF interactions over 30 seconds in addition to the cumulative end result of platelet-thrombus effect, thus the DPFA is capable of generating a significant number of novel platelet parameters and not just thrombus formation.

Our understanding of the contribution of platelets to the pathophysiology of PET and utero-placental disease is still currently limited. Despite this, a therapy targeted at platelets prevents disease in select subgroups\textsuperscript{31,42,43}. There currently exists no single biomarker with sufficient power to predict the development of these serious conditions early in pregnancy, at a time where therapy such as aspirin could be prescribed to potentially encourage healthy placentation, and thus prevent short and long-term complications for both mother and fetus\textsuperscript{18-22,65}. Women most at risk of these conditions remain ‘hidden’ within routine antenatal care\textsuperscript{53}. By firstly properly characterising platelet changes in conditions complicated by established utero-placental disease we may understand the contribution of platelets to the development of the condition, in addition to characterising the changes in platelet behaviour that may be considered a predictive tool when applied in early pregnancy.

It is logical to investigate platelet function under arterial flow conditions because in pregnancy and utero-placental disease, the vessels in the placenta undergo considerable dynamic vascular changes and remodelling, where the sub-endothelium and VWF is continually exposed. In utero-placental disease and specifically PET, widespread overall endothelial damage is a feature. Thus the DPFA is a vascular assay that in effect recreates the conditions platelets are exposed to in the placentas and maternal vasculature of healthy pregnancies and pregnancies complicated by utero-placental disease. This has never been investigated before in pregnancy by other research groups. The aim of this chapter was to investigate a number of novel multi-parameters of platelet behaviour in healthy pregnancy compared with utero-placental disease using the dynamic platelet function assay (DPFA).
6.3. Study Design

Patients with a singleton pregnancy and with a diagnosis of either Gestational hypertension (GH), pre-eclampsia (PET) or intrauterine growth restriction (IUGR) detected from 24 weeks' gestation and beyond were recruited in the Rotunda Hospital over the course of the study time-frame. Dynamic platelet function results for these groups of patients were compared to results for the next available third trimester singleton healthy controls, who were confirmed to have had a normal pregnancy outcome after delivery. In addition, non-pregnant female participants were recruited in RCSI, St. Stephen’s green, and platelet function results were compared to healthy third trimester patients to investigate normal pregnancy-related changes in platelet function. The details regarding the study design and recruitment have already been described in previous chapters.

6.4. Materials and Methods

6.4.1. Dynamic Platelet Function Testing methods

6.4.3.1. Phlebotomy

Blood samples were taken according to the protocol as previously described. Only 10ml venous blood samples were required for this assay. The first 5ml of blood drawn was sent to the Rotunda laboratory for a full blood count to confirm a normal platelet count. The remaining 5 ml of blood was used for the dynamic platelet function assay. Immediately prior to sampling, the laboratory was informed to enable adequate and timely preparation. The blood samples were transported in a padded blood transport box to avoid activation of the platelets. Blood samples were kept at room temperature with gentle rocking and used within 1 hour of
phlebotomy. Whole blood cell counts were recorded for each donor, using a Sysmex-KX21N haematology analyser (Sysmex Corp., Kobe, Japan), to confirm normal platelet count prior to analysis.

6.4.3.2. The DPFA assay

In brief, the chips used for the assay were prepared before the blood samples were obtained. Platelets were fluorescently labelled and blood was drawn through silicone tubing at a defined rate which enabled it to be perfused through the flow chamber, and across the VWF-coated surface. Platelets were imaged by video fluorescence microscopy in real-time as they tether, adhere, roll, and form thrombi on the VWF surface (figure 6.1). The sequences of fluorescence images were then processed and analysed by the Irish Centre for High-End Computing (ICHEC). ICHEC maintains a portal system on which all platelet data were uploaded.
Figure 6.1 Platelets (green) are covering the von Willebrand factor (VWF) surface of the dynamic platelet function assay (DPFA) at the end of the assay run and this is the typical appearance of the final frame (frame 500) (reproduced with permission by Professor Dermot Kenny). This parameter is called the % end-surface coverage. Platelets have been propelled across the chip under high speed arterial flow and have adhered and aggregated to the surface.
6.5. Data collection

Clinical data was collected as outlined in the material and methods. Patient data was anonymized and each donor was identified via a unique identifier. Platelet parameters were linked with unique patient identifier codes for analysis by ICHEC.

6.6. Statistical analysis

Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample independent t-test for continuous data. Continuous data were checked for non-normality and for outliers. Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). Data management and statistical analysis were performed using SAS Version 9.3®, GraphPad PRISM Version 6® and SPSS Version 22®.

6.6.1. Statistical analysis for dynamic platelet parameters

An advanced tracking system developed by ICHEC was used to generate results of the various platelet parameters above. Each blood sample from each participant was analysed in triplicate. This was done in order to address the inter-individual variability of the assay that may be related to blood condition or experimental error. All runs from each cohort were then analysed, and runs outside the mean ± 2SD were removed before each donor's parameters were averaged over 3 or less eligible runs. This process facilitated detection and removal of extreme outliers to determine a normal reference range. Normality was assessed using a D’Agostino and Pearson omnibus normality test. A two-tailed unpaired or unpaired Welch's t-test was used where appropriate on each parameter derived from the assay's image analysis. All the data is presented as mean ± standard error of the mean (mean ± SEM) unless otherwise stated.
6.6.2. Key DPFA outputs

The parameters are defined as the main parameters of platelet adhesion/tracks (tethering), translocation (roll) and stasis (sticky platelets that lead to thrombus or % end surface coverage). Our research group has demonstrated the clinical utility of this system to provide such information. The six key DPFA-measured platelet “motional” parameters captured from sequences of fluorescence video frames over 500 frames in total are described below:

The key parameters of the DPFA are:

1. The platelets that interact with VWF (nTracks),
2. The number of platelets that roll on VWF (nTrans),
3. Stable adhesion of individual platelets (nStatic)
4. The velocity of platelets that roll (nVel)
5. Unstable platelet interactions (where a platelet interacts with VWF >10 frames but <490 frames, i.e. the platelet may behave in a ‘stop- start’ way)
6. The surface coverage of the chip at frame 500 (% surface coverage)
6.7. Clinical Results:

A total of 102 pregnant patients and 32 non-pregnant participants were recruited to the study of DPFA (figure 6.2). The pregnant patients consisted of cases with a singleton pregnancy and with a diagnosis of either gestational hypertension (GH), pre-eclampsia (PET) or IUGR (EFW<10th centile), and results of these groups of utero-placental disease were compared to healthy third trimester controls. A total of 14 patients were initially recruited and later excluded due to various reasons as outlined in figure 6.2. This resulted in final groups for analysis consisting of non-pregnant females (n=32), normal third trimester pregnant controls (n=22), GH (n=16), PET (n=27) and IUGR (n=23)

The clinical outcomes for each of the conditions GH, PET and IUGR compared with normal pregnancy controls are outlined in the next chapter that correlates clinical findings with DPFA results.
Figure 6.2 Study Design of the patients recruited for the dynamic platelet function assay (DPFA).

*PPROM Prelabour premature rupture of membranes, **SSRI selective serotonin reuptake inhibitor
6.7.1. Dynamic Platelet function analysis (figures 6.3-6.6)

Compared with non-pregnant females, healthy pregnant patients appear to have significant differences in a number of different platelet parameters, and these parameters generally appear to be reduced in pregnancy (figure 6.3). For utero-placental disease compared with healthy pregnancy, a pattern of further reductions in parameters of platelet function appear depending on the clinical subtype: for IUGR, the only significant finding is a reduction in the number of static platelets (p<0.05) (figure 6.4); for GH a significant reduction is observed in 4 parameters: platelet tracks, platelet translocation, platelet stasis and unstable platelet interactions (p<0.05 respectively) (figure 6.5), and for PET, a greater statistically significant reduction in platelet parameters are observed for the same 4 parameters: platelet tracks (p<0.01), platelet translocation (p<0.01), static platelets (p<0.05) and unstable platelet interactions (p<0.01) (figure 6.6).

This implies that differences in differing platelet motions on VWF appear based on clinical presentation on the spectrum of utero-placental disease: dynamic platelet behaviours appear to be reduced in healthy pregnancy compared with non-pregnant females, and further reductions in parameters appear in IUGR, GH and PET. The most statistically significant reductions in platelet parameters were observed in the PET group compared with healthy pregnant controls. Recalling that for agonist-induced aggregation, platelet aggregation was also reduced. Using the DPFA, multiple subtle parameters and behaviours of platelets on VWF also appear to be reduced in pregnancy and utero-placental disease.
Figure 6.3 In healthy pregnancy (n=22), (A) platelet tracks, (B) platelet translocation (C), platelet speed and (F) percentage end surface coverage of platelets at the end of the assay are all significantly reduced compared with non-pregnant controls (n=32) (p<0.001). This indicates that pregnancy results in overall less platelet interaction with VWF surface. An unpaired t-test and unpaired t-test with Welch’s correction were used where appropriate to detect statistically significant differences between the groups. Data is represented as mean ± SEM.
Figure 6.4 In Intrauterine Growth restriction (IUGR, n=23) only one parameter, (D) the number of static platelets (platelets that stably adhere to the VWF), was found to be significantly reduced compared with healthy pregnancy controls (n=22) (p<0.05). This indicates in IUGR there are less ‘sticky’ platelets compared with healthy pregnancy. An unpaired t-test and unpaired t-test with Welch’s correction were used where appropriate to detect statistically significant differences between the groups. Data is represented as mean ± SEM.
Figure 6.5 In gestational or pregnancy-induced hypertension (PIH, n=16) (A) platelet tracks, (B) platelet translocation, (D) platelet stasis and (E) unstable platelet interactions were significantly reduced compared with healthy pregnant controls (n=22). This indicates that in hypertension in pregnancy, platelets interact less with the VWF. An unpaired t-test and unpaired t-test with Welch’s correction were used where appropriate to detect statistically significant differences between the groups. Data is represented as mean ± SEM.
Figure 6.6 In pre-eclampsia (PET n=27) (A) platelet tracks, (B) platelet translocation (D), platelet stasis and (E) unstable platelet interactions were significantly reduced compared with healthy pregnancy controls (n=22, p<0.01). This indicates that in PET platelets interact the least with the VWF surface compared with all other pregnancy groups. An unpaired t-test and unpaired t-test with Welch’s correction were used where appropriate to detect statistically significant differences between the groups. Data is represented as mean ± SEM.
6.8. Explanation of DPFA results in pregnancy and utero-placental disease

6.8.1. Healthy pregnancy compared to non-pregnancy controls

A number of dynamic platelet parameters including platelet tracks, platelet translocation, platelet velocity and % end-surface coverage are reduced in healthy pregnancy compared with non-pregnant controls.

A total of 32 non-pregnant women (RCSI, St. Stephen's Green staff) volunteered to have dynamic platelet function testing, and results were compared to 22 healthy third trimester pregnancy controls. Dynamic platelet function was found to be significantly altered in pregnancy. Within the healthy pregnant cohort a significant decrease in the number of platelet tracks (271 ± 20 versus 426 ± 20; p<0.0001); platelet translocation (165 ± 10 versus 290 ± 14; p<0.0001), and a reduction in velocity on VWF (2.4 ± 0.2 μm/sec versus 4.8 ± 0.4 μm/sec; p<0.0001) was observed compared to non-pregnant controls.

There were no significant differences observed in the total number of static platelets (91 ± 8 versus 93 ± 6), or in the numbers of unstable platelet interactions on VWF (93 ± 14 versus 123 ± 11) for pregnant patients compared to non-pregnant controls. Nonetheless, it was identified that pregnancy was associated with a significant decrease in the percentage of platelet surface coverage (i.e. the number of platelets visible on the last frame, representing an end-point of the first initial platelet-to-surface (VWF) interactions, and a cumulative platelet effect) (5.5 ± 0.3 % versus 7.9 ± 0.5 %; p<0.0001) (figure 6.3). The significant differences in multiple platelet parameters observed in pregnancy may represent pregnancy-specific alterations in platelet function.
6.8.2.  Intrauterine growth restriction (IUGR)

The number of static platelets on VWF in IUGR is reduced compared with healthy third trimester controls with an appropriate-grown fetus.

Dynamic platelet function of 23 pregnancies complicated by IUGR was compared to 22 third trimester controls with an appropriately-grown pregnancy. There were no significant differences in the number of platelet tracks (238 ± 21 versus 271 ± 20), platelet translocation (160 ± 15 versus 165 ± 10) or platelet velocity (2.3 ± 0.1 μm/sec versus 2.5 ± 0.2 μm/sec) for IUGR pregnancies compared to healthy third trimester pregnancy controls. However, in the IUGR group there was a statistically significant reduction in the number of static platelets (68 ± 6 versus 91 ± 8; p<0.05). There were no significant differences noted in either the total number of unstable platelet interactions on VWF (72 ± 8 versus 93 ± 14) or in the percentage of platelet surface coverage (4.9 ± 0.3 % versus 5.4 ± 0.3 %) (figure 6.4).

6.8.3.  Gestational Hypertension (GH)

The number of platelet tracks, platelet translocation, static platelets and unstable platelet interactions on VWF is significantly reduced in GH compared with healthy normotensive third trimester controls.

A total of 16 patients with gestational hypertension (GH) were recruited from 24 weeks’ gestation and beyond and DPFA results were compared to 22 third trimester normotensive controls. When compared with healthy pregnant controls, GH was associated with significantly reduced platelet tracks (208 ± 18 versus 271 ± 20; p<0.05), platelet translocation (133 ± 12 versus 165 ± 10; p<0.05), static platelets (59 ± 5 versus 91 ± 8; p<0.05) and unstable platelet interactions with VWF (52 ± 7 versus 93 ± 14; p<0.05). No significant difference was observed in
velocity (2.2 ± 0.2 μm/sec versus 2.5 ± 0.2 μm/sec) or in the percentage of platelet surface coverage (4.8 ± 0.3 versus 5.4 ± 0.3 %) (figure 6.5).

6.8.4. Pregnancy with preeclampsia (PET)

The number of platelet tracks, platelet translocation, static platelets and unstable platelet interactions on VWF is most significantly reduced in PET compared with healthy normotensive third trimester controls.

A total of 27 patients with a diagnosis of preeclampsia (PET) were recruited from 24 weeks’ gestation and beyond and dynamic platelet function results were compared to 22 healthy third trimester controls. The following parameters were significantly reduced in PET compared with normal pregnancy: platelet tracks (187 ± 10 versus 271 ± 20; p<0.01); platelet translocation (117 ± 8 versus 165 ± 10; p<0.01); platelet stasis (61 ± 5 versus 91 ± 8; p<0.05) and unstable platelet interactions with VWF (59 ± 7 versus 93 ± 14; p<0.05). No significant difference was observed in velocity (μm/sec 2.3 ± 0.2 versus 2.5 ± 0.2 μm/sec), compared to controls. There was also a trend towards a decreased percentage of platelet surface coverage (4.7 ± 0.3 versus 5.4 ± 0.3 %) in PET, but this was not found to be statistically significant (figure 6.6).
6.9. Conclusion

The dynamic platelet function assay (DPFA) is a sophisticated and near-biological test of how platelets actually interact through initial vessel contact. This study demonstrates (i) significant differences in multiple novel platelet behaviors in pregnancy compared with non-pregnant controls and (ii) further differences in utero-placental disease compared with heathy pregnant controls.

In normal pregnancy a significant reduction in the total number of platelets interacting with the VWF (platelet tracks) was observed in addition to a reduction in the total number of translocating platelets, the velocity of the interactions and the degree of surface coverage of platelets at the end of the assay. Platelets are demonstrated to interact less with the VWF surface in third trimester pregnancy compared with non-pregnant controls.

In utero-placental disease platelets appear to interact even less with VWF. Platelets appear to only be less ‘sticky’ in IUGR. Static platelets can be visualised with the naked eye as appearing on the screen as the platelets flow over the surface under arterial shear-flow, but these platelets stick down to the VWF surface almost immediately and do not translocate off again. The other parameters do not appear to be affected in IUGR.

The parameters platelet tracks, translocation, stasis are reduced in GH and PET compared with normal third trimester controls. For the hypertensive groups, unstable platelet interactions with the VWF were also found to be reduced. This particular parameter defines platelets that interact with the surface across more than 10 frames but less than 490 frames, in other words these platelets can be visualised as translocating along the surface, adhering for a short-time but then they translocate along the surface again. This is a novel platelet parameter and may indicate unstable platelet behaviour in hypertension in pregnancy. Overall, it
is as if platelets are ‘ignoring’ the VWF surface in utero-placental disease, and specifically in PET. This indicates a platelet dysfunction in the condition.

These significant differences in multiple novel platelet parameters may be reflective of pregnancy-specific and disease-specific platelet differences in subtle platelet parameters that are not identifiable using standard aggregometry assays. There appears to be a trend in a reduction of platelet behaviours based on clinical sub-type of utero-placental disease.

To further investigate the reasons for this, in the next chapter, I performed a number of sub-analyses and correlations to determine the effect of potential maternal demographic and clinical confounding variables on platelet function results.
Chapter 7. **Sub-group analysis of the DPFA results**
Platelet function, as assessed with a novel dynamic platelet vascular assay, is altered in utero-placental disease compared with healthy pregnancy controls. Platelets appear to interact less with the VWF assay surface particularly for the conditions GH and PET. A sub-group analysis did not reveal any differences based on maternal demographic variables and dynamic platelet parameters. An experiment using a single pregnant control indicated that labetalol or Dexamethasone use in pregnancy may affect platelet function. A further sub-group analysis based on severity of clinical presentation of PET, indicated that early-onset disease < 34 weeks' gestation is associated with further significant reductions in platelet behaviour on VWF compared with later-onset disease. Platelet function assessed using the DPFA may be a marker for utero-placental disease.
7.2. Introduction

7.2.1. Maternal demographic variable effect on platelet function

In the preceding chapter, significant differences were found in a number of novel dynamic platelet parameters in utero-placental disease compared with healthy pregnant controls. To further investigate the differences based on clinical presentation, I performed a number of sub-analyses. A number of demographic variables such as increasing BMI, older age categories, smoking and hypertension have been shown to affect platelet function outside of pregnancy. Our research group recently demonstrated using this specific assay that platelet behaviour was different in males and females, with differences based on age. There is evidence for the effect of vasculopathy, such as the micro-angiopathy on arterial shear stress and platelet function. Since a number of patients had a history of chronic hypertension, I investigated whether the presence of microvascular disease was associated with changes in DPFA results. I determined the effect of all of the above maternal variables on DPFA results for GH, PET and IUGR.

7.2.2. Results

Refer to the clinical results tables (tables 7.1-7.3) at the end of this chapter for reference. No differences in platelet behaviour using the DPFA were noted for age, BMI, ethnicity, parity or smoking status (p>0.05). Given the association between a pregnancy affected by utero-placental disease and the development of later-onset cardiovascular disease, I also sought to investigate any confounding effect on platelet function for those who were documented to have had a history of utero-placental disease, or a current history of essential hypertension. There were no differences in platelet function noted for those
patients with such a history compared to those without (p>0.05). Diabetes mellitus and gestational diabetes were both strict exclusion criteria for this study.

The lack of effect of these maternal demographic factors on platelet function results in pregnancy in this study indicate that platelet functional changes appear to be true and may be a reflective of the utero-placental condition, rather than any confounding maternal factors.

7.3. The effect of medication use on DPFA platelet function in utero-placental disease

7.3.1. Analysis of DPFA results in relation to antihypertensive medication use in pregnancy

The beta blocker labetalol is the usual first line therapy for hypertension in pregnancy and the majority of the cases in this study were treated with this antihypertensive medication. Although principally a beta blocker, labetalol also has alpha blocking properties. Platelets have an alpha receptor. I have already demonstrated a lack of effect of labetalol on platelet aggregation assessed either by spontaneous platelet aggregation (SPA) or agonist-induced platelet aggregation. I hypothesised that the alpha blocking properties of labetalol might affect more subtle platelet functions that can be assessed with the DPFA. To this end I compared DPFA results for pregnant patients treated with and without labetalol and additional antihypertensive medications.
7.3.2. Results

A total of 44% of patients with utero-placental disease were prescribed antihypertensive medications overall (4% (1/23) IUGR, 69% (11/16) PIH, 63% (17/27) of PET cases) (tables 7.1 and 7.2). Labetalol use alone comprised of 66% of all cases. Compared to patients treated with antihypertensive medications (n=15), there were no differences in platelet translocation (117 ± 49 versus 132 ± 46 p = 0.3033) or platelet stasis (112 ± 37 versus 106 ± 38 p = 0.6146) for those who were conservatively managed (n=37). It is also worth mentioning that a similar proportion of patients were prescribed antihypertensive medications within the PET and GH groups, and yet significance in platelet parameters overall was observed mainly for the PET group, therefore the dynamic platelet function results are not likely due to antihypertensive medication effect alone at the time of platelet function testing.

Next, I compared platelet function results for patients on dual therapy (labetalol and adalat) versus single therapy (labetalol); and for patients on triple therapy (labetalol, adalat and α-methyl-dopa) versus single therapy (labetalol). Though the numbers were notably small for statistical comparisons between these groups, nonetheless there were no differences noted in platelet translocation and stasis for poly-pharmacy compared to single-pharmacy (p>0.05).
7.3.3. The effect of medication use on DPFA platelet function in 500 non-pregnant donors

To further investigate if labetalol affected the DPFA results, I analysed data from a large database of over 500 non-pregnant patients at risk of cardiovascular disease from our research group with the assistance of ICHEC and RCSI statisticians to determine if the use of beta blockers outside of pregnancy resulted in any differences in platelet parameters assessed using the DPFA. After excluding unstable patients at risk of MACE and patients with acute presentations to Beaumont Hospital, I analysed data for patients attending the out-patients who were documented as having been prescribed a beta-blocker.

7.3.3.1. Results

In 35 patients (men and women) treated with beta blockers compared to 15 who were not, no differences were noted in a number of platelet parameters (figure 7.1). Results were also stratified according to gender with no differences noted for men and women (p=0.88).

The results of this analysis indicate that the use of antihypertensive medication, specifically labetalol, does not appear to affect platelet function in men and non-pregnant females. The analysis of the pregnancy data also fails to identify a confounding effect of labetalol on novel platelet parameters assessed using the DPFA.
Labetalol use does not appear to affect platelet function as assessed using the DPFA in men or non-pregnant women. Of 50 non-pregnant females and males from a database of 500 patients with cardiovascular disease, a total of 35 patients attending the outpatients were taking a beta blocker and 15 were not. An analysis of variance on four parameters (static platelets, translocating platelets, velocity, and percentage end surface coverage) did not reveal any significant differences in those on beta blockers compared to those without.
7.3.4. An experiment to test the effect of Labetalol and Dexamethasone on dynamic platelet function in pregnancy using the DPFA

To further test the hypothesis of a medication effect of dynamic platelet function, I performed an additional experiment investigating the differences in dynamic platelet function with labetalol use, where I directly added soluble labetalol to a whole blood sample of a healthy patient in the third trimester. I performed this experiment with the help of BDI at Beaumont hospital. I also assayed the effect of the corticosteroid Dexamethasone on dynamic platelet function on the same patient sample. On the basis of a prior study that showed that Bethamethasone use in pregnancy can affect platelet count and MPV, I generally avoided platelet function testing for at least 48 hours after steroids had been administered for utero-placental disease. The exact effect of steroids on platelet function has not previously been shown using platelet functional assays. I hypothesised there was likely some effect, though possibly subtle, of antihypertensive medications and corticosteroids on platelet haemostatic function that may not be apparent using gross platelet functional assays.

7.3.4.1. Methods

To investigate the effect of Labetalol and corticosteroids administered for fetal lung maturation on platelet function I performed an experiment on a single normal pregnant control subject in third trimester. The sample was divided into two, and half of the sample was prepared separately with Labetalol and Dexamethasone, and the remainder was untreated and results were compared. Fifty µl of intravenous Labetalol hydrochloride (5,000µg/ml solution) was added to 950µl of anti-coagulated blood to achieve a desired therapeutic blood concentration of 250µg/l. For Dexamethasone, 31.25µl of Dexamethasone 4ml injection was added to 968.75µl blood to achieve plasma concentration of 0.125mg/hr. For the prepared samples of Labetalol and Dexamethasone, each sample was assayed
and run in triplicate. The blood was maintained at 37°C in polypropylene tubes and was intermittently agitated with a blood rocker for the duration of this experiment to avoid platelet aggregation.

7.3.4.2. Results

In total for this study, corticosteroids were administered in 6%, 26% and 48% of cases of GH, PET and IUGR respectively (tables 7.1-7.3). Although this experiment consisted of a single donor, there were some changes in dynamic platelet parameters noted. There was less platelet translocation and less platelet coverage on the last slide for the sample treated with Labetalol, with a trend towards reduced platelet stasis (figure 7.2). For the sample treated with Dexamethasone, all three platelet parameters were significantly reduced.

This experiment suggests both Labetalol and Dexamethasone may have subtle effects on platelet function, with a trend to reduced platelet function for both. Future studies with larger numbers and incremental concentrations will be required to make any firm conclusions regarding the effect of these medications on platelet function.
Figure 7.2 An Experiment of the effect of anti-hypertensive medications and steroids on dynamic platelet function as assessed with the DPFA. Compared with an untreated sample (ctrl), (i) platelet translocation, (ii) stasis and (iii) end surface coverage (no. of stably adhered platelets) are reduced when the sample is treated with Labetalol or Dexamethasone. A single normal pregnancy control in the third trimester was used in this experiment.
7.4. Effect of severity of clinical disease (early versus late-onset GH/PET) on DPFA results

Recalling that for the study of agonist-induced platelet aggregation, significant differences in platelet aggregation were found in cases of the most severe subtypes of disease. When cases of IUGR were further complicated by hypertension and PET, platelet function was significantly reduced globally in response to all agonists. When PET was detected earlier in pregnancy, at < 34 weeks’ gestation, I found that platelet aggregation in response to the agonist ADP was reduced. To further test whether changes in dynamic platelet parameters were also reflective of severity of disease, or could explain the differences between the conditions, I performed a final sub-group analysis of the results of the dynamic platelet function assay based on the presentation of early versus late onset PET.

7.4.1. Results

A total of 37% (10/27) of all cases of PET were diagnosed and treated at less than 34 weeks’ gestational age, whereas almost all of the cases of IUGR were detected < 34 weeks’ gestation. Therefore I performed a sub-group analysis for the hypertensive group only. The most significant differences in DPFA parameters were observed in the PET group overall and so there was an opportunity to examine this group more closely to determine if there were clinical indications as to why this may have been. To determine the effect of severity of PET based on early versus late-onset disease, a comparative analysis for dynamic platelet function within the PET group (n=27) was performed. When compared to later-onset disease (≥34 weeks’ gestation), early-onset PET (≤34 weeks’ gestation) was associated with significantly less translocating platelets (91 ± 26 versus 126 ± 48; p = 0.048). This indicated that less platelet interaction had occurred with the VWF surface, indicating reduced platelet responsiveness in early-onset PET.
No difference was noted in static platelets (105 ± 34 versus 109 ± 40 p = 0.7653). Therefore for pregnancies diagnosed with PET for this study, platelet function was even more significantly reduced when PET occurred earlier in a pregnancy compared to later-onset PET. This may confirm the clinical severity of early-onset disease in addition to supporting the concept that early and late-onset utero-placental disease is not necessarily part of a homogenous pathogenesis. Differences in dynamic platelet parameters, not identified by standard aggregometry assays, might indicate differing patterns of platelet parameters based on clinical sub-type on the spectrum of utero-placental disease.
7.5. Summary of overall clinical results

Tables 7.1-7.3 at the end of this chapter accompany the following brief outline of the maternal demographic and clinical data for the patients recruited to the dynamic platelet function assay study.

There were no significant differences in age between the groups, and although mean BMI was higher in the hypertensive groups compared to normal pregnancy and IUGR groups, this finding was not significant. A higher proportion of PET cases were black African compared with normal pregnancy controls (15% versus 5%, p=0.448). Assisted reproduction was a feature of 9% and 11% of IUGR and PET pregnancies respectively, compared with 5% of normal pregnancy controls. All cases of gestational hypertension were spontaneously conceived.

A history of fetal growth restriction was recorded in 26% of cases of IUGR compared with 9% of PET/GH cases. Nearly half of all cases of GH had a history of PET/GH, compared with 19% of cases of PET. Essential hypertension complicated 13% of GH and 26% of cases of PET. Essential hypertension was not a feature of IUGR cases. With regards to smoking status, a higher proportion of pregnancies complicated by IUGR had a history of maternal smoking compared with normal pregnancy controls (22% compared with 9% pregnancy controls respectively, p=0.226). Smoking was not an exclusion criterion overall as it contributes to the development of utero-placental disease but smoking effect on platelet function will be further evaluated below to determine any confounding effect on platelet function analysis. There were no significant differences noted for smoking status within each of the groups.

Cases of IUGR were diagnosed at an earlier gestational age (mean gestational age of 30+2/7 (range 20-39+6/7) compared with cases of GH and PET (37+1 (32-40+6/7) and 36+1 (28-40+6/7 respectively, p<0.001). A total of 37% (10/27) of cases of PET were diagnosed less than 34 weeks’ gestational age.
Antihypertensive medications were prescribed in 44% of cases overall (4% (1/23) IUGR, 69% (11/16) GH, 63% (17/27) of PET cases). Single-pharmacy with labetalol was prescribed in 66% of all cases. Poly-pharmacy consisting of dual antihypertensive therapy with labetalol and adalat, and triple therapy with labetalol, adalat and α-methyldopa were prescribed in one and two cases respectively of GH, and in two cases and one case respectively of PET. In 31% (5/16) cases of GH and 37% (10/27) cases of PET, patients were recruited before antihypertensive medications were administered.

As expected the platelet counts were slightly lower in cases of PET compared with normal pregnancy controls (0.04). There were no cases of HELLP syndrome or fulminating PET observed for this cohort. The average level of proteinuria (g/24hr) was 720g (300-3000g).
Summary of demographic and clinical differences between the groups of utero-placental disease compared with healthy pregnancy (tables 7.1-7.3)

Table 7.1 Overall patient demographic data for patients recruited to the Platelet Function Assay 3: DPFA, demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by gestational hypertension †Normal Control: pregnant controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. *p-value=<0.05. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery.

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Normotensive 3rd trimester (n=22)</th>
<th>Gestational Hypertension (n=16)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>32 (19-47)</td>
<td>29 (17-37)</td>
<td>0.24</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.2 (19-34)</td>
<td>29 (22-36)</td>
<td>0.23</td>
</tr>
<tr>
<td>Caucasian Ethnicity</td>
<td>91% (20/22)</td>
<td>94% (15/16)</td>
<td>0.62</td>
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<tr>
<td>Nulliparous</td>
<td>36% (8/22)</td>
<td>44% (7/16)</td>
<td>0.45</td>
</tr>
<tr>
<td>Hx Uteroplacental disease</td>
<td>0%</td>
<td>50% (8/16)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Smoker</td>
<td>9% (2/22)</td>
<td>6% (1/16)</td>
<td>0.62</td>
</tr>
<tr>
<td>Steroids</td>
<td>0%</td>
<td>6% (1/16)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>0%</td>
<td>69% (11/16)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

**Phlebotomy:**

<p>| | | | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Gestational age(weeks)</td>
<td>36+1 (28-40+7)</td>
<td>37+2(33-40+6)</td>
<td>0.24</td>
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<tr>
<td>Platelet count</td>
<td>171 (107-266)</td>
<td>157 (118-234)</td>
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<tr>
<td>Haematocrit</td>
<td>31 (26-45)</td>
<td>30 (22-37)</td>
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**Obstetric Outcome:**

<p>| | | | |</p>
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<tr>
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</thead>
<tbody>
<tr>
<td>Gestation at delivery( wks)</td>
<td>40+2 (37-41+6)</td>
<td>38+6 (36-40+7)</td>
<td>0.001*</td>
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</tbody>
</table>

**Mode of delivery‡**

<p>| | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Vaginal delivery</td>
<td>50%</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>Elective caesarean section</td>
<td>41%</td>
<td>38%</td>
<td></td>
</tr>
<tr>
<td>Emergency caesarean section</td>
<td>9%</td>
<td>18%</td>
<td>0.45**</td>
</tr>
</tbody>
</table>

| Birth-weight (g) | 3610 (3010-4400) | 3258 (2040-4860) | 0.13    |
Table 7.2 Overall patient demographic data for patients recruited to the Platelet Function Assay 3: DPFA, demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by pre-eclampsia

†Normal Control: pregnant controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. *p-value=<0.05. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery.

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Normotensive 3rd trimester (n=22)</th>
<th>Pre-eclampsia (n=27)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Maternal Age (years)</td>
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<td>BMI (kg/m²)</td>
<td>25.2 (19-34)</td>
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<td>Nulliparous</td>
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<td>70% (19/27)</td>
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<tr>
<td>Hx Uteroplacental disease</td>
<td>0%</td>
<td>50% (8/16)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Smoker</td>
<td>9% (2/22)</td>
<td>11% (3/27)</td>
<td>0.59</td>
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<td>Steroids</td>
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<td>26% (7/27)</td>
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<tr>
<td>Antihypertensive use</td>
<td>0%</td>
<td>63% (17/27)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Phlebotomy:**

| Gestational age(weeks)         | 36+1 (28-40+7)                           | 36+2 (29-40+6)       | 0.74    |
| Platelet count                 | 171 (107-266)                            | 141 (64-277)         | 0.04*   |
| Haematocrit                    | 31 (26-45)                               | 32 (16-48)           | 0.87    |

**Obstetric Outcome:**

| Gestation at delivery( wks)    | 40+2 (37-41+6)                           | 37+2 (29-40+6)       | <0.001  |
| Mode of delivery†              |                                          |                      |         |
| Vaginal delivery               | 50%                                      | 41%                  |         |
| Elective cesarean section      | 41%                                      | 11%                  |         |
| Emergency cesarean section     | 9%                                       | 48%                  | 0.05    |
| Birth-weight (g)               | 3610 (3010-4400)                         | 2827 (1008-4410)     | <0.001* |
Table 7.3 Overall patient demographic data for patients recruited to the Platelet Function Assay 3: DPFA, demonstrating some expected clinical differences between healthy pregnancies and pregnancies complicated by IUGR. †Normal Control: pregnant controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight. Demographic data for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers.*p-value=0.05. ¥Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). ** p-value= any difference in mode of delivery.

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>Control† Normotensive 3rd trimester (n=22)</th>
<th>IUGR (n=23)</th>
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<td>Nulliparous</td>
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<td>16% (7/23)</td>
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<td>Smoker</td>
<td>9% (2/22)</td>
<td>22% (9/23)</td>
<td>0.22</td>
</tr>
<tr>
<td>Steroids</td>
<td>0%</td>
<td>48% (11/23)</td>
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<tr>
<td>Antihypertensive use</td>
<td>0%</td>
<td>4% (1/23)</td>
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<td>Phlebotomy:</td>
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<td>Gestational age(weeks)</td>
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<td>Hct</td>
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<tr>
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<td>36+3 (30-39+6)</td>
<td>&lt;0.002*</td>
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<td>Mode of delivery†</td>
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<tr>
<td>Vaginal delivery</td>
<td>50%</td>
<td>48%</td>
<td></td>
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<tr>
<td>Elective cesarean section</td>
<td>41%</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>Emergency cesarean section</td>
<td>9%</td>
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<td>0.92</td>
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<tr>
<td>Birth-weight (g)</td>
<td>3610 (3010-4400)</td>
<td>2100 (1090-2970)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
7.6. Conclusion

Main findings

The dynamic platelet function assay (DPFA) is a sophisticated vascular platelet assay. This study demonstrates (i) significant differences in multiple novel platelet behaviors in normal pregnancy compared with non-pregnant controls and (ii) further differences in utero-placental disease compared with healthy pregnancy controls.

As outlined in the previous main results chapter of the DPFA assay, in normal pregnancy compared with non-pregnant controls, platelet tracks, translocation, velocity and percentage platelet coverage on VWF at the end of the assay were all significantly reduced. When the results of same healthy pregnant patients were next compared to patients with a diagnosis of IUGR, the only parameter that was significantly altered was the total number of static platelets, which was reduced in IUGR. In GH and PET four parameters were reduced compared with healthy pregnancy controls: platelet tracks, translocation, static platelets and unstable platelet interactions. Platelets appear to be less reactive to the VWF surface in hypertensive conditions in pregnancy compared with normal controls. These results are novel.

For normal healthy pregnancy, we demonstrated a significant reduction in platelet interactions with VWF, as shown by significantly less platelet tracks and overall platelet-mediated thrombus at the end of the assay. As previously discussed, another research group examined platelet-mediated thrombus formation on collagen surfaces under arterial shear rate and also found reduced platelet interaction and thrombus formation with the surface in pregnancy. In contrast to this study, where platelet-thrombus on VWF was assessed after 20 seconds, platelet-thrombus in the aforementioned study was assessed on collagen after 2 minutes. Collagen is highly thrombogenic, and does not replicate the initial platelet-
vessel interactions in the arterial system in the way that VWF does. The additional problem with using collagen as the surface of flow-based assays is that there are many different sources of collagen available including human, equine and murine, and there are also many different types of collagen (type 1, type II, type III etc.)\textsuperscript{234}. In the DPFA, purified human VWF is used and the VWF surface is well-characterised and uniformly coats the surface used to test the platelet interactions\textsuperscript{283,284}. Nonetheless some similarities were identified between the two studies, where platelet-mediated vascular interactions appear to be reduced in pregnancy.

A possible explanation for this may be due to a reduction in haematocrit (HCT) in pregnancy. Haemodilution is part of the normal physiological changes in pregnancy and results in a reduction in the concentration of red blood cells in circulation\textsuperscript{215}. Interestingly, we observed that platelet translocation speeds were significantly reduced in pregnancy. It could be that in pregnancy, platelets travel at a slower pace and that this is a compensatory mechanism to counteract the reduced platelet interaction with VWF?

For utero-placental disease, platelet behavior was significantly altered across a range of platelet interactions on VWF. Pregnancy with IUGR was associated with a significant reduction in only the number of static platelets (i.e. those platelets that travel less than 1.5 times the average radius of the platelet). Whereas pregnancies with GH and PET resulted in multiple changes in platelet translocation behavior on VWF, including reductions in platelet tracks, translocating platelets, static and unstable platelet interactions with VWF. Changes in platelet function were most significant for the PET group, and a subsequent sub-group analysis indicated further reductions in platelet behaviour within the PET group alone comparing early-onset (< 34 weeks’ gestation) versus later-onset disease (> 34 weeks’ gestation). The finding of statistically significantly reduced platelet translocation within the PET group compared to the GH group is intuitive, as patients with PET are generally symptomatic and represent a more severe and acute clinical entity.
These changes in platelet function may be representative of gross maternal platelet function impairment in response to defective placentation.

A possible explanation for the differences in platelet parameters in hypertensive pregnancies compared with normal pregnancy controls was explored. A significant proportion of hypertensive patients in this study were taking anti-hypertensive medications at the time of blood sampling, however a similar proportion of patients in each of the GH and PET groups were prescribed anti-hypertensive medications. When comparing all platelet parameters for patients on antihypertensive medication compared to those not treated, there were no differences noted for antihypertensive medication use. Although an experiment indicated there may be changes in platelet function based on antihypertensive or corticosteroid use, further larger studies are required. The results of the differences in dynamic platelet parameters in different diseases in pregnancy cannot be explained by medication use alone.

Overall, the results of this study are consistent with the concept that pregnancy results in significant differences in platelet behavior on VWF. Utero-placental disease is associated with further impairment of platelet function. This impairment is most apparent in established PET. Findings of a significant functional impairment, or ‘reduced’ platelet function, are observed for PET even after controlling for maternal factors and antihypertensive medication use.

Utero-placental disease is described as a spectrum of placental disease. Different patterns of novel platelet behaviors in conditions that encompass this spectrum using the dynamic platelet assay have been identified. IUGR, GH and PET are clinical manifestations of a placental pathology that occurs as early as the first trimester, and in most cases, becomes clinically apparent in the third trimester. We have described platelet function in these conditions therefore in the third trimester compared with healthy gestation-matched normal pregnancies. The DPFA platelet parameters could be markers for disease in pregnancy.
Chapter 8. **Correlation of Placental Histopathology and results of Platelet Function**
8.1. Concise summary

I have demonstrated changes in platelet function in utero-placental disease using three different assays of platelet function. Since the placenta may be a factor in these changes, I investigated the association between placental histopathology and the results of the three platelet function assays and found differences in the three assays of platelet function based on macro and microscopic placental changes.
8.2. Introduction

Platelets have been shown to promote healthy placental development\textsuperscript{4}. This process is likely occurring as a result of platelet activation, platelet growth factor release and subsequent promotion of angiogenesis in the developing placenta. Early changes in placentas that go on to develop utero-placental disease appear to indicate altered platelet activation\textsuperscript{51,52,100}. It is not clear if this is a cause or a consequence of utero-placental disease. Studies have shown that maternal platelet function, as assessed using various techniques, is also altered in utero-placental disease, though as outlined in this thesis, results have largely been inconsistent to date. While there have been some direct placental studies in relation to platelet activation\textsuperscript{29,51,52}, it is not clear that any of these studies have investigated platelet function in relation to the final placental histopathological changes or have correlated the findings with clinical outcomes. The root cause of utero-placental disease is the placenta\textsuperscript{1}. Given that the maternal circulation extends into the placenta, it could be inferred that changes in platelet function in the maternal circulation may reflect some of the vascular changes occurring in the placenta. It may further follow that different placental lesions in utero-placental disease may reflect different changes in platelet function.

The main objective of this thesis was to investigate platelet function using a range of assays in utero-placental disease in the mother compared with third trimester controls. As outlined in the results chapters, I found that spontaneous platelet aggregation and agonist-induced platelet aggregation were significantly reduced in gestational hypertension (GH), pre-eclampsia (PET) and fetal growth restriction (IUGR) compared with healthy pregnancy controls. I also found that there were significant differences in novel multiple dynamic platelet parameters based on the sub-type of utero-placental disease. Because I assayed these patients in the third trimester and close to delivery, the platelet function changes in the maternal circulation could be reflective of changes in the final placental vascular conditions in utero-placental disease. Therefore a secondary aim of this thesis was to
correlate platelet function changes with placental histopathological changes in utero-placental disease to determine any differences in function based on placental findings that may indicate severity of disease. To the best of my knowledge maternal platelet function has not been correlated before with placental findings after delivery.

Ultimately the placenta is the root cause of utero-placental disease and platelet changes may be a cause or a consequence of the changes occurring in the placenta. A more complete understanding of the maternal platelet functional changes in relation to specific placental changes may identify parameters that may become useful screening tools or treatment targets in utero-placental disease.

The establishment of an optimally functioning feto-placental unit is vital in achieving a normal pregnancy outcome\(^1\). Placental disease occurs early in pregnancy, but it is usually later in gestation that the clinical consequences become apparent. After delivery of a complicated pregnancy, an assessment of the placenta can confirm general and specific causes of placental disease. This is an important aspect in the management of pregnancy complications, because this assessment has the potential to impact on clinical care by explaining the cause of adverse pregnancy outcome and/or identify placental patterns that may be associated with future recurrence risk. Indeed, in as high as 50% of cases of stillbirth, placental insufficiency is a feature\(^11,12\).

During normal placentation, embryo-derived trophoblasts invade approximately 100 maternal spiral arterioles converting them into low resistance, high-flow vessels to support the requirements of the developing fetus\(^2\). This process is suboptimal in utero-placental disease, such that the invasion barely occurs in the myometrial layer, and the vessels remain high-resistance vessels and with subsequent reduced placental perfusion\(^3\). The normal vascular remodelling of the placenta requires careful controlled activation of the haemostatic system and platelets are believed to play a key role through platelet activation. A balance must
be achieved between the vascular changes that can predispose to thrombus or haemorrhage, either of which would be detrimental to the early-developing fetus.

Several lines of evidence suggest that platelets are involved in the complex process of placentation, and a number of recent studies now provide direct evidence for the role of defective platelet function in the subsequent development of utero-placental disease. In animal and human models, platelets have been shown to promote the actual remodelling of the endothelial cells lining maternal spiral arteries\textsuperscript{4,48,49,96,99,100}. Histological examination of placentas has shown that maternal platelets are actually deposited in the trophoblast aggregates lining these arteries. The platelet-derived vascular remodelling is believed to be mediated by a possible chemokine gradient produced by platelets in spiral arteries, where trophoblasts have been shown to express the chemokine receptor for a ligand released by platelets (CCR1 expression for platelet-derived RANTES)\textsuperscript{48}. Soluble factors, such as VEGF released from the activated platelets, as a whole have been shown to enhance the invasive capacity of isolated trophoblasts in vitro\textsuperscript{94,95}. VEGF is strongly expressed in the developing placenta\textsuperscript{95}. Thus platelet function in the healthy developing placenta likely has a pro-angiogenic role as opposed to a haemostatic role.

What is interesting is that platelets appear to be activated in the placenta, but do not actually form thrombus. While platelet activation has been linked with early placental development, platelet adhesion and aggregation have not directly been shown. However many studies have shown gross thrombus formation in placentas of pre-eclampsia. Thrombus and subsequent arrest of blood flow would be detrimental to the fetus. Some studies have suggested a platelet inhibitory mechanism in pregnancy is counteracting platelet thrombus formation. Pregnancy-specific glycoproteins (PSG’s) are highly expressed in the developing placenta\textsuperscript{96}. PSG1 in human platelets was found to inhibit the platelet receptor αIIbβ3, and therefore inhibit fibrinogen-mediated platelet aggregation and thrombosis\textsuperscript{96}. This fits with the results of platelet aggregation by our research group, where we found that platelet aggregation was significantly reduced in response to numerous
agonists in healthy pregnancy compared with non-pregnant females. This response may be a compensatory mechanism in pregnancy: platelets may be required for their growth factor promotion in the placenta, but platelet activation does not lead to platelet aggregation possibly through raised PSG1. Interestingly, in pregnancies complicated by utero-placental disease decreased levels of PSG have been documented and this may contribute to thrombosis formation in this condition\textsuperscript{109,110}. These findings suggest that platelets promote healthy placentation, possibly through growth factor release, but do not appear to form thrombus, thus supporting a non-hemostatic platelet function in maternal vascular remodelling.

The process of placentation however is a vulnerable one. The balance appears to require some degree of platelet activation. Uncontrolled or excessive platelet activation could lead to thrombus formation. A pro-thrombotic process has long been suggested as a feature of placental disease. There has been some recent direct evidence to support this concept and the role of platelet-mediated thrombus formation. A mouse model suggested that when the protease-activated receptor 4 (PAR 4), which is the receptor for platelet thrombin, was deficient in mothers this restored normal development in embryos\textsuperscript{100}. PAR 4 is highly expressed on platelets and promotes thrombin generation\textsuperscript{155}. Our research group recently found that platelets responses to thrombin were reduced in healthy pregnancy compared with non-pregnant females\textsuperscript{203}. For this study I found that a reduced PAR receptor response through thrombin-induced platelet aggregation (using TRAP as the agonist) was further significantly reduced in utero-placental disease compared with healthy pregnancy. Given that PAR is the receptor for thrombin generation it may be that a reduced response to PAR could be happening in normal pregnancy with further reductions in utero-placental disease. This may suggest that altered responses to platelet-mediated thrombin generation through PAR may play a role in defective placentation.
Recent studies have also shown that damaged placenta (i.e. syncytiotrophoblasts) in PET can secrete large numbers of tissue factor bearing extracellular vesicles (EVs) that in turn can cause platelet activation, aggregation and thrombus formation\textsuperscript{51,52}. These studies further support a role for aspirin in pregnancies destined to develop PET and utero-placental disease, and provide a more direct association between platelet activation and defective placentation.

Utero-placental disease occurs early in pregnancy, but it is at a much later stage that the condition becomes clinically apparent in the mother and fetus. As previously outlined, most of the studies of platelet function in pregnancy have concentrated findings in the third trimester, when the disease is detected in the mother or fetus, and with overall inconsistent results. To the best of our knowledge, none of these studies examined placental histopathology changes in relation to platelet function results. One study examined the relationship between mean platelet volume (MPV) and placental grading, detected ultrasonographically in the third trimester\textsuperscript{316}. The placental grading system, or ‘grannum grading’ assesses degree of placental calcification as a measure of placental ‘ageing’, where higher grades have been associated with a greater degree of intervillous placental thrombosis\textsuperscript{317}. The study found that MPV was positively correlated with increasing placental grade\textsuperscript{316}. The authors hypothesised that higher MPV values might be indicative of placental micro-thrombus formation, as denoted by ultrasonographic evidence of increased placental calcification and grade. Younger platelets have a higher mean platelet volume and can activate and lead to thrombus formation. This in turn could indicate utero-placental disease however results of clinical outcomes were not available for this study.

The root cause of utero-placental disease is the placenta. As the maternal circulation is an extension of the conditions occurring in the placenta, platelet changes in the maternal circulation are possibly reflecting changes occurring in the maternal spiral arterioles in the placenta. The main aim of this thesis was to comprehensively investigate platelet function in utero-placental disease using a number of different physiological assays of platelet function. A secondary aim was
to correlate platelet function changes in relation to placental lesions associated with utero-placental disease, as a reflection of the possible vascular and platelet changes occurring in both systems. The objective of this analysis was to firstly (i) characterise the clinical and pathological placental features in pregnancies complicated by utero-placental disease and (ii) to correlate platelet function changes with placental lesions in utero-placental disease. To the best of my knowledge this has not been previously done before. The hypothesis is that changes in maternal platelet function are reflective of utero-placental insufficiency and may be evident as placental histopathological changes.
8.3. Study Design

Pregnant patients were recruited at the Rotunda Hospital to participate in studies of platelet function as previously outlined. Platelet function in pregnancies complicated by utero-placental disease (gestational Hypertension (GH), pre-eclampsia (PET), or intrauterine Growth Restriction (IUGR)) was compared to that of normal pregnancy controls. Clinical outcomes and placental histopathology results were later correlated with platelet results.

Three different platelet function modalities were tested: spontaneous platelet aggregation, agonist-induced platelet aggregation and shear-induced platelet aggregation. These assays assessed the haemostatic function of platelets: adhesion, activation and aggregation. Specific protocols were strictly adhered to for each of the platelet function tests (appendix 2.1).
8.4. Materials and Methods

8.4.1. Platelet function testing

A brief description of the assays will follow.

8.4.1.1. Spontaneous platelet aggregation

When platelets are centrifuged in-vitro in platelet-rich plasma (PRP), through gentle agitation, spontaneous platelet aggregation can occur. This process is spontaneous platelet aggregation (SPA). In patients at risk of cardiovascular disease, this process may be a risk factor. Spontaneous platelet aggregation was assessed using a method of light-transmission aggregation (LTA). In brief, platelet-rich plasma (PRP) was obtained by centrifuging the whole blood sample, and a further centrifugion of PRP created a sample of platelet-poor plasma (PPP). PPP and PRP were added to four wells each of the platelet well-plate to assess spontaneous platelet aggregation, that is, aggregation in the absence of agonists. The sample was spun in the platelet reader the degree of light transmitted correlated with degree of spontaneous platelet aggregation. In principle, the mean SPA for each patient was calculated based on the difference in spontaneous platelet aggregation from time 0 to time 18 minutes. The overall mean SPA for each of the test groups was calculated using the formula described in the materials and methods chapter, and was compared to the results for the control group.
8.4.1.2. **Agonist-induced platelet aggregation:**

LTA is a standardised method of assessing haemostatic platelet function. Platelet aggregation was assessed using LTA where a number of different platelet agonists at multiple concentrations of each are thought to reflect physiological effect. The 96-well plate was used to test platelet rich plasma samples, which were spun at high speed to generate aggregates which produced optical densities which were subsequently read and converted units of platelet aggregation. Dose-responses of the platelets to increasing doses of each agonist were generated.

8.4.1.3. **Shear-induced aggregation: dynamic platelet function assay (DPFA):**

The differing shear forces in the circulation may result in platelet adhesion and aggregation to the vascular endothelium. In the arterial system high shear force resides and can promote platelet binding to VWF via the GPIb platelet receptor. This is an initial irreversible action, however it can signal downstream continued platelet activation and recruitment, and subsequently platelet aggregation. This is a physiological assay that mimics the actual circulatory conditions that platelets are exposed to in-vivo. This assay may in turn mimic the conditions occurring in the maternal, fetal and placental circulatory systems.

Multiple platelet interactions on VWF under arterial shear-force were captured using a camera. Small volumes of whole blood samples (150µml) were used. Platelets were fluorescently labelled. A microfluidic parallel plate channel permitted the ease of blood flow across the chamber and platelets were easily captured in real-time over the VWF. The first 500 frames were analysed using sophisticated software, and multiple different behaviours of platelets were captured. This assay represents the most physiological assay of platelet function to date and is a further development of a bio-chip point-of-care utility.
Placentas from pregnancies complicated by utero-placental disease were evaluated as part of a standard protocol at the Rotunda hospital. Placental evaluation was undertaken by experienced Consultant Perinatal Pathologists. Evaluation included both macroscopic and microscopic assessments. The pathologists were provided information regarding the indication for assessment, including delivery details and gestational age at delivery. Placentas were assessed grossly for macroscopic changes, thickness and volume and umbilical cord changes. The placental birth weight ratio was also calculated (PBWR) and correlated with platelet function. Microscopic evaluations were undertaken from the placenta at various sites, and specific sites of interest at 1-1.5cm intervals. Full thickness sections including fetal and maternal cut surfaces were evaluated. Microscopic assessments were categorised into (i) villous developmental abnormalities, (ii) maternal vascular pathologies (iii) fetal vascular abnormalities or (iv) inflammatory lesions. These categories of placental lesions were compared between groups of utero-placental disease and were then correlated with platelet function results for each of the groups separately.
8.4.3. Placental classification (refer to results table 8.1)

8.4.3.1. Villous developmental abnormalities

The terminal villi are the site of nutrient and oxygen transfer to the fetus and are the border between maternal and fetal blood. Branches of fetal umbilical arteries carry fetal blood to the villi; the blood circulates through the capillaries of the villi and returns back to the fetus through the umbilical veins. The chorionic villus is connective tissue that contains blood vessels. In utero-placental disease the terminal villi are more mature and show changes consistent with utero-placental insufficiency. The typical finding of chronic utero-placental insufficiency in IUGR is evidence of accelerated villous maturation (AVM)\(^1\). Distal villous hypoplasia (DVH), a subgroup of placental villous abnormalities, may also be present. Together, AVM and DVH constitute abnormalities in placental villous development that are characteristic of placental insufficiency\(^318,319\). Platelets circulate through both maternal and fetal vessels in the villi and thus changes in the terminal villi may be reflective of changes in platelet function in both systems.

Chorangiosis is the abundance of blood vessels within the chorionic villi. It is associated with gestational diabetes and smoking. Given smoking is strongly associated with fetal growth restriction, this finding may be apparent in IUGR placentas.

8.4.3.2. Maternal vascular pathology

Vascular changes in the maternal vessels include fibrinoid deposition, infarction and retro-placental haemorrhage. These changes are often found in conjunction with placental developmental abnormalities. Micro-thrombus formation in the placenta may be due to platelet activation and aggregation.
8.4.3.3. Fetal vascular pathology

Vascular changes in the fetal side of the placenta include cord abnormalities such as cord knots, hypercoiling and thrombus formation in the cord and chorionic plate (fetal thrombotic vasculopathy). Platelets may be involved in fetal thrombotic lesions.

8.4.3.4. Inflammatory lesions

Infection (congenital or acquired, e.g. chorioamnionitis) and inflammation of the villi are causes of IUGR. For this study, known causes of congenital infection were excluded. Villitis of unknown etiology (VUE) and chronic histiocytosis are features of IUGR. VUE it is thought to represent t-lymphocyte mediated graft-versus host disease to the fetus. The lesions are associated with a recurrence rate of utero-placental disease of 10-15%. Aspirin has been trialled as prevention of recurrence of histiocytosis with some success. This implicates platelets in inflammatory and infective lesions in the placentas. Inflammatory conditions outside of pregnancy such as rheumatoid arthritis and infective processes including sepsis are associated with platelet activation.

8.4.3.5. Fetal nucleated erythroblasts

The presence of fetal nucleated erythroblasts suggests a response to chronic hypoxia. The ischaemic responses in the placenta in utero-placental disease trigger release of vasoactive substances that may alter platelet function. Ischaemic processes in the fetal circulation may alter platelet function in the fetal compartment.
The main aim of this secondary analysis was to (i) firstly compare the main placental histopathological changes for pregnancies complicated by PET/GH compared with IUGR, and (ii) correlate platelet function changes (SPA, agonist-induced aggregation and dynamic platelet behaviour) with (a) macroscopic placental changes (placental weight and placental birth weight ratio) and (b) the five main categories of placental histopathological changes in utero-placental disease (villous abnormalities, maternal vascular lesions, fetal vascular lesions, inflammatory/infective lesions and the presence of nucleated fetal erythroblasts).

8.5. Statistical analysis

Demographic data and results of placental histopathology for study participants were compared using the Chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). Data management and statistical analysis were performed using SAS Version 9.3®, GraphPad PRISM Version 6® and SPSS Version 22®.

For platelet function results, specific statistical analyses were used depending on the type of assay: for spontaneous platelet aggregation, the study was powered (80%) to detect a 5% change in spontaneous platelet aggregation for pregnancies complicated by utero-placental disease. An advanced statistical analysis was developed and used to calculate the responses of platelets to the agonists using three rules: total aggregation model profiles; dose-response with half maximal concentrations were quoted (EC50). For the DPFA assay, sophisticated tracking software has continually been developed by the Irish Centre for High-End
Computing (ICHEC), to validate the tracking system using refined probability algorithms.

8.6. Results

Over the course of the study period, 252 patients with a singleton pregnancy and with a diagnosis of either gestational hypertension (GH), pre-eclampsia (PET) or intrauterine growth restriction (IUGR) were recruited to the various platelet function studies from 24 weeks’ gestation and beyond at the Rotunda Hospital. Platelet function results were compared to the next available healthy singleton third trimester pregnancies (who were subsequently confirmed to have a normal pregnancy outcome). A number of patients were excluded from the final analysis as they either did not fulfil the inclusion criteria, or experimental error was noted at the time of analysis. This resulted in a total of 58 normal third trimester pregnancies, 30 pregnancies with GH, 54 pregnancies with PET and 67 pregnancies with IUGR for analysis. Overall, a total of 60 pregnancies that were complicated by utero-placental disease had placental histopathology performed (figure 8.1), and results were correlated with each of the three platelet functional assay results.

8.6.1. Clinical outcome

Comprehensive demographic and platelet function data has already been presented individually in each of the chapters of the results of platelet function. The main differences in demographic data and delivery outcomes between the groups are as follows: for pregnancies complicated by gestational hypertension, women had a higher BMI, and for pregnancies complicated by PET, a higher proportion of women were non-Caucasian. A total of 70% and 65% of pregnancies complicated by GH and PET respectively were taking antihypertensive
medications at the time of recruitment to the various platelet function assays. For pregnancies complicated by IUGR, 22% of patients admitted to smoking during the pregnancy. Corticosteroids were administered for fetal lung maturity in 13%, 31% and 63% of cases of GH, PET and IUGR respectively.

In terms of delivery outcomes, although there were no differences in gestational age at delivery for hypertensive compared with IUGR pregnancies, a significantly lower birth weight was apparent in the latter group (2629±871 compared with 1985±542 g, p=0.001).

Results of placental histopathology are presented firstly as overall comparisons between each of the groups of utero-placental disease (GH, PET and IUGR). Results are then presented as differences within the groups for each of platelet function assays, determined by placental histopathology.
Figure 8.1 Study Design comprising the total number of pregnant patients recruited to the three different platelet function assays and the number of cases that had placental histopathology performed.
8.6.2. Comparison of placental lesions in pregnancies complicated by GH/PET compared with IUGR

Regarding differences in placental lesions for pregnancies complicated by GH and PET compared with IUGR, the main significant differences were for placental villous developmental abnormalities and inflammatory processes: for IUGR, villous abnormalities and inflammatory lesions (mainly of the histiocytic type) occurred more frequently compared with hypertensive pregnancies (p<0.001 and p=0.03 respectively) (table 8.1). This was an expected finding. Histiocytosis can recur in pregnancy and this has implications for future pregnancy management. There were 3 cases of chorangiosis in the IUGR group. A significantly higher proportion of smokers comprised this group. Specifically, distal villous dysmaturity occurred more frequently in hypertensive pregnancies compared with IUGR pregnancies (p<0.001). What is interesting is that this feature has specifically been associated with increased fetal hypoxia during labour. Overall for this study, pregnancies complicated by gestational hypertension and pre-eclampsia had a higher rate of emergency caesarean section in labour compared with pregnancies complicated by IUGR (p<0.001).

There were no differences between the groups for maternal vascular pathological changes, although infarction was more commonly a feature of pregnancies complicated by hypertension (n/s). While the converse held true for fetal thrombotic vasculopathy processes: fetal thrombotic features occurred more frequently in IUGR pregnancies (n/s). In one case of IUGR there was umbilical cord occlusive thrombosis. Although placental/birth weight ratios (PBWR) did not differ significantly between the groups, there was a slightly higher PBWR ratio in pregnancies complicated by GH and PET corresponding with a higher mean birth weight for this group compared with the IUGR group.
Table 8.1 Clinical and placental features in pregnancies complicated by hypertensive disorders (gestational hypertension (GH) and pre-eclampsia (PET)) compared with IUGR demonstrate significantly more placental villous developmental abnormalities overall in IUGR compared with GH/PET, though distal villous dysmaturity (which has been associated with fetal hypoxia) was found to be greater in the hypertensive group. *p<0.05

<table>
<thead>
<tr>
<th>Placental Abnormality</th>
<th>GH/PET n=24</th>
<th>IUGR n=36</th>
<th>P-value</th>
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<tbody>
<tr>
<td><strong>Placental development abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accelerated villous maturation</td>
<td>15(21%)</td>
<td>36(100%)</td>
<td>&lt;0.001*</td>
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<tr>
<td>Distal villous dysmaturity/ immaturity</td>
<td>13(54%)</td>
<td>9(25%)</td>
<td>&lt;0.001*</td>
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<td>Chorangiosis</td>
<td>0</td>
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<td><strong>Maternal vascular pathology</strong></td>
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<td>Infarction</td>
<td>8(33%)</td>
<td>10(27%)</td>
<td>0.77</td>
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<td>Retroplacental hemorrhage</td>
<td>4(17%)</td>
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<td>Perivillous fibrinoid deposition</td>
<td>3(12.5%)</td>
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<td>True cord knots</td>
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<td>Cord hypercoiling</td>
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<td>Fetal thrombotic vasculopathy</td>
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<tr>
<td>Nucleated fetal erythroblasts</td>
<td>4(17%)</td>
<td>5(14%)</td>
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<td><strong>Maternal haematological indices</strong></td>
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<tr>
<td>Mean platelet volume (MPV)</td>
<td>12.4±3.5</td>
<td>9.5±1.7</td>
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<td>Platelet count</td>
<td>214±51</td>
<td>248±57</td>
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<td>Haematocrit</td>
<td>0.33±0.08</td>
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<td><strong>Perinatal outcome</strong></td>
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<td>Gestational age at delivery</td>
<td>37±3</td>
<td>36±3</td>
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<td>Birthweight (g)</td>
<td>2629±871</td>
<td>1985±542</td>
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<td>363±121</td>
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</tbody>
</table>
8.6.3. Spontaneous Platelet Aggregation and Placental Histopathology results

8.6.3.1. Spontaneous platelet aggregation and macroscopic placental changes

A negative correlation was found for SPA and placental weight ($r=-0.23$, $p=0.12$) (figure). A significant positive correlation was found for SPA and PBWR ($r=0.33$, $p=0.03$) (figure). It is likely this represents the GH/PET group, with a higher PBWR and higher overall SPA compared with the IUGR group.

Correlation of placental weight and spontaneous platelet aggregation

![Correlation graph](image)

Figure 8.2 There is a negative correlation between placental weight and spontaneous platelet aggregation
Correlation of placental/birth weight ratio and spontaneous platelet aggregation

Figure 8.3 There is a positive correlation between placental/birth weight ratio and spontaneous platelet aggregation
### 8.6.3.2. Spontaneous platelet aggregation and microscopic placental changes

Platelet function results in relation to placental lesions are outlined in table 8.2. There were no differences found in mean spontaneous platelet aggregation (SPA) results according to the classification of placental pathology in utero-placental disease.

**Table 8.2: Mean spontaneous platelet aggregation (SPA) in relation to placental lesions in utero-placental disease.** SPA is presented as mean ± standard error of mean. *p-value* is any difference in mean SPA. Comparisons between the groups were made using Mann-Whitney two-tailed t-tests for non-normal data.

<table>
<thead>
<tr>
<th>Placental Abnormality</th>
<th>SPA Presence of lesion</th>
<th>SPA Absence of lesion</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placental development abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=45</td>
<td>1.1±0.8</td>
<td>0.11±3.4</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Maternal vascular pathology</strong></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1.7±5.9</td>
<td>0.66±0.94</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Fetal vascular pathology</strong> **</td>
<td></td>
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<tr>
<td></td>
<td>1.3±0.8</td>
<td>-0.34±3.8</td>
<td>0.61</td>
</tr>
<tr>
<td><strong>Inflammatory lesions</strong></td>
<td></td>
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<tr>
<td></td>
<td>0.9±0.8</td>
<td>1.5±1.5</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Histocytosis</strong></td>
<td></td>
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<tr>
<td></td>
<td>1.4±1.8</td>
<td>0.98±0.87</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Nucleated fetal erythroblasts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5±0.93</td>
<td>-0.3±1.03</td>
<td>0.35</td>
</tr>
</tbody>
</table>
8.6.4. Agonist-induced platelet aggregation and Placental Histopathology results

8.6.4.1. Agonist-induced aggregation and macroscopic placental changes

The statistical modelling approach for agonist-induced platelet aggregation is designed to pick up on different aspects of the dose-response curves. The expected patterns of platelet responses for the given agonists have been described in relation to the different clinical presentations of utero-placental disease compared with healthy pregnancy. The approach to the analysis is very general. It describes a combination of various types of differences between individuals in responses to agonists and this takes into account inter-individual and intra-individual variability. Unlike a single spontaneous platelet aggregation result, direct correlations of general dose-response curves with continuous data such as placental weight and birth weight ratio is not feasible. Instead, the differences in aggregation based on microscopic differences in placental histopathology grouped by the presence or absence of the lesion was achieved.

8.6.4.2. Agonist-induced aggregation and microscopic placental changes

In the hypertensive group, of the placentas analysed, 12 cases had evidence of AVM however no differences were noted for overall dose response curves for cases with compared to without placental AVM (n=5). There were also 6 cases of maternal vasculopathy compared to 8 cases without, and again no differences were noted for placental lesion sub-type in the hypertensive group.
As already mentioned, all cases of IUGR had evidence of accelerated villous maturation (AVM). Some specific lesions had too few numbers for analysis, and results are only reported for where there were at least 5 cases with the lesion in a sub-group.

Further analysis of agonist-induced platelet aggregation in IUGR according to placental lesion revealed (i) significantly increased aggregation in response to arachidonic acid alone for the presence of fetal vascular lesions (n=5) compared to without (n=39) (p=0.001); (ii) further significant increases in platelet aggregation in response to not only AA (p=0.01) but also adenosine-diphosphate (ADP) (p=0.005) and collagen (p<0.001) for the presence of infectious or inflammatory lesions in IUGR (n=11) compared to cases without (n=33). No differences were found in platelet aggregation results for IUGR placentas with evidence of maternal vascular lesions (n=13) or the presence of nucleated fetal erythroblasts (n=5) compared to placentas without these lesions (n=31 and n=39 respectively).

Although numbers are small for analysis, sub-categories of placental lesions including fetal thrombotic vasculopathy and inflammatory and infectious lesions may be associated with increased platelet aggregation and micro-thrombus formation at a placental level.
8.6.5. Dynamic Platelet Function and Placental Histopathology results

8.6.5.1. Dynamic platelet parameters and macroscopic placental changes

The platelet parameters analysed were the number of platelet tracks, the number of static platelets, the number of translocating platelets and the % end surface coverage (platelet coverage on the final 500th frame). A significant difference in dynamic platelet parameters according to macroscopic placental changes was firstly noted for the number of platelet tracks: (i) birth weight positively correlated with platelet tracks ($r=0.41$, $p=0.01$) and (ii) placental birth weight ratio ($r=0.66$, $p<0.001$). A further positive correlation was noted for the number of static platelets and placental birth weight ratio ($r=0.38$, $p=0.03$).

Correlation of birth weight with number of platelet tracks

![Graph showing correlation between birth weight and number of platelet tracks](image)

Figure 8.4 There is a positive correlation between birth weight and the number of platelet tracks on the DPFA
There is a positive correlation between placental/birth weight ratio and the number of platelet tracks on the DPFA.

There is a positive correlation between placental/birth weight ratio and the number of static platelets on the DPFA.
8.6.5.2. Dynamic platelet parameters and microscopic placental changes

Differences in differing dynamic platelet parameters were found for each of the subcategories of placental histopathological changes. Though numbers were small for analysis, (i) for the presence of placental villous developmental abnormalities \((n=9)\) compared to without \((n=6)\), there were significantly less platelets tracks \((353\pm41 \text{ vs } 458\pm49)\), (ii) for the presence of maternal vascular abnormalities \((n=5)\) compared to without \((n=10)\), no differences in dynamic platelet parameters were found, (iii) for the presence of fetal thrombotic vasculopathy \((n=2)\) compared to without \((n=13)\) there were significantly less static platelets \((68\pm11 \text{ vs } 128\pm8)\), (iv) for the presence of inflective or inflammatory placental lesions \((n=2)\) compared to without \((n=13)\), there were significantly more translocating platelets \((68\pm11 \text{ vs } 128\pm8)\), and significantly greater % end surface coverage \((3.8\pm0.1 \text{ vs } 2.6\pm0.19^*)\) (\textbf{table 8.3}).
Table 8.3 Dynamic platelet function parameters in relation to placental lesions in utero-placental disease. Means ± SEM are reported as the presence of the lesion compared with the absence of the lesion. *p-value <0.05. Mann-Whitney two-tailed t-tests were used to compare groups of non-normal data. Definitions of dynamic platelet parameters: ** nTracks is the numbers of platelets that interact with VWF over a period of 500 frames ***Stasis is the number of stably adhered platelets defined as platelets that have not moved more than 1.5 times their diameter over the image acquisition time of 500 frames, ****nTrans is the number of platelets that are translocating or moving over 500 frames, ***** % end surface coverage is defined as the percentage of the visible surface covered by adhered platelets in the imaged area of the final 500th frame relative to the background.

<table>
<thead>
<tr>
<th>Platelet parameter</th>
<th>Placental villous developmental abnormalities (n=9)</th>
<th>Maternal vascular pathology (n=5)</th>
<th>Fetal vascular pathology (n=2)</th>
<th>Infection/Inflammation (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTracks**</td>
<td>353±41 vs 458±49*</td>
<td>338±48 vs 402±46</td>
<td>218±15 vs 128±20*</td>
<td>548±122 vs 363±34</td>
</tr>
<tr>
<td>Stasis***</td>
<td>123±10 vs 121±11</td>
<td>133±10 vs 109±11</td>
<td>68±11 vs 128±8*</td>
<td>135±25 vs 121±8.4</td>
</tr>
<tr>
<td>nTrans****</td>
<td>112±9.8 vs 144±15</td>
<td>113±15 vs 128±9.2</td>
<td>88±15 vs 125±9</td>
<td>196±9.5 vs 113±8*</td>
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<tr>
<td>%end coverage*****</td>
<td>2.8±0.2 vs 2.7±0.4</td>
<td>2.7±0.3 vs 2.7±0.2</td>
<td>2.8±0.3 vs 2.4±0.2</td>
<td>3.8±0.1 vs 2.6±0.19*</td>
</tr>
</tbody>
</table>
8.7. Conclusion

Although platelet functional changes have been shown to be associated with utero-placental disease, few studies, if any, have documented platelet changes in relation to actual placental histopathological findings after delivery. In the first three results chapters I demonstrated significant differences in platelet function according to the sub-type of utero-placental disease: gestational hypertension, pre-eclampsia and intra-uterine growth restriction using three different platelet function assays. The purpose of this final analysis was to identify if these changes in maternal platelet function were reflective of changes occurring in the vasculature of the placenta in utero-placental disease.

To summarise the findings of this chapter, significant differences were found in macroscopic placental changes (placental weight and placental birth weight ratio, PBWR) for changes in platelet aggregation assessed by spontaneous platelet aggregation and agonist-induced aggregation. No differences were found however for microscopic placental changes and platelet aggregation using these first two assays. However, when using the dynamic platelet function assay (DPFA), significant differences were noted in both macro and microscopic placental changes.

Regarding overall placental histopathological changes between the groups (GH/PET vs IUGR), what is interesting is that for the GH/PET group, a significant finding was that of distal villous dysmaturity/immaturity (DVI), a classification of placental villous developmental abnormalities. DVI may be associated with increased fetal distress, and for this study for the GH/PET group a higher proportion of cases were delivered by emergency caesarean section compared with the IUGR and healthy pregnancy groups (<0.001). GH/PET was interestingly associated with higher PBWR, and an increased PBWR has also been associated with adverse outcome in some studies. It has not been previously shown before if this is associated with changes in platelet function. To the best of my knowledge
this is the first study identifying patterns of platelet changes that may be reflective of placental changes and ultimately adverse pregnancy outcome.

Regarding the platelet functional assays, although no significant differences were found in mean values of spontaneous platelet aggregation (SPA) according to placental classification, SPA was found to be negatively correlated with placental weight, and positively correlated with PBWR. Increasing PBWR has been suggested to imply the placenta has ‘outgrown’ the demands of the fetus and has been suggested to be associated with adverse outcome. For this study, the average birth weight for pregnancies complicated by GH/PET was higher than that of pregnancies with IUGR. Placental birth weights were not significantly different between the groups. This may suggest that in GH/PET, the relatively ‘smaller’ placenta is outgrowing the demands of the ‘bigger’ fetus and this may be reflected in changes in platelet function.

Although not significant, the average placental weight was less in the IUGR pregnancies, and overall results for SPA in IUGR were significantly less than normal pregnancy controls. It is likely these changes in SPA are largely reflective of the IUGR subtype of utero-placental disease. Whereas regarding agonist–induced platelet aggregation some differences were further noted according to placental lesion in IUGR, where the presence of fetal thrombotic vasculopathy or the presence of infection/inflammatory lesions (e.g. histiocytosis) was found to be associated with increased platelet aggregation to AA and AA, ADP and collagen respectively. The relationship between agonist-induced platelet aggregation and placental sub-classification in IUGR has never been shown before. Though these numbers are small for analysis, the findings suggest that platelet aggregation may be increased in the placental circulation in IUGR and this may contribute to the pathogenesis of IUGR. Furthermore, when IUGR was complicated by infection or inflammatory processes, platelet aggregation was increased in response to the three potent platelet agonists: AA, ADP and collagen. What is interesting is that for pregnancies complicated by histiocytosis, this has a high chance of recurring in a subsequent pregnancy, and some limited studies have suggested using aspirin to
prevent recurrent IUGR in subsequent pregnancies\textsuperscript{321}. This current study further supports the rationale for this.

For the final assay, the dynamic platelet function assay, some novel differences were found in a number of the platelet parameters according to macro and microscopic placental changes that have never been shown before. Birth weight and PBWR were found to positively correlate with platelet tracks and platelet stasis. For the main maternal platelet function analysis according to disease, platelet tracks were found to be less for GH/PET compared with normal pregnancy controls. It is difficult to explain the finding of increased PBWR and increased tracks, however it is worth pointing out that placental results were not available for normal pregnancy controls, and thus the results were only quoted for any sub-type of utero-placental disease. Placental birth weight ratio was found to be negatively associated with % end surface coverage. For both GH/PET and IUGR, % end surface coverage was found to be significantly reduced compared with normal controls, and as the IUGR had a lower PBWR it is likely this group contributed to these findings.

Regarding the differences in the dynamic platelet parameters for each of the subcategories of placental histopathology, some differences were noted, although for some analyses the small group numbers for analysis would preclude any robust statistical conclusion. The classical placental lesion in utero-placental disease is that of placental villous developmental abnormalities and this can be present in both IUGR and GH/PET. For the presence of the lesion compared to without, the only significant difference in dynamic platelet function was that of significantly less platelet tracks. This particular platelet parameter was found to be more significantly reduced in PET compared with normal pregnancy controls rather than in IUGR or GH. Given that we have already discussed, that of the placental villous developmental findings, the GH/PET group had a higher proportion of cases specifically with the subtype of DVI, it could be inferred that for this category overall, the significant reduction in platelet tracks may be due to the
PET group. A reduction in platelet tracks may indicate increased micro-thrombus formation in this particular lesion in the placenta.

Similarly for fetal vascular changes (more commonly a feature of the IUGR group in this study), there were less overall static platelets. For the main platelet function analysis of the IUGR group as a whole compared with normally grown pregnancies, platelet stasis was found to be reduced. Platelet stasis and fetal vascular pathological changes appear to correlate in IUGR. The findings of increased translocating platelets and increased % end surface coverage in cases of infection or inflammation may indicate an increased thrombotic tendency when utero-placental disease is complicated by these factors. Platelet function has been shown to be affected by sepsis and inflammatory conditions such as rheumatoid arthritis\textsuperscript{34}. This is a novel finding for similar processes that may be occurring in utero-placental disease. Though again it must be pointed out that the numbers for sub-group analyses were small and therefore larger studies are required to investigate the relationship between novel platelet parameters and placental histopathological categories in utero-placental disease. However overall these findings may suggest that platelet function changes assessed using physiological in-vivo assays may be reflective of differing vulnerabilities based on utero-placental disease sub-type and these changes may not be apparent in standardised aggregometry assays.

The DPFA is an in-vivo platelet assay and correlates with physiological vascular conditions. The vascular conditions within the placenta are an extension of the circulatory conditions in the maternal circulation, which is where we obtained platelets for analysis. The parameters assessed using this technique are novel and have never been tested before in relation to specific placental changes in utero-placental disease. Differences in subtle platelet parameters were found according to whether the lesions were on the maternal, fetal or inter-villous interfaces that were not evident when using standardised techniques of assessing platelet function. It may follow that specific placental changes may represent a cause, consequence, or both of altered platelet function in the placenta depending
on the instigating factor. I have already outlined that platelet function is integral in the development of a healthy placenta, and that placentas in pregnancies complicated by PET have now been shown to directly activate platelets.

The DPFA is a vascular platelet assay that is based on the haemostatic function on platelets: the conditions that are created with this assay mimic those of a damaged vessel, and the platelet effects measured are the interactions of the platelets that should recognise the surface as highly thrombogenic. Although the conditions in the maternal vessels in the placenta likely represent the conditions in the general maternal circulation, the conditions in the fetal vessels are likely to be different. In some cases of utero-placental disease, it may be only within the fetal side of the placenta that gross changes of thrombosis may be evident when the placenta is analysed after delivery. A future study of subtle platelet vascular functions, using a modification of the DPFA to recreate the likely fetal and placental vessel conditions present in utero-placental disease, may uncover further differences in platelet function in the condition that may contribute to adverse pregnancy outcome. Adoptions of this assay may become useful in investigating causes of stillbirth of unknown aetiology in the future.

While the subtle dynamic platelet parameter changes in relation to platelet function are promising, it could be said that in this study, the most clinically relevant finding perhaps is that of the significant findings of platelet count and platelet volumetric changes in relation to placental weight and PBWR, which appear to be largely driven by the hypertensive group. As discussed this group had a significantly higher rate of emergency caesarean section, with a higher birth weight than their IUGR counterparts. This may suggest that although a pregnancy complicated by GH or PET may appear to have an appropriately grown fetus, there still may be a risk of hypoxia and fetal distress in labour. Though this study is not adequately powered for clinical outcome, maternal platelet functional changes may indicate an increased risk of fetal distress and adverse delivery outcome. This further supports the role for continuous monitoring of fetal heart rate in labour for all cases of utero-placental disease, not just in the setting of fetal growth restriction.
In conclusion platelet function may further serve as a marker for adverse pregnancy outcome. Different platelet functional changes as detected using a physiological vascular assay appear to be present based on the sub-type of utero-placental disease. This may indicate that platelet function is altered in different ways depending on the instigating lesion in the placenta, and may be reflective of different clinical presentations on the spectrum of utero-placental disease. Larger studies are required to correlate platelet changes, placental changes and clinical outcome. A better understanding of these processes, though more advanced vascular assays, may help improve outcomes by the early identification of utero-placental conditions where platelet function is suboptimal, and where targeted therapy may improve outcome for mother and fetus.
Chapter 9. The effect of Blood Group on Dynamic Platelet Function Results
9.1. Concise summary

It has recently been suggested that patients with blood group O may be at an increased risk of haemorrhage. Platelets express ABO antigens and several lines of evidence suggest that platelets interact differently with red blood cells and VWF depending on blood group type. The dynamic platelet function assay (DPFA) is designed to assess platelet-VWF interactions, and therefore differences in platelet behaviour on VWF identified with this assay could be due to a blood group effect. The relationship between blood group and platelet function in pregnancy has not been studied to date. To see if blood group had any effect on my results I compared results of the DPFA in pregnant patients with blood group O compared with non-O blood types. I found differences in platelet behaviour between the two groups. This is a novel finding. These differences may indicate for the first time a possible mechanism behind a risk of obstetric haemorrhage with blood group O that may involve platelet function on VWF.
9.2. Introduction

There is a strong association between ABO blood group and the risk of thrombosis or bleeding. Several lines of evidence have suggested blood group determines platelet interaction with VWF. Blood group ‘O’ has been associated with increased risk of haemorrhage. Individuals with blood group O have been shown to have lower VWF levels. Whereas AB blood group types are associated with increased levels of VWF, and in contrast to blood group O types, are at increased risk of thrombosis.

Platelet-mediated thrombosis occurs when platelets translocate and adhere to VWF. Blood group antigens are expressed on VWF but also on the platelet receptors that bind to VWF. Several lines of evidence suggest there is a functional difference in how platelets interact with VWF according to blood-group phenotype because of the presence of certain blood group antigens. What is not clear is whether obstetric haemorrhage or thromboembolic disease in pregnancy is influenced by ABO blood group and whether this is in turn related to platelet-VWF binding. In this thesis I have shown that platelet-VWF interactions are different in pregnant patients compared with the non-pregnant females, with further differences when pregnancy is complicated by utero-placental disease.

A recently published retrospective study of over 125,000 deliveries in a single tertiary centre spanning 9 years (2005-2014), found that after multivariate analysis, patients with blood group O compared with non-O blood types had significantly higher odds of postpartum haemorrhage (OR 1.14; 95% CI 1.05-1.23, p<0.001), and higher odds of a reduction in haemoglobin (Hb) of at least 2g/l at delivery (OR 1.07; 95% CI 1.04-1.11, p<0.001). Compared with women with non-O blood types, however this did not translate into an increased requirement for blood transfusion products (p=0.58). Nonetheless, the authors suggested that women with blood group O may be at greater risk of obstetrical haemorrhage.
In a further abstract presented at the 2015 International Society of Thrombosis Haemostasis (ISTH) meeting, the effect of blood group in relation to risk of post-partum haemorrhage specifically in factor XI-deficient women was examined\textsuperscript{328}. Factor XI (FXI) deficiency is a rare autosomal dominant bleeding disorder. Bleeding risks tend to be trauma or surgery related, however the risk of haemorrhage in pregnancy in these patients is unclear. A total of 115 women with FXI were identified from a database of 5 hospital trusts in the UK. The women were further stratified into ‘bleeders’ and ‘non-bleeders’ based on a documented history of bleeding tendency. The overall rate of PPH rate in FXI-deficient women was 16.2% compared to local rates of only 5%. Of those with PPH and FXI, 22% of cases occurred in ‘bleeders’ compared to 10% in ‘non-bleeders’, and 26% of PPH cases occurred in blood group O’s compared to only 8% in Non-O’s. Women with combined blood O and bleeding phenotype had the highest PPH rate of 33%. The authors concluded that while for most cases of FXI deficiency in pregnancy, women can anticipate a straightforward delivery, FXI deficiency in association with a documented history of bleeding and/or blood group O may infer a higher risk of excessive bleeding peri-partum.

The relationship between blood group and platelet function is unknown. Although limited studies in the literature suggest that blood group may be associated with obstetric haemorrhage\textsuperscript{327,328}, the actual effect of blood group phenotype on platelet function has not been investigated in pregnancy to date. In this thesis, differences in dynamic platelet function were found in normal pregnancy compared with non-pregnant females, with further differences identified in utero-placental disease. As post-partum haemorrhage can sometimes be an unpredictable and catastrophic feature of an otherwise low-risk pregnancy, I sought to investigate differences in dynamic platelet function in relation to blood group type based on O versus non-O and whether this was related to estimated blood loss at delivery.
9.3. Study Design

Over the course of two years (2012-2014) patients with a singleton pregnancy and with a diagnosis of either gestational hypertension (GH), pre-eclampsia (PET) or intrauterine growth restriction (IUGR) detected from 24 weeks’ gestation and beyond were recruited in the Rotunda Hospital for platelet function testing. Results of the dynamic platelet function assay (DPFA) for these groups of patients with utero-placental disease were compared to results for the next available third trimester singleton healthy controls, who were confirmed to have had a normal pregnancy outcome after delivery. The study design and recruitment has already been described in previous chapters.

9.4. Materials and Methods

9.4.1. Data collection

Demographic data and clinical outcomes were obtained for all cases. Specifically for this study, blood group was documented prior to DPFA assessment and was later analysed for differences in dynamic platelet function in pregnancy. Specific clinical outcomes of interest in this study included the estimated blood loss (EBL) at delivery in addition to rate of postpartum haemorrhage (PPH).
9.4.2. **Platelet function testing: dynamic platelet function assay (DPFA):**

This assay has been described in detail elsewhere. In brief, multiple platelet interactions on a plate coated with VWF under arterial shear-force were captured using a camera. Small volumes of whole blood samples (150µml) were used. Platelets were fluorescently labelled. A microfluidic parallel plate channel permitted the ease of blood flow across the chamber and platelets were easily captured in real-time over the VWF. The first 500 frames were analysed using sophisticated software, and multiple different behaviours of platelets were captured. This assay represents the most physiological assay of platelet function to date and is a further development of a bio-chip point-of-care utility.

9.4.3. **Statistical analysis**

Demographic data were compared using the chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for non-normality and for outliers. Fisher’s exact test was used to compare categorical data in the case of small category numbers (n<5). Data management and statistical analysis were performed using SAS Version 9.3, GraphPad PRISM Version 6 and SPSS Version 22.

For the DPFA assay, sophisticated tracking software has continually been developed by the Irish centre for high-end computing (ICHEC), to validate the platelet tracking system using refined probability algorithms. Differences in dynamic platelet parameters between groups were compared using Mann Whitney two tailed t-tests for non-normality.
9.4.4. Results

9.4.4.1. Clinical results

A total of 102 pregnant patients were recruited for DPFA testing over the study time-frame. A total of 14 pregnant patients who were initially recruited were later excluded due to various reasons as outlined previously. This resulted in a final pregnant group of 88 for analysis of this particular study of the effect of blood group on dynamic platelet function results (consisting of normal third trimester pregnancy controls (n=22), GH (n=16), PET (n=27) and IUGR (n=23)).

The pregnancy group as a whole (n=88) were divided into two groups based on whether they were blood group O (n=41) or non-O (n=47). The specific breakdown of the blood group types and clinical outcomes are presented after the main dynamic platelet function blood group results (table 9.1)
9.4.5. Dynamic platelet function in relation to blood group phenotype in pregnancy

When compared to patients with blood group O (n=41), there were significant differences noted in platelet function results for patients with non-blood group O (n=47) (figure 9.1). Platelet tracks (201±12 versus 205±18, \(p=0.02\)), translocating platelets (124 ± 51 versus 158 ± 72, \(p=0.01\)), unstable platelet interactions (35±2 versus 42±2, \(p=0.02\)) static platelets (113±45 versus 134±52, \(p=0.03\)) were all reduced in blood group O pregnant patients compared with non-O blood types.

These findings may indicate that platelet function in pregnant patients with blood group O is impaired. Platelets in patients with blood group O do not appear to recognise the VWF surface and this may indicate a functional impairment and increased tendency to haemorrhage. Further research is required to clarify and investigate the clinical utility of these novel findings in larger clinical trials.
Pregnant patients who were blood group O (n=41, dark grey boxes) had significantly less platelet tracks (p=0.02), platelet translocation (p=0.01), unstable platelet interactions (p=0.02) and static platelets (p=0.03) compared with pregnant patients with non-O blood group (n=47, orange boxes). Differences in platelet parameters in pregnant patients with blood group O assayed with the DPFA may indicate and increased risk of obstetric haemorrhage.
To further investigate if the differences in dynamic platelet parameters based on blood group translated into a difference in either (i) estimated blood loss at delivery (EBL) or rate of postpartum haemorrhage (PPH), I correlated these clinical outcomes according to blood group type and platelet function. Overall the breakdown of blood group type for the pregnant group as a whole (n=88) include: blood group ‘O’ (47%); blood group ‘A’ (32%); blood group ‘B’ (15%), and blood group ‘AB’ (6%). These are outlined in table 9.1 in relation to the clinical groups. There were no significant differences in blood group type between the groups of utero-placental disease and healthy pregnancy.

A greater estimated blood loss at delivery was recorded for PET cases (424ml range 200-800ml) compared with normal pregnancy controls (331ml, range 150-1150ml), although this was not found to be significant. There was no correlation noted between any blood group type and risk of post-partum hemorrhage. For patients with a postpartum hemorrhage (PPH, EBL documented as ≥500ml at delivery) (n=14) versus no PPH (n=74), there was no difference in dynamic platelet function as defined by platelet translocation and stasis. In relation to differences in EBL at delivery based on blood group type, there were also no significant differences in EBL at delivery noted for blood group O versus ‘non-O’ types (p=0.741), and neither was a correlation noted between any blood-group type and EBL(p=0.630), (figure 9.2).

Although a functional impairment in platelet behavior on VWF for blood group O versus non-O blood types is observed in this study in pregnancy, this did not translate into a clinically significant difference in blood loss at delivery. These changes in subtle platelet behaviours may explain why a subpopulation of patients, and possibly patients with blood group O are at increased risk of unanticipated and unexplained post-partum hemorrhage (PPH).
Table 9.1 The differing blood group types based on clinical presentation of 88 pregnancies, with no significant differences in blood group type based on clinical groups. For the pregnancy group as a whole (n=88) blood groups were: blood group ‘O’ (47%); blood group ‘A’ (32%); blood group ‘B’ (15%), and blood group ‘AB’ (6%). In other words 47% of pregnant patients were blood group O and 53% were non-O blood type and there were no significant differences in blood group type between the groups of uteroplacental disease. The PET group had a greater estimated blood loss (EBL) at delivery although this was not found to be significant when compared to the other clinical groups.

<table>
<thead>
<tr>
<th>Blood group details</th>
<th>Healthy pregnancy Control n = 22</th>
<th>Intra-uterine growth restriction n = 23</th>
<th>Gestational hypertension n = 16</th>
<th>Pre-eclampsia n = 27</th>
<th>P value**</th>
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</thead>
<tbody>
<tr>
<td>n=88</td>
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<tr>
<td>Estimated blood loss at delivery (mls)</td>
<td>331 (150-1150)</td>
<td>330 (150-800)</td>
<td>328 (200-700)</td>
<td>424 (200-800)</td>
<td>0.980</td>
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<td>B−</td>
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<td>55% (12/22)</td>
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<td>35% (8/23)</td>
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<td>6% (1/16)</td>
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<td>Blood group O versus 'non-O'</td>
<td>41% (9/22)</td>
<td>48% (11/23)</td>
<td>44% (7/16)</td>
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Figure 9.2 Estimated blood loss at delivery (EBL) in 88 post-partum patients was not significantly different based on blood group-type. No significant differences in EBL at delivery were noted when patients were stratified based on blood group O versus ‘non-O’ blood types (p=0.741), and neither was a correlation noted between any blood-group type and EBL(p=0.630).
9.5. Conclusion

It has recently been suggested that blood group O may be associated with an increased risk of obstetric haemorrhage\textsuperscript{327,328}. The mechanism for this is not clear though there is evidence to suggest that the presence of the O blood group antigen receptor expressed on platelets may be a factor\textsuperscript{325-327}. There may be differences in the way that platelets expressing blood group O antigen behave and interact with other blood cells, and the vasculature, particularly VWF, compared to platelets not expressing the O antigen.

In this study multiple platelet parameters on VWF were analysed in a total of 88 pregnant women and platelet function results were compared for those with blood group O compared to those who had non-O blood types. Significant differences in a number of platelet behaviours were identified for blood group O versus non-O: compared with non-O blood types, pregnant patients with blood group type O have significantly reduced numbers of platelet tracks, translocating platelets, static platelets, unstable platelet interactions and % end-surface coverage at the end of the experiment. This indicates for patients with blood group O who are pregnant, platelets interact less with VWF compared with pregnant patients with non-O blood group type.

Given that platelet-mediated thrombus formation on VWF is the haemostatic function of platelets, an impairment of this function for patients with blood group O as outlined in this study may indicate an increased risk of obstetric haemorrhage. These platelet parameters are novel and further research is currently being undertaken by our research group to determine the significance of these parameters.

Although differences in dynamic platelet function were identified for blood group O versus non-O in pregnancy, this did not translate into an increased rate of postpartum haemorrhage for blood group O. It may be inferred from this study that
for the majority of cases of blood group O a straightforward delivery can be anticipated, but perhaps this may be mediated through some compensatory platelet mechanism occurring to prevent obstetric haemorrhage.

A limitation of this study is the small numbers of pregnant patients for analysis and the study is underpowered to attribute changes in novel dynamic platelet parameters to estimates of blood loss at delivery, which is also largely an inaccurate measure. Future studies assessing VWF levels in pregnancy in relation dynamic platelet function would enable more direct correlations with platelet function, VWF and blood group in pregnancy. Nonetheless this is the first study of its kind investigating a relationship between blood group and platelet function in pregnancy where differences were found for blood group O versus non-O.

Although for this study blood group O’s were not found to have a higher rate of PPH, in support of an association between blood group O and obstetric haemorrhage, a recent large retrospective study found ‘O’s’ were more likely to have a PPH and a reduction in Hb, though they did not require more transfusions than ‘non-O’s’.327 Though this was a retrospective study, the results suggest that there must be some overriding event in patients with blood group O that prevents catastrophic haemorrhage in pregnancy, because while this group may haemorrhage more frequently, they don’t do so to a degree that requires blood volume replacement.

We have shown for the first time differences in dynamic platelet function identified based on blood group type in pregnancy which may explain in part these observed clinical differences. Women with blood group O have significantly less overall platelet adhesion to VWF and may thus be at increased risk of haemorrhage at delivery.

This study suggests that assessing in-vivo platelet function using a physiological assay prior to delivery may identify a sub-group of women at increased risk of PPH, which may optimize the prompt availability of blood products in select cases.
There may also be a role for dynamic platelet function assessment in patients with known thrombocytopenia, whether inherited or acquired prior to delivery, and particularly from an anaesthetic viewpoint in terms of suitability for regional anaesthesia. Future larger prospective studies of DPFA function in pregnancy in relation to all obstetric outcomes and specifically in relation to obstetric haemorrhage are promising.

Additional studies of dynamic platelet function in relation to blood group and menorrhagia may have significant implications in gynaecological care. Also the assessment of blood group in relation to bleeding risk in neonates and particularly those that are preterm is also a promising venture and the assay is suitable for use in this particular group because it only requires microliters of blood for processing.
Chapter 10. Discussion
10.1. Main findings

Data presented in this thesis consolidate platelet function in utero-placental disease using standardised and newly developed platelet function assay technologies, addressing some of the inconsistencies of results of previous platelet function studies in pregnancy, and contribute novel concepts which have the potential to optimise outcomes in utero-placental disease.

The relationship between platelets and utero-placental disease, specifically pre-eclampsia, has been postulated for decades and is now supported by recent direct evidence of altered platelet function in the condition\(^{51,52}\). The work of this thesis agrees with this concept, further supporting the rationale for aspirin use to prevent utero-placental disease\(^{31,42,43}\). The major challenge with this preventive strategy is that the current guidance of the blind administration of a uniform dose of aspirin is clearly suboptimal because it is associated with only a moderate reduction in the development of the disease. It is evident that for the large part, women with utero-placental disease often have little in common; representing a very heterogeneous group and consequently the condition itself is not homogenous in its clinical presentation. Thus a ‘one-for-all’ treatment is not optimal.

The work of this thesis supports the concept of differences in platelet function in utero-placental disease using a number of different platelet assays, with differences evident based on clinical sub-type. More sophisticated and accurate shear-flow platelet assays, for example the final assay tested in thesis, the Dynamic Platelet Function Assay (DPFA), may be the answer to address the unmet need of testing aspirin responses in pregnancy. Tailored dosing of aspirin, through more accurate platelet function testing may be the key to individualised care. A better understanding of platelet function in the condition itself may help
inform how to better optimise outcomes and this has formed the rationale for the work of this thesis.

I firstly found that platelet function, assessed as spontaneous platelet aggregation was significantly reduced in utero-placental disease (gestational hypertension (GH), pre-eclampsia (PET) and intrauterine growth restriction (IUGR)) compared with healthy pregnancy controls. I next assessed platelet function using a more complex assay that measured platelet aggregation in response to a number of different agonists and at a range of concentrations of each agonist. This assay was the light-transmission aggregometry assay (LTA) and is a standardised method of assessing platelet function, where dose-responses of platelet aggregation were described, and differences in the appearance of the curves (platelet responses to agonists) was found based on clinical condition. These first two assays assessed in-vitro platelet aggregation using optical techniques and in relatively static experimental conditions. Large volumes of blood were required to accommodate the various agonists. This would preclude these assays for use within a large prospective study of platelet function in pregnancy. Also these measures of assessing platelet aggregation, while capable of providing information on aspirin compliance, are not widely recommended for testing aspirin response in the general population.

The final assay in this thesis was a novel microfluidic in-vivo platelet vascular assay that assessed multiple parameters of platelet interactions in whole blood under arterial shear flow. This assay required only microliters of blood samples. It is a newly developed chip-based assay, and the surface on which the platelets were tested and visualised was designed to mimic a damaged vascular bed (VWF). Again for this assay significant differences were found firstly in pregnancy compared with non-pregnant females, and secondly in utero-placental disease compared with healthy pregnancy. This assay is one step further to a point-of-care assay. Unpublished data from our research group indicate the DPFA may be capable of identifying unique subtle platelet responses to aspirin in patients with cardiovascular disease, cancer and in pregnancy.
Overall the differences in platelet function in pregnancy cohorts assessed using three different platelet assays in this thesis described different features of the haemostatic function of platelets: platelet adhesion, activation and aggregation. The differences appeared to be a real feature of pregnancy and pregnancy-related complications, not attributable to experimental technique or maternal demographic variables. Across the three assays, further subtle differences in platelet function were identified for the more severe sub-types of utero-placental disease and particularly for agonist-induced platelet aggregation in IUGR, where the subsequent development of hypertension superimposed on the primary condition resulted in further platelet functional changes.

Differences were also found in specific platelet functions based on differing macro and microscopic placental lesions of utero-placental disease; to the best of my knowledge platelet function and placental histopathology have never been correlated before. Novel findings of differences in dynamic platelet parameters in pregnancy based on blood group type, where platelet function for patients with blood group O appears to be very different from non-O blood types, were also found. These findings suggest that women with blood group O may be at increased risk of obstetric haemorrhage. The DPFA might be a useful measure to predict this.

It is clear from the findings of this thesis of three different static and in-vivo platelet assays that platelet function is altered in the three different conditions on the spectrum of utero-placental disease (gestational hypertension (GH), pre-eclampsia (PET) and intrauterine growth Restriction (IUGR)) compared with normal pregnancy. Some similar results of platelet function appeared to be present for these clinical entities, but some differences were also obtained. This indicates that utero-placental disease is a heterogeneous condition, and more than likely requires an individualised approach to screening, diagnosis and therapy.
This is relevant in current obstetric and neonatal clinical practice because more mothers are continuing to develop the condition, and as a direct consequence more babies will be born prematurely. The infants exposed to a diseased utero-placental environment will themselves grow up with increased risk of cardiovascular and metabolic complications as adults. When they themselves become mothers, the problem will continue to spiral out of control. There is an opportunity to revolutionise antenatal care.

10.2. Strengths and limitations of the study

There are several strengths to this study worth mentioning in the context of previous publications of platelet function in pregnancy. The study was a prospective case-control study of a number of different platelet function assays in a large pregnant population over 2 years. The clinical site at the Rotunda Hospital facilitated the steady stream of suitable patients for recruitment. All patients were closely followed up for pregnancy outcome and for the subsequent development of complications, such that patients were appropriately diagnosed and assigned into select study groups, or were excluded if appropriate. A strict list of inclusion and exclusion criteria was adhered to, to limit the possibility of maternal confounding demographic or clinical variables on platelet function results. A specific protocol for blood draw was adhered to, with a specialised mechanism for avoiding inadvertent platelet aggregation by ensuring the blood flowed freely and without agitation. Blood samples were analysed within 60 minutes after blood draw, at either the clinical site or the research site. Care was taken when handling the blood samples during transport.

The platelet assays tested in this thesis were assays of spontaneous platelet aggregation, agonist-induced platelet aggregation and shear-induced platelet aggregation, where the assays increased in complexity respectively. The three assays were tested in the three clinical conditions of utero-placental disease, GH,
PET and IUGR with some similar and different results obtained. Haematological, maternal demographic and clinical data including placental histopathology were taken into consideration when interpreting results of the three assays.

Platelet aggregation is a standardised method of assessing haemostatic platelet function\textsuperscript{127}. The dynamic platelet function assay (DPFA) is a newly developed assay that takes into consideration the influence of the conditions within the circulation that can affect platelet function, and is thus a more physiological assay\textsuperscript{283,284}. The relevance of this assay is that for a large cohort of patients at risk of cardiovascular disease our research group has now identified differences in the platelet parameters tested with this assay that cannot be identified with aggregation assays, and these findings may be associated with major adverse cardiovascular events (MACE). The DPFA has also identified patterns of aspirin-platelet responses. The differences in DPFA results I obtained in pregnancy and utero-placental disease for this thesis may suggest this assay could be a useful screening test in pregnancy or could be used in the future to individualise aspirin treatment in pregnancy. Each of these assays has not been tested before in utero-placental disease, and thus a major strength of this thesis is the large body of data on different novel platelet function parameters in the condition.

A weakness of this study is the lack of longitudinal data particularly from the first trimester, and therefore we missed an opportunity to study platelet changes during a subclinical period before established disease. Information on platelet function early in complicated pregnancies might afford the opportunity for the development of a robust predictive test of adverse pregnancy outcome. To achieve a PET rate of 5-7% together with an IUGR rate of 10%, this would require the recruitment of at least 500 patients from first through to third trimester. This was out of the scope of this particular thesis time-frame. Nonetheless I felt it was important to properly characterize platelet function in pregnancy, to more accurately describe platelet function, and hence to provide a robust rationale for a large prospective study of dynamic platelet behavior in pregnancy. There were also significant gaps in the
literature that required attention, in terms of varying study designs and protocols and a lack of physiological assays.

10.3. Spontaneous Platelet Aggregation

To put in the context of previous publications, I will address each assay individually. Firstly, for this thesis I found spontaneous platelet aggregation (SPA) to be less in pregnancy and further reduced in hypertensive conditions in pregnancy (GH/PET), with the most significant reductions in IUGR.

There are some limited studies of SPA in pregnancy in the literature\textsuperscript{285-291}. It was recognised about 50 years ago that platelets have the ability to ‘spontaneously’ adhere and aggregate together when gently stirred\textsuperscript{169,170}. The discovery that heparin, when used for thrombo-prophylaxis in pregnancy could lead to spontaneous platelet aggregation and heparin-induced thrombocytopenia syndrome (HITS), led a number of authors to investigate SPA in pregnancy in the 1980’s\textsuperscript{285,288}. These studies were primarily concerned with the type of anticoagulant used and the effect on SPA rather than any clinical outcome. In contrast to the results of this thesis, results from these studies indicated that SPA was increased in normal pregnancy compared to non-pregnant controls; however it must be pointed out that these findings were in the context of unfractionated heparin rather than citrate\textsuperscript{285,286,288}. In the study by Burgess et al, when examining the graphs for SPA when sampled in citrate, it appeared SPA was reduced in gestational hypertension\textsuperscript{286}, a finding similar to this study, where for GH compared with healthy normotensive controls I also found that SPA was reduced.

A recent study by a Japanese research group was undertaken of SPA in twin versus singleton pregnancies. SPA in twin pregnancies was found to be significantly increased in the second trimester compared with singletons. The authors hypothesised this could be related to the increased placental mass in multiple pregnancies\textsuperscript{290}. In this study I did not examine SPA in twins, although I did
correlate placental weight and placental/birth weight ratio (PBWR) with SPA in singletons and found that in fact SPA was negatively associated with placental weight. The placental weight was overall less in the IUGR group so perhaps this group contributed to these findings. For PBWR I found a positive correlation with SPA. This is a novel finding, and likely represented changes in SPA in the respective subgroups of utero-placental disease and associated placental volumes. The same research group also recently reported that SPA was reduced in first trimester compared with non-pregnant controls\textsuperscript{289}. These findings are similar to those of our research group who reported that SPA was also reduced in the first trimester compared with non-pregnant controls, whereas we also reported that SPA incrementally increased as pregnancy advanced\textsuperscript{291}.

It has also been suggested that SPA is influenced by a number of clinical variables such as age, BMI, platelet count and haematocrit (hct)\textsuperscript{170}. For this study I did not find any correlation with age, hct or platelet count and SPA, however I did find a positive correlation between BMI and SPA, and this has been shown before. For this study smoking status overall was associated with less SPA than non-smoking, which is at odds with previous studies that smoking is associated with increased SPA. Given that a significant proportion of patients with IUGR were smokers I analysed this group further and found no differences in smokers versus non-smokers. The findings of this study suggest SPA is a feature of utero-placental disease and does not appear to be influenced largely by other variables in pregnancy.

Spontaneous platelet aggregation has more recently been associated with an increased incidence of thrombosis and recurrent ischaemic events in patients who survived a recent myocardial infarction\textsuperscript{172}. It has also been proposed as a possible predictive marker for mortality and adverse events in these patients\textsuperscript{168,172}. In patients with metastatic cancer spontaneous platelet aggregation has also been shown to be significantly increased\textsuperscript{33,35}. Pregnancy is a diabetogenic state, and is also associated with significant changes in the cardiovascular system\textsuperscript{77}.
Cardiovascular disease and utero-placental disease are linked. SPA may have predictive value in pregnancy.

The main strength of this study is the fact that a standardised method of testing spontaneous platelet aggregation was used, light-transmission aggregometry, which was the same method used for agonist-induced platelet aggregation, but without the addition of the agonists. For this method platelet adhesion and aggregation was tested after 18 minutes after gentle stirring and the amount of light transmitted by the platelets that aggregated together after that specified time, was converted via an equation into optical units. In contrast to the aforementioned studies in pregnancy above, which described SPA based on a method of counting platelets before, and then counting the single platelets remaining after the experiment as a measure of the percentage of platelets that had aggregated, LTA is the standardised method of assessing platelet aggregation.

I was interested in testing this assay in pregnancy for a number of reasons: firstly as highlighted above there is very little information in the literature regarding SPA in pregnancy; secondly the concept that SPA may be a feature in some individuals and may be associated with adverse cardiovascular outcome seemed attractive in terms of a suitable screening test for utero-placental disease, and thirdly the fact that this assay did not require the addition of agonists would also make this a good candidate as a potential screening tool for utero-placental disease.

The lack of a clear association with clinical parameters known to be associated with utero-placental disease and spontaneous platelet aggregation for this study implies (1) that we cannot clinically predict the patients who will have platelet dysfunction related to utero-placental disease, and (2) there isn’t any confounding variable that could have explained our results of reduced spontaneous platelet aggregation in utero-placental disease, and specifically the reductions in SPA observed in IUGR. Differences in spontaneous platelet aggregation appear to be a real feature of differing phenotypes of utero-placental disease and this study adds
some further evidence to the currently limited literature that SPA is different in pregnancy and utero-placental disease.

10.4. Agonist-induced Aggregation

In the second study of this thesis I found that agonist-induced platelet aggregation was significantly reduced in utero-placental disease. This implies platelets are less responsive or reactive to agonists in the condition compared with uncomplicated pregnancies implicating platelet function is defective. To summarise: for GH platelet aggregation was reduced in response to three out of the five agonists: AA, COL, and TRAP; for PET, platelet aggregation was reduced in response to four agonists: AA, COL, TRAP and epinephrine and for IUGR, platelet aggregation was reduced in response to all five agonists: AA, COL, TRAP, epi and ADP. Where platelet function in GH and PET appears to be affected by 3 and 4 agonists respectively, for IUGR there appears to be a highly statistically significant global ‘hypo-reactivity’ of platelets in response to all five agonists and at all concentrations of each agonist.

As detailed in the introduction chapter, a myriad of platelet function studies have been undertaken to date in pregnancy. The problem with interpreting previous results is that different publications describe different functions of platelets, with inconsistent results. Studies of platelet activation (determining the expression of platelet surface markers following activation, or quantifying the release of platelet granule release) were inconsistent though there appears to be more some evidence for increased platelet activation in PET\textsuperscript{86,179,187,188,189,190,191}. Studies of platelet aggregation described methods of versions of light transmission aggregometry using platelet rich plasma, versions of whole blood aggregation using platelet counting or electrical impedance methods, assessments of
pharmacological responses of platelet activation by measuring thromboxane, and there are very limited studies of shear-induced platelet aggregation in pregnancy.

Specifically regarding studies of platelet aggregation in pregnancy, studies have reported either no change in aggregation or increased aggregation in response to a limited number of agonists. Regarding studies of aggregation in PET, there are a number of authors who agree with the findings of reduced platelet aggregation in response to some similar agonists that were used in this study. I found that in PET platelet aggregation was reduced in response to AA, Coll, TRAP and epinephrine, but not ADP, when compared with healthy pregnant controls. In a few limited studies of platelet aggregation in IUGR, results appear similar to the results of this study; platelet aggregation is reduced in IUGR.

The problem with interpreting previous studies of platelet aggregation in pregnancy is numerous-fold: studies describe varying gestational ages at entry; some studies were not longitudinal in nature, and not all studies compared results in pregnancy to a control group of non-pregnant female subjects. No two studies of platelet aggregation in pregnancy described the same platelet function methods. The issues with some of the study designs of these studies were that some studies included men as control subjects, and this may have affected platelet function results, as our group recently found that there are differences in platelet function based on age and gender. One study included patients in early or threatened pre-term labour; infection and inflammation can affect platelet function. Other studies consisted of either very small study numbers for analysis (in some cases less than ten patients), or included a broad range of gestational ages for entry.

The greatest limitation in interpreting these studies is that the methods of testing platelet aggregation were not standardised. The standardised method of assessing light transmission aggregometry is by assessing the response of platelets to multiple submaximal concentrations of agonists, beginning from very
low concentrations to higher concentrations. Platelet aggregation in response to agonists in humans is characterised by a sigmoidal relationship. Computer programs will generate a sigmoidal curve based on platelet aggregation between the primary aggregation response rate and the log dose of the aggregating agent used. However this requires multiple increasing doses of agonists to generate such a response. By estimating the shape of the curve, an individual’s platelet aggregometry response to that agonist can be calculated. Individuals respond to agonists in a characteristic way, and dose-response curves of platelet–agonist responses have been validated in healthy people. Our research group demonstrated reduced platelet responsiveness in pregnancy, although the responses increased as pregnancy advanced\textsuperscript{203}. For this study I demonstrated further general and specific reductions in platelet responsiveness to agonists depending on the clinical subtype of utero-placental disease.

Results from this assay contribute to the knowledge of platelet function in utero-placental disease and confirm the findings of some previous researchers, albeit by using a standardised comprehensive method of assessment of platelet function. An interesting finding in this study was that it appears that when IUGR is complicated by superimposed GH/PET, platelet function appears to be further globally impaired, indicating that platelet function may already be deteriorating in a pregnancy complicated by IUGR that will later develop hypertension. This may suggest a strong indication for aspirin for IUGR. This may also suggest that platelet function could be a useful predictive tool for the deterioration of established utero-placental disease.

Another finding was that different agonists appear to be affected based on differing disease sub-type, most notably ADP, TRAP and Collagen. This may support a rationale for the investigation of additional antiplatelet therapies for utero-placental disease prevention (i.e. clopidogrel for the ADP pathway; thrombin antagonists for the TRAP pathway, or collagen antagonists for the GPIV pathway). Larger randomised controlled trials will be required to determine safety profiles for these medications in pregnancy.
Previous in-vitro methods of platelet function testing in pregnancy have described gross platelet aggregation, or reactivity in response to agonists, and have not taken into account the cardiovascular effects on platelet function. The final assay tested in this thesis, the DPFA, is an assay that mimics the vascular environment that platelets are exposed to in-vivo.

10.5. Dynamic Platelet Function Assay

For the final assay tested in this thesis, dynamic platelet function assay (DPFA) I found significant differences in multiple novel platelet behaviors in pregnancy compared with non-pregnant controls and further differences in utero-placental disease compared with healthy pregnancy controls. In normal pregnancy a significant reduction in platelet translocation on VWF was identified compared with non-pregnant controls. In utero-placental disease platelet translocation behavior on VWF was further reduced compared to normal healthy gestation-matched pregnant controls, and this reduction appeared to increase in significance from IUGR to GH, with the most significant reductions observed for PET. These novel changes in platelet motions have been shown for the first time in pregnancy in the work of this thesis, using a sophisticated physiological shear-based assay. The interpretation and clinical implications of these parameters are still undergoing further interpretation by our research group.

For normal healthy pregnancy, we demonstrated a significant reduction in platelet interactions with VWF, as shown by a significantly less platelet tracks. Another research group recently used collagen as a surface in a type of shear-flow based assay and found that platelet mediated thrombus formation on the surface after 2 minutes was less in pregnancy compared with non-pregnant volunteers. The limitation of using collagen as a surface in a microfluidic assay is that it is difficult to obtain a uniform reproducible surface there are many different types of collagen and many different sources available (human, equine etc.). Collagen also
promotes stable platelet-platelet aggregation, and thus the study in question only measured one facet of platelet aggregation (i.e. thrombus formation) at the end of the assay, whereas the DPFA can inform on multiple cumulative platelet motions prior to the development of platelet-mediated thrombus, all of which can be assessed using sophisticated software. Our research group have characterised the VWF surface accurately.

For utero-placental disease, I have demonstrated for the first time, using a near-physiological assay of platelet function under arterial shear, that platelet behavior is significantly altered across a range of platelet motions, captured in real-time on VWF. Pregnancy with IUGR was associated with a significant reduction in only the number of static platelets (i.e. those platelets that have stuck down stably to the VWF). Whereas pregnancies with GH and PET resulted in multiple similar changes in platelet translocation behavior on VWF, such as reductions in platelet tracks, translocating platelets, static and unstable platelet interactions with VWF. These changes in platelet function were most significant within the PET group, and for early-onset (<34 weeks’ gestation) versus later-onset PET (>34 weeks’ gestation), which is not surprising considering PET represents a more severe maternal clinical entity within the spectrum of utero-placental disease. These changes in platelet function may be representative of gross maternal platelet function impairment in response to defective placentation.

The most significant reduction in platelet interactions as assessed with the DPFA were found in PET group. After controlling for antihypertensive medication use, these findings did not appear to be altered. A further experiment of the effect of labetalol and also Dexamethasone on a number of dynamic platelet parameters in this thesis did indicate that there may be pharmacological influences on platelet function, though further larger studies are required to investigate this further, and using titrated doses of medications. The results of the differences in dynamic platelet parameters in different diseases in pregnancy cannot be explained by medication use alone.
Overall, the results of this study are consistent with the concept that pregnancy results in significant differences in platelet behavior on VWF, with further differences in utero-placental disease, and most significantly in PET. These different sequences of novel platelet behaviors in clinical conditions on the spectrum of utero-placental disease have never been described before and require further investigation.

What is clear is that from a large body of unpublished data from our research using this assay in patients at risk of cardiovascular disease, there are significant differences across these very platelet parameters that may identify patients at further risk of complications due to defective platelet function. The massive challenge in managing these patients is that there currently is no tool to identify patients with abnormal platelet behaviour that may be contributing to further athero-sclerotic or bleeding processes. Much like pregnant patients, these patients are treated with the same low-dose aspirin for primary and secondary cardiovascular disease prevention. And much like pregnant patients who go on to still have adverse pregnancy outcomes, these high-risk patients still die from intracranial and gastric haemorrhaging or stent thrombosis, from either over and under medication with antiplatelet therapy.

The difficulty with assessing antiplatelet therapy response in pregnancy is that even for non-pregnant patients at risk of cardiovascular disease, there is still an unmet need for an accurate measure of assessing how a large heterogeneous population is responding to a ‘one-for-all’ antiplatelet treatment. It is clear that people have very different risk factors outside of and within pregnancy. The dynamic platelet function assay may be the key to addressing this unmet need for a way to assess aspirin response, and unpublished data from our research group has shown that both non-pregnant and pregnant patients have a heterogeneous response to aspirin. The opportunity for future studies following on from this work will be discussed shortly.
The DPFA may thus provide a platform for testing aspirin effect in pregnancy in larger studies. In a pilot study of aspirin effect in pregnancy on 7 pregnant patients attending the rotunda hospital, significant differences and variability in aspirin effect on a number of the platelet parameters was identified. Larger studies of the effect of aspirin in pregnancy are warranted.

10.6. Interpretation

The question remains as to whether these findings of significant differences in platelet function as measured by the three different platelet function assays tested in this thesis represent a cause or consequence of utero-placental disease? Exaggerated platelet activation has long been thought to accompany early pre-clinical utero-placental disease\textsuperscript{26,44,45,47}. The rationale for aspirin use in current pregnancies at risk of utero-placental disease implies an indirect pathogenic role of platelets early in the development of this condition. Platelets have recently been shown to promote normal placental development, where activated platelets have been localised within maternal spiral arterioles\textsuperscript{48,49}. Despite the constant remodelling and exposure of the highly thrombogenic collagen lining these vessels, it is surprising that platelets, though activated, uniquely do not appear to aggregate or promote thrombus in the normally developing placenta\textsuperscript{4}. Our research group recently observed the most significant reduction of platelet reactivity to the agonist collagen in healthy first trimester pregnancies\textsuperscript{203}. It is therefore possible that defective platelet behaviour or activation with unwanted thrombus formation in response to endovascular sub-endothelial collagen is occurring early during the establishment of the utero-placental unit in such pregnancies, and that his may well explain the mechanism by which aspirin counteracts this. Our findings in this study of significantly reduced reactivity of platelets in IUGR diagnosed in third trimester in this study may possibly be reflective of an adaptation or reversion back to first trimester conditions, in an attempt to promote angiogenesis and improve utero-placental perfusion.
Another hypothesis is that this finding of platelet hypo-reactivity or reduced dynamic platelet functions may represent a function of the ‘platelet-release-reaction’, whereby platelets have been shown to have the ability to preferentially release either pro or anti-angiogenic material within a given micro-environment. There have been consistent lines of evidence showing that s-Flt levels are increased in PET and IUGR. As s-Flt is the soluble protein receptor for growth factors vascular endothelial growth factor (VEGF) and placental growth factor (PLGF), the upregulation of s-Flt therefore explains the observation of reduced levels of these factors in these conditions. The pro-angiogenic vascular endothelial growth factor (VEGF) is predominantly stored in platelets. It is possible that the global hypo-reactivity of platelets in this study is mirroring preferential platelet-release of VEGF. The method of platelet function that we used in this study (platelet aggregation) possibly represents an ‘end’ result of a potentially large number of platelet-growth-factor-release mechanisms. Though our study is unable to biologically support this concept, we observed an interesting finding of reduced platelet reactivity in response to the agonist ADP alone when IUGR was complicated by abnormal UA Doppler waveform. ADP has been shown to promote the preferential release of VEGF from platelets, perhaps suggesting a therapeutic role for ADP-driven platelet growth-factor release in severe IUGR.

Animal studies have supported a role for VEGF therapy as a growth stimulant in IUGR. An ongoing randomized controlled trial (The EVERREST trial) of VEGF gene therapy for severe early-onset fetal growth restriction diagnosed between 20-26+6 weeks’ gestation is currently recruiting. It has been proposed that delivery of adenovirus containing VEGF gene to the utero-placental circulation leads to local over-expression of VEGF, alteration of uterine artery vascular tone and improved angiogenesis and uterine blood flow. Given that most of the VEGF in our circulation is stored and released by platelets, it is plausible that platelets may play a role in the reconciliation of placental-mediated IUGR and the promotion of fetal growth through platelet growth factor release.
A unique finding in this study is that of significantly reduced platelet reactivity for the study of agonist-induced platelet aggregation, in those fetuses with a prenatal diagnosis of IUGR but with a subsequent normal birth weight and outcome, which may lend further support to a possible pro-angiogenic role of platelets in pregnancy. Dynamic in-utero growth spurts were described in as many as 17% of IUGR fetuses in the large prospective PORTO study.\textsuperscript{330}

Although overall for spontaneous platelet aggregation and agonist-induced platelet aggregation significantly reduced platelet aggregation was found specifically in IUGR, this group did not appear to have the most significant differences in platelet adhesion, as assayed with the DPFA. The PET group showed the greatest difference in dynamic DPFA platelet parameters. The various assays may be identifying specific differences in platelet function based on pathways of platelet haemostasis according to the clinical presentation of each of these entities. Future larger prospective studies are required to investigate if these platelet changes that have been identified in the third trimester of complicated pregnancies are present to the same degree in the first trimester. Not only with the potential as a screening tool in utero-placental disease, future research may even focus on extrapolating possible platelet-derived growth factors as an effective intervention in early-onset disease in pregnancy. In addition, future point-of-care tests of platelet function may evolve to identify novel modifiable markers of utero-placental disease. Such measures may also provide a platform for enabling individualised dosing of aspirin in pregnancies deemed at high-risk for developing utero-placental disease to optimise outcomes.
10.7. Future studies

PROPHETS study

A prospective study of platelet function in pregnancy

In 2015 I co-wrote a grant submitted to the Health Research Board (HRB) for a Health Research Award called the PROPHETS study (PRediction Of Pre-eclampsia using a sHEar- flow plaTelet aSSay). The aim of the study was to serially investigate platelet function in a large cohort of low-risk nulliparous women in the first trimester at three time points in the pregnancy. Our hypothesis was that differences in platelet function could predict the subsequent development of pre-eclampsia and utero-placental disease. The purpose of the grant was to fund the set-up of the dynamic platelet function assay (DPFA) at the clinical research site at the Rotunda Hospital. We are currently developing the DPFA into a point-of-care technology that will enable the ease of implementation of such a study within the large clinical setting of the Rotunda Hospital.

I was shortlisted for the HRB Research Training Fellowship for Healthcare Professionals in 2014 for a study of the DPFA in utero-placental disease.

RCSI Seed funding (short-listed 2015/2016)

A feasibility study of the biologic effect of low-dose aspirin on dynamic platelet function in pregnancy

I recently submitted an RCSI Seed funding grant with Jonathan Cowman (PhD) on a study testing aspirin response with the DPFA in pregnancy. The primary aim of this pilot study was to characterize aspirin-related platelet changes in each trimester in pregnancy to enable the identification of a subset of women who might benefit from a higher dose of aspirin or even additional antiplatelet therapy. Results from this study will support a proposal for a future large randomised
controlled trial of individualised antiplatelet dosing regimens based on dynamic platelet functional changes in pregnancy.

**Study proposals drafted (2015)**

**The relationship between Platelet Function and Patent Ductus Arteriosus**

I recently co-drafted a study proposal of a prospective study of platelet behaviour in mother-fetus pairs delivered less than 33 weeks’ gestation using the dynamic platelet assay to determine the relationship between platelet function and Patent Ductus Arteriosus (PDA) in preterm neonates.

The rationale for this study is that in approximately 70% of neonates delivered < 29 weeks’ gestation the PDA fails to close in the first few days of life, and the mechanism by which ductal closure occurs at a cellular level is not clearly understood. A recent murine study revealed platelet-mediated thrombus formation is intimately involved in constriction of the ductus arteriosus\textsuperscript{331}. Studies in humans are scarce. Given the haemostatic role of platelets in thrombus generation, it is possible that defective platelet behaviour within the DA is contributing to the development of PDA for certain neonatal cohorts. The DPFA may therefore be a useful screening tool in neonates to help determine risk of PDA.
10.8. Conclusion

In conclusion, data from this thesis indicate significant differences in platelet function in three conditions of utero-placental disease (gestational hypertension, pre-eclampsia and intrauterine growth restriction) compared with healthy pregnancy assessed using three different assays of platelet function. The platelet function assays increased in complexity from the first assay which was a test of spontaneous platelet aggregation, to the second assay of agonist-induced aggregation, and to the final assay which was a microfluidic shear-based platelet function assay designed to mimic a damaged blood vessel.

Platelet biology appears to be different based on clinical presentation on the spectrum of utero-placental disease. Where diseases overlap or represent more severe clinical entities, platelet function is significantly altered across the three assays. Placental correlation with platelet function results revealed some novel findings that may further support the rationale for platelet involvement in the development of the disease. Some novel findings of the impact of blood group on dynamic platelet function results were identified, where blood group O may be associated with an increased risk of obstetric haemorrhage.

Future studies incorporating platelet function by use of the DPFA in pregnancy may address the unmet need of a predictive tool for the development of adverse outcome. Further developments of the DPFA into a point-of-care chip are ongoing with promising implications for widespread use within large clinical populations. Furthermore, results from our pilot data suggest the DPFA may be a useful tool to test aspirin response in pregnancy and future study proposals are ongoing. The DPFA may become a platform for testing additional anti-platelet therapies in pregnancy. The DPFA may also have implications for use in neonatal cohorts owing to the fact that only a very small sample of blood is required. The DPFA may have additional future clinical use in gynecology and anaesthetics. The work of this thesis has contributed to the knowledge of platelet function in pregnancy.
and utero-placental disease, and has provided a platform for many research proposals and research questions.
References


8. Fetal Growth Restriction- Recognition, Diagnosis and Management. Institute of Obstetricians and Gynaecologists, Royal College of Physicians of
Ireland and Health Service Executive National Clinical Practice Guideline 2014; No. 29(March 2014).


55. GBD 2013 Mortality and Causes of Death, Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240


87. Lok CA, Snijder KS, Nieuwland R, van Der Post JA, de Vos P, Faas MM. Microparticles of pregnant women and preeclamptic patients activate endothelial


211. Nissel H GC, Berglund M, Karlberg KE, Lunell NO, Sylven C. Platelet aggregation in vitro and ex vivo in normal pregnancy, pregnancy-induced hypertension and preeclampsia. Hypertension in pregnancy: official journal of the


233. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides:
collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. The Biochemical journal. 1995;306(Pt 2):337-44.


273. Gralnick HR, Williams SB, Coller BS. Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. Blood. 1984;64(4):797-800.


305. Polger J, Eichler P, Greinacher A, Clemetson KJ. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation


Society for Pediatric Pathology and the Pediatric Pathology Society. 2010;13(6):459-64.


Appendix 1.1

Patient Information Leaflet and Consent Form

Protocol Title: What is the role of platelets in pregnancies complicated by Intrauterine Growth Restriction, Preeclampsia and Gestational hypertension?

Principal Investigators:
- Dr Sieglinde Müllers, SPR Obs/Gynae Rotunda Hospital –RCSI unit
- Professor Fergal Malone:, Rotunda Hospital - RCSI unit.
- Professor Dermot Kenny, RCSI, St.Stephens Green.

Telephone No: 01- 8786070.

We invite you to take part in a research study, which will be carried out at the The Rotunda Hospital. We are examining the role of platelets (clotting cells) in different types of pregnancies.

This research is being done, in an attempt to understand platelet function in pregnancies complicated by intrauterine Growth Restriction (IUGR), preeclampsia (PET) and Gestational hypertension (GH). We wish to compare platelet activity in complicated pregnancies to normal pregnancies. The study may show doctors how to better manage patients with growth restricted pregnancies.

Participation includes a blood test during your pregnancy. Blood samples will be obtained at the Rotunda Hospital and the analysis will be carried out and at the Rotunda Hospital and Royal College of Surgeons in Ireland (RCSI), York Street St. Stephen’s Green.

The only risk associated with participating in this study is that of taking a blood sample, which may cause bruising at the point the needle goes into your arm. Some patients may feel light-headed or dizzy. As previously stated obtaining a blood sample is needed as part of the routine investigation so there will only be one needle prick required. A qualified healthcare professional will obtain the blood.

We will respect completely your right to confidentiality. The samples will be processed without being labelled with your name or medical history. Regarding medical details obtained they will be discarded after the study has been completed and published. Your medical records will not leave the Rotunda Hospital.
Before you make a decision to participate or not, please read carefully the information provided and take the time to ask questions. If you wish to discuss any aspect of the study with any of the study researchers or with family and friends, or your GP please take the time to do so. Please ensure that you clearly understand the risks and benefits of participating in the study.

You may change your mind at any time and decide to withdraw before the start of the study or even after the study has commenced. You do not have to justify your decision to any person involved in the study. Your decision to participate or not in the research will have no effect on your care in the hospital. If you have any further questions about the study, you may contact the main study investigator at the number below:

Name: Dr. Sieglinde Müllers
Address: Dept. of Obstetrics and Gynaecology, RCSI Unit, Rotunda Hospital, Parnell Square, Dublin 1.
Tel: 01-8786070
Appendix 1.2

CONSENT FORM

Please tick the appropriate answer.

☐ I confirm that I have read and understood the Patient Information Leaflet attached, and that I have had ample opportunity to ask questions all of which have been satisfactorily answered.

☐ Yes ☐ No

☐ I understand that my participation in this study is entirely voluntary and that I may withdraw at any time and without giving reason, which will have no impact on my care.

☐ Yes ☐ No

☐ I understand that other research scientists may view any experimental data arising from my participation in this study with delegated authority from the Rotunda hospital or RCSI and that my identity and medical details will remain anonymous.

☐ Yes ☐ No

☐ I understand that my identity will remain confidential at all times and that all data will be destroyed on completion of the study.

☐ Yes ☐ No

☐ I am aware of the potential risks of this experimental study.

☐ Yes ☐ No

☐ I have been given a copy of the Patient Information Leaflet and this Consent form for my records.

☐ Yes ☐ No

____________________________
Patient Signature and dated

____________________________
Name in block capitals

To be completed by the Principal Investigator or his/her nominee.
I the undersigned have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved; the experimental nature of the treatment, as well as the possible benefits and have invited him/her to ask questions on any aspect of the study that concerned them.
Appendix 1.3

DATA INPUT

Date:  
Time:  

(CIRCLE)

Normal control  Gestational Hypertension  PET  IUGR

Demographic data:

Name:  

MRN:  Study No: IU_______

Age:  Phone no:  

Ethnicity:  Primip  Y/N

Height:  Weight:  BMI:  

IVF  Y/N

LMP:  EDD:

Smoking:  Y  N  No of cigarettes/day:  

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Factors for Assay

Fasting time: 

Gestation at blood draw: 

Time blood collected: 

Date: 

Recent bloods (Date tested): 

Plt count _______ Hct ________ Blood group ___

Past Obstetric History:

Gravidity: 

Parity: 

Miscarriages: 

Recurrent: 

History of IUGR/GH/PET: 

Y / N

If Y 

Birth weight _______ Gestation at delivery __________

Past medical history:

Cardiac disease ____________

PET ________________

HTN ________________

Renal disease ___________

Inflammatory disease __________

SLE __________________________
<table>
<thead>
<tr>
<th>Fam history:</th>
<th>HTN Y/N</th>
<th>Diabetes Y/N</th>
</tr>
</thead>
</table>

**Use of Aspirin**
- Y
- N
  - If yes, time since discontinued: _________________

**Use of NSAIDS**
- Y
- N
  - If yes, time since discontinued: _________________

**Other medications**: ____________________________________________

<table>
<thead>
<tr>
<th>Gestation at diagnosis of IUGR/GH/PET</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal Dopplers Y/N</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive Y/N</td>
<td></td>
</tr>
<tr>
<td>Type(s) of antihypertensive</td>
<td>________________________________</td>
</tr>
<tr>
<td>Steroids Y/N</td>
<td></td>
</tr>
<tr>
<td>GA at steroid administration</td>
<td></td>
</tr>
</tbody>
</table>

- If PET Proteinuria (g)________________________
- Abnormal LFTS y/n
- Subsequent development of fulminating PET/HELLP Y/N
## Delivery details

**GA at delivery**

**MOD** : VD, Elective CS, Em CS

**Birth weight (g)**

**Birth weight centile**

**EBL (mls)**

**NICU** Y/N

**Indication for NICU**

**Placental histopathology** y/n

**AVM** y/n

**Placental thrombosis/vasculopathy** y/n

**Placental histocytosis** y/n

**Other**

______________________________
Appendix 2.1

Detailed protocol and standard operating procedure (SOP) for the light-transmission aggregometry platelet assay which incorporates the methodology for the first two platelet function assays described; spontaneous platelet aggregation and agonist-induced aggregation.

These specific instructions apply to a Victor 3TM Multiplate reader (Perkin Elmer, Wellesley, MA, USA) and Wallac software machinery but can be adapted and applied to most plate readers.

The preparation of the plate reader and the syringe for blood draw is time consuming and it is recommended before undertaking blood sampling to factor in for this.

2.11 Set up of the plate reader

- First switch on the Wallac software (Large green switch at the back)
- Switch on the computer after first switching on the Wallac so that it can detect the machinery.
- Click on the Wallac Manager Program icon located on the desktop and wait for the program to initiate. The initiation may take several minutes.
- Ensure the default pre-set protocol is applied before you begin. The settings include
  - Temperature- 37°C
  - Wavelength- Absorbance @ 572nm
o Shake pattern- OrbitaL

o Shaking diameter- 1mm

o Protocol duration- 18 minutes including 5 standard time-points (0,3,9,15,18 minutes)

- Within the program firstly click on the temperature tab

- Set the heating plate to ‘ON’

- Set the temperature to 370C

- Click the ‘APPLY’ button

- After approximately one minute confirm this have been set up by checking the temperature graph. It will display the current Wallac temperature line (blue line) and the temperature it is aiming for (horizontal dotted line). The blue line grows slowly and it will be seen rising towards the dotted horizontal line as the heating plate warms up.

- During this warm up period perform any calibrations required (e.g. pH meter).

**2.12 Buffer preparation**

- The buffer solution used is called JNL (an artificial solution of salts, dextrose and pH buffers), used to match the physiological environment for platelets

- The JNL ingredients are stored in the refrigerator labelled JNL A, B, D, E and ACD (table 2.11). Remove all ingredients and check for cloudiness, impurities and the expiry date (they each last 4 weeks when stored in the refrigerator) and require refrigeration at all times.

- Obtain a clean, dry 200ml beaker and using a sterile pipette each time, add the following to the beaker
- 5ml JNL-A
- 5mls JNL-B
- 5mls JNL-D
- 0.5mls JNLE (use a 1ml sterile pipette for accuracy)

- Add deionised water to make up a final solution of approximately 40-45mls

- Allow time for the solution to reach warm temperature as it will affect the pH measurements in the next step.

- The solution prepared so far according to the steps above will have a pH of approximately 8.8. Using the pH meter, add ACD, drop by drop, until the pH reaches 7.35, and stir the solution continuously to ensure homogeneity.

- Add further deionised water to make up a final solution of 50ml.

- It is important to check the pH meter at all times during preparation of the buffer solution as an unstable pH reading might indicate bacterial contamination of the JNL ingredients.

- Use the solution on the same day.
<table>
<thead>
<tr>
<th>JNL</th>
<th>Component</th>
<th>FW</th>
<th>Amount</th>
<th>FC</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60mM Dextrose</td>
<td>180.2</td>
<td>5.4g/500ml</td>
<td>6mM</td>
<td>10X</td>
</tr>
<tr>
<td>B</td>
<td>1.3M NaCl</td>
<td>58.44</td>
<td>37.99g/500ml</td>
<td>130mM</td>
<td>10X</td>
</tr>
<tr>
<td></td>
<td>90mM Na Bicarb</td>
<td>84.01</td>
<td>3.78g/500ml</td>
<td>9mM</td>
<td>10X</td>
</tr>
<tr>
<td></td>
<td>100mM Na citrate (tribasic, dehydrate)</td>
<td>294.1</td>
<td>14.7g/500ml</td>
<td>10mM</td>
<td>10X</td>
</tr>
<tr>
<td></td>
<td>100mL Tris base</td>
<td>121.14</td>
<td>6.06g/500ml</td>
<td>10nM</td>
<td>10X</td>
</tr>
<tr>
<td></td>
<td>30mM KCL</td>
<td>74.56</td>
<td>1.12g/500ml</td>
<td>3mM</td>
<td>10X</td>
</tr>
<tr>
<td>D</td>
<td>8.1 Mm KH2PO4 (monobasic anyhydrate)</td>
<td>136.1</td>
<td>0.55g/500ml</td>
<td>0.81mM</td>
<td>10X</td>
</tr>
<tr>
<td>E</td>
<td>90Mm MgCL26H2O</td>
<td>203.3</td>
<td>1.83g/100ml</td>
<td>0.9mM</td>
<td>100X</td>
</tr>
</tbody>
</table>
ACD contains 38 Mm citric acid anhydrous (FW= 192.12g/M, 0.73 g/100ml), 75 mM sodium citrate tribasic dehydrate (FW= 294.1G/100ml), 124 Mm dextrose (also known as D-glucose FW=180.16, 2.23g/100ml)

2.13 Preparation of the syringe for the phlebotomy

- Obtain a 50ml syringe
- Remove the sodium citrate 3.2% solution from the fridge.
- Draw up the correct dose of sodium into the syringe, such that the amount of sodium citrate in the tube should equate to one tenth of the final volume of blood obtained.
- For a female patient, 30mls of blood is required for the platelet function testing, therefore 3ml of 3.2% sodium citrate will be drawn up
- For a male patient, 40 ml of blood is obtained so 4mls of sodium citrate will be required.
- Put the sodium citrate container back into the fridge.
- Remove any air bubbles before proceeding to ensure that the correct amount of blood will be drawn up at the time of blood sampling.
- It is recommended that the syringe containing the sodium citrate is gradually warmed up to at least room temperature so as to try to best replicate the conditions that platelets are exposed to in-vivo and not to shock the platelets into inadvertent aggregation during blood draw.
2.14 Procedure for making up fresh stock of agonists

This is not required every day, only when agonist stocks are depleted. The agonists required for the platelet function testing included Arachidonic acid (AA), Collagen (Col), Adenosine Di-Phosphate (ADP), Epinephrine (Epi), Thrombin-related Activating Peptide (TRAP). It is recommended to always check the expiry date for each agonist which should be clearly indicated on the manufacturer’s bottle or from the date assigned on the Eppendorf container by the person who prepared it. AA, ADP, Col and Epi were supplied by Brennan & Co. TRAP was supplied by Sigma Aldrich

- Remove the vial with its powered agonist from the fridge. The exception is TRAP, which in its concentrated form is kept frozen.

- Make up new ampules of agonist using deionised water:

**For AA, Col and ADP:**

- Open the glass ampule first (the metal capping first and then the grey soft rubber bung), taking care to avoid losing any of the fine powder.

- Measure 0.5mls deionised water accurately using a calibrator

- Add the water to the ampule and replace the runner bung.

- Shake the ampule to ensure the powder is fully dissolved including any powder stuck to the rubber bung such that you should be left with a clear colourless fluid (Col is slightly cloudy).

- Label it with the date is was re-constituted.
- For Col and ADP: store in the fridge.

- For AA: divide the re-constituted AA into 54ìL aliquots in Eppendorf 0.5ml “safe-lock” tubes so that they can be frozen and individually used when required. One vial of freshly made AA will make 9 Eppendorf’s. Store these in the freezer in an upright position. Note the product information by Biodata AA states that it lasts for 24 hours under refrigeration and cannot be refrozen, so only use one sample for each test.

**For Epi:**

- Follow the steps above for ADP to make up one ampule of full-strength Epi of 1Ml.

- Dilute this down to the assay-strength of 200ìM in a plastic 1.5ml “safe-lock” tube. This is done by transferring 200ìl into the safe-lock tube and adding 800ìL deionised water (i.e. a 1:4 dilution).

- Label and date and store in the fridge

**For TRAP:**

- Take an ampule of frozen full-strength TRAP form the freezer (10Mm IN 20 ìL).

- Add 380 ìL distilled water (making a total of 400 ìL at 500ìM strength, the desired strength). Transfer this to 1.5mL plastic “safe-lock” tube. Add 600ìL
distilled water to make a total volume of 1000Ìl, at strength 200ìM, which is our final desired strength.

- It is easiest to keep ADP, Col and Epi together in a small container in the fridge so that they don’t get mixed up with the unprepared ampules.

- TRAP and AA should be kept frozen when not in use.

2.15 Preparing the 96-Well plates for platelet function testing

For platelet aggregometry testing (spontaneous platelet aggregation and light-transmission aggregometry) a plate with plastic wells is used to test the platelet samples. The wells are 96 Perspex-bottomed plastic wells (12 columns of 8). Of these, the test used 48 wells (6 columns, each with 8 rows) per patient. Each well, when fully prepared, will have the various concentrations of reagents at a volume of 20ìL. The wells should all have a starting volume of 20ìL and should all appear to be equal and uniform. It is imperative that each new plate for each new sample has the reagents accurately measured and inserted carefully in to the various wells.

- When adding liquid to a well at any point, always touch the pipette tip to the bottom edge of the well and inject slowly.

- Avoid air bubbles where possible as this will affect results of platelet aggregation.

- Avoid contact with the side of the wells.
- Avoid spraying liquid above the wells, as droplets can contaminate nearby wells.

- As you work, try to hold the plate at an angle so that any fluid will sit at the bottom edge of the well, allowing you to see the tip of the pipette easily.

Add JNL to appropriate wells. This is described for columns 1-6 (columns 7-12 will correspond respectively).

- Pour the JNL into a clean dry dispensing trough.

- Dispense JNL using ‘reverse pipetting’: over-fill the pipettes by fully dispensing the pipette plunger to the 2nd stop prior to immersing the tips in the JNL. This draws up more than the required amount into each pipette.

- Then dispense the JNL into the wells by pressing the plunger but only to the first stop without over-pressing it. Keep your thumb down at the first stop as you withdraw from the wells. Then obtain more JNL for the next set of wells.

- Using the multi-channel pipette:

  - First add 10Ìl of JNL to the upper 7 wells of column 4 (rows A-G). Leave the H-well empty.

  - Add 20 JNL to rows A-G of columns 3,4,5,6. Leave H-well empty again. Note at this stage column 4 now has 30 iL JNL.

  - Add 20 IL JNL to rows A-F of columns 1 and 2 (now leaving both the G and H-wells of columns 1 and 2 empty).
• Add 20 JNL to well H6.

• Add 10 JNL to wells G1 and G2.

Return the remaining JNL to its beaker and cover and store away in the fridge.
The final volumes of JNL in the plate layout before you start to add the test samples should be as follows in the **table 2.12**:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td><strong>A</strong></td>
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<td>20</td>
<td>20</td>
<td><strong>30</strong></td>
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<td>20</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td><strong>30</strong></td>
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</tr>
<tr>
<td><strong>E</strong></td>
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</tr>
<tr>
<td><strong>G</strong></td>
<td>10</td>
<td>10</td>
<td>20</td>
<td><strong>30</strong></td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>
The next steps involve the addition of the prepared agonists AA (Column 1):

- Reverse pipette 20µl of (thawed) AA into well H1.

- Reverse pipette 30µl of AA to well G1. G1 already had 10 µL of JNL so it will now have 40 µl of fluid. Mix thoroughly by drawing the solution up and down with a 20 µl pipette. This must be done by normal pipetting. This is done about 10 times ensure adequate mixing of the AA and the JNL while avoiding air bubbles.

- Remove 20 µl from G1 and transfer to F1. F1 will now have 40µl. Again, use normal pipetting by drawing the fluid in and out 10 times to ensure mixing.

- Remove 20µl from F1 and transfer it to E1 and mix as above.

- Repeat this 20 µL transfer along the rest of column 1, mixing well each time and avoiding air bubbles.

- Discard the final 20µL drawn from well A1.

- Each well in column 1 now has 20 µL of AA at various dilutions.

- Visually they should all appear similar in terms of the quantity of fluid present and the appearance and the fluid should coat the well’s plastic base giving it an oily appearance.
Col (Column 2):

- Reverse pipette 20µL Col into H2

- Reverse pipette 30µL Col into well G2.

- G2 already had 10 µL of JNL, so it will now have 40 µL of fluid.

- Mix these thoroughly be drawing up and dispensing with a 20 µL pipette. This is done by normal dispensing 10 times to ensure adequate mixing of the Col and the JNL while avoiding air bubbles.

- Remove 20µL from G2 and transfer it to F2. Again this is done by normal pipetting.

- Remove 20µL from F2 and transfer it to E2, then mix as above.

- Repeat this 20 µL transfer along the rest of the column 2, mixing well each time and avoiding air bubbles.

- Discard the final 20 µL drawn from well A2.

- Each well in column 2 should now have 20µL of Col at various dilutions. Visually they should all appear similar in terms of quantity of fluid present and the appearance, as described for AA.
ADP (Column 3):

- This is done exactly as for AA above except only 20 µL of ADP is added to well G3.

- ADP is added to column 3

- Reverse pipette 20µL ADP into H3

- Reverse pipette 20 µL ADP into G3

- G3 already had 20 µL JNL to it will now have 40 µL fluid. Mix thoroughly by normal pipetting.

- Remove 20 µL from G3 and transfer to F3. Again, draw the in and out 10 times to ensure adequate mixing.

- Repeat this 20 µL transfer along the length of column 3, mixing well each time to avoid air bubbles.

- Discard the final 20 µL drawn from well A3.

- Each well in column 3 should have 20 µL of ADP at various dilutions with a similar appearance to each well.
Epi (column 4):

This is done similarly to the others except that (a) only 10µL of Epi is added to well G4, (b) only 10µL of liquid is transferred between the wells, and (c) once mixed, remove 10 µL from well G4 and A4 so they all end up with 20µL of liquid. The difference was devised to get quarterly dilutions.

- Reverse pipette 20µL Epi into well H4.
- Reverse pipette 10µL of Epi into well G4.
- G4 already had 30µL of JNL so it will now have 40µL fluid. Mix these thoroughly by normal pipetting again using a 10µL pipette. This is done about 15 times to ensure adequate mixing of this sample.
- Remove 10µL from G4 and transfer it to F4. Again, draw the fluid in and out about 15 times to ensure mixing.
- Repeat this 10µL transfer along the length of column 4, mixing well and avoiding air bubbles.
- Discard the final 10µL drawn from well A4.
- Well G4 to A4 will now each contain 10µL of liquid. This needs to be reduced to 20µL. Withdraw and discard a further µL from these wells so they all end up with a final volume of 20µL (no need to touch well H4 since it already had the exact 20µL volume).
- All the wells in column 4 should now have 20µL of Epi at various concentrations.
TRAP (Column 5):

This is identical to ADP.

- Reverse pipette 20µL TRAP into H5.

- Reverse pipette 20 µL TRAP into G5.

- G5 already had 20 µL JNL to it will now have 40 µL fluid. Mix thoroughly by normal pipetting.

- Remove 20 µL from G5 and transfer to F5. Again, draw the n and out 10 times to ensure adequate mixing.

- Repeat this 20 µL transfer along the length of column 5, mixing well each time to avoid air bubbles.

- Discard the final 20 µL drawn from well A5.

- Each well in column 5 should have 20 µL of TRAP at various dilutions with a similar appearance to each well.

Column 6 will just have the JNL as control. These wells will all already have 20µL of fluid. Thus all the wells (in column 1-6) should now all have 20iL of fluid. They should all have a similar appearance in terms of volume and as a clear colourless ball of fluid at the base of the well (except for the oily appearance of the wells in column 1 with AA).
The addition of raw unmixed agonists is summarised below in **Table 2.13**:

<table>
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<tr>
<th></th>
<th>1(AA)</th>
<th>2(Col)</th>
<th>3(ADP)</th>
<th>4(Epi)</th>
<th>5(TRAP)</th>
<th>6</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td>D</td>
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<td></td>
<td></td>
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<tr>
<td>E</td>
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<td>F</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td><strong>30</strong></td>
<td><strong>30</strong></td>
<td>20</td>
<td><strong>10</strong></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>
To do two patient samples simultaneously on a single plate (i.e. to do a double plate) repeat the above JNL and agonist preparations for columns 7 to 12.

2.16 Collecting a blood sample

Prepare the necessary equipment

- A warmed syringe with the correct amount of sodium citrate, with no air bubbles
- Tourniquet
- 19G butterfly needle
- 5ml syringe
- Alcohol swab
- Cotton ball
- Sticking plaster
- Attach the 5ml syringe to the butterfly.
- Place the tourniquet on the upper arm and clean the sample site with alcohol.
- Insert the needle until a flashback of blood is seen.
• Remove the tourniquet keeping the needle in place in the vein at the same time.

• Slowly draw 5ml of blood and discard.

• Remove the syringe and attach the 50ml syringe.

• Slowly draw the blood sample to the exact amount required (a total of 30 ml for women).

• Blood should flow slowly and freely to avoid shear stresses on the platelets, as this can lead to inadvertent platelet aggregation.

• Remove the needle; apply cotton wool and then a plaster.

• Gently rock the syringe about 5 times back and forth to ensure adequate mixing of the citrate and the blood.

• Record the date and time that the blood was taken.

• Leave the blood to rest 15 to 30 minutes before centrifugation.

2.17 Centrifugation of the blood sample

• Transfer the blood in 5ml aliquots into plastic test-tubes (6 tubes used for women, 8 tubes for men). Do this by tilting the test-tubes and allowing the blood to slowly run from the top of the test-tube down along the side of the tube into its base.
• Each tube should have the same amount of blood in them. This is essential to ensure the centrifuge is balanced and spins without agitation. Use plastic pipettes to correct any discrepancies in volume.

• Screw on the caps of the tubes and transfer them into the centrifuge.

• Place the test-tubes in a balanced fashion along the centrifuge.

• Close the lid and set it to spin at 150g for 10 minutes.

• Press the start button firmly.

• Check the centrifuge starts by looking at the display.

• Once finished, slowly and carefully remove the test-tubes and sit them vertically into a holding rack.

2.18 Collecting platelet-rich plasma (PRP)

• Using a plastic pipette, remove the PRP from each test-tube, avoiding any red blood cells and the thin layer of white blood cells.

• Hold the test-tube vertically and try to keep the pipette tip just below the surface of the PRP to avoid collecting other blood cells.

• If you get too close to the other cells, you can see the different type of fluid being drawn into the pipette. Stop and quickly squeeze out a few drops so that the contaminating cells are removed.
• Add the PRP to a clean 50ml plastic test-tube by running the PRP along the side of the tube.

2.19 Collecting platelet-poor plasma (PPP)

Transfer 1ml of PRP into an Eppendorf centrifuge and spin at high speed to remove any platelets. This will give you 1ml of platelet-poor plasma (PPP).

Add 180Ìl PPP to well H6, G6, F6, E6 using reverse pipetting (table 2.14). Column 6 is the control column and the first 4 rows (A6-D6) will have PRP added. Refer to the table below, Recalling that the length of column 6 only contains JNL (i.e. no agonists were added to this column); this column is used to calculate the spontaneous platelet aggregation (SPA, platelet aggregation without the addition of agonists). The difference in the mean PRP and PPP platelet aggregation results will be factored into the following equation to calculate SPA:

The mean SPA was calculated using the following formula:

\[
\{1-(SPA\ T18 / SPA\ T0)\} \times 100, \text{ where}
\]

SPA Time 0 = average PRP (the average of the values at time 0 for D6, C6, B6, A6) – average PPP (the average of the values for H6, G6, F6, E6).

SPA Time 18 = average PRP (the average of the values at time 18 for D6, C6, B6, A6) – average PPP (the average of the values for H6, G6, F6, E6).
Table 2.14. The 96-well plate for testing platelet aggregation is represented in a table format above (48 wells used per patient sample). Only column 6 was used to assess SPA (this column contained the same volume of 20µL JNL buffer solution along the whole length of the column). For the agonist-induced platelet assay, various agonists at varying concentrations were added to the remaining columns 1-5. The mean values for spontaneous platelet aggregation (SPA), PRP (A6-D6) and PPP (H6-G6) were calculated according to the equation described above.
2.191 Obtain measurements

- Measure the amount of PRP obtained. 8.64mls is the minimum required for full testing.

- Prepare the PPP and transfer 180iL into well H6, G6, F6, and E6 as described above.

- Run the Wallac’s set up wizard to set it to the 3-9-15-18 program. Choose columns 1 to 6 to be “measured” if only one patient sample is being tested and set the rest to “empty”.

- Enter in the sample details, such as the identity of the sample, PRP volume, platelet count etc. (platelet count is not adjusted).

- Gently pour the PRP into a clean dry trough.

- At this stage you should be ready to start. CHECK ALL IS OK BEFORE PROCEEDING (i.e. the Wallac is set at the correct temperature of 37 degrees and the program details are correctly entered).

- Using reverse pipetting, add 180 iL PRP to the remaining wells, one column at a time. Do this by holding the pipettes almost horizontally across the tops of the well and allow the PRP to run from the top of each well, down the wall of each well, into the base where it will mix with the agonists.

- Do this quickly as aggregation begins immediately once the PRP has been added to the first well, but be careful and accurate.

- Quickly place the plate in the Wallac.
• Click the start button.

• Once completed (25 minutes), save the data to the appropriate file and date.

• All data will be displayed as raw values in light absorption units (see appendix). Ensure each sample has been labelled with a correct 4 digit study number, e.g. for sample number 1 in the IUGR study group, this will be saved as ‘IU01’. A 4-digit label is required for each sample to be converted from raw units into platelet aggregation values using the conversion file.

The values are converted using a pre-determined macro conversion file in excel into %aggregation using the following equation:

\[
\% \text{ aggregation} = 100 \times \frac{(\text{PRP-Well})}{(\text{PRP-PPP})}
\]
Log values of agonists used to calculate dose-response curves

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>COL</th>
<th>ADP</th>
<th>EPI</th>
<th>TRAP</th>
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</table>
• prepare any other samples for other tests

• Ensure an up to date batch of fresh agonists are available for the next study sample and that any remaining sample is discarded carefully as clinical, or if being stored for additional testing, ensure it is placed in the freezer immediately.
**Appendix 2.12.**

Example raw data for victor plate reader for platelet aggregation for pre-eclampsia study patient no. 6 (PE06)

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<th>Plate</th>
<th>Repeat</th>
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<th>Start temp</th>
<th>End temp</th>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
<td>0.371 0.338 0.331 0.300 0.336 0.335</td>
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<tr>
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<td>0.331 0.325 0.318 0.262 0.281 0.139</td>
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<tr>
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<th>Absorbance @ 572 (A)</th>
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<tr>
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<tr>
<td>0.319 0.333 0.312 0.297 0.332 0.332</td>
<td></td>
</tr>
<tr>
<td>0.311 0.330 0.197 0.291 0.327 0.331</td>
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</tr>
<tr>
<td>0.263 0.315 0.172 0.277 0.232 0.094</td>
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<tr>
<td>0.177 0.274 0.148 0.243 0.140 0.094</td>
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<td>0.131 0.190 0.168 0.193 0.171 0.102</td>
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<td>0.243 0.299 0.251 0.262 0.324 0.297</td>
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## Appendix 2.2

### Dynamic platelet function Assay study protocol

#### 2.21 Reagents and buffers

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<thead>
<tr>
<th>Reagent</th>
<th>Abbreviation</th>
<th>Source</th>
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<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
<td>Sigma Ireland</td>
</tr>
<tr>
<td>Von willebrand factor</td>
<td>VWF</td>
<td>Professor Robert Montgomery, Milwaukee, USA</td>
</tr>
<tr>
<td>2’-Deoxy-N6-methyladenosine 3,5-diphosphate diammonium salt</td>
<td>MRS2179</td>
<td>Sigma Ireland</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
<td>Sigma Ireland</td>
</tr>
<tr>
<td>Trizma® base</td>
<td>Tris Base</td>
<td>Sigma Ireland</td>
</tr>
<tr>
<td>3.2% trisodium citrate</td>
<td>CIT</td>
<td>Sigma Ireland</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
<td>Location</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------</td>
<td>-------------------------------</td>
</tr>
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<td>PSA</td>
<td>Adhesive Research, Limerick</td>
</tr>
<tr>
<td>Polymethymethacrylate</td>
<td>PMMA</td>
<td>Ensinger Plastics, UK</td>
</tr>
<tr>
<td>3,3’-Dihexyloxacarbocyanine iodide</td>
<td>DIOC&lt;sub&gt;6&lt;/sub&gt;</td>
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</tr>
<tr>
<td>Microscopic slides (24 x50mm)</td>
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</tr>
<tr>
<td>19-gauge butterfly needle</td>
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</tr>
<tr>
<td>Polypropylene syringe</td>
<td>N/A</td>
<td>Beckman Dickinson, Oxford,</td>
</tr>
<tr>
<td>Parallel plate flow chambers</td>
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<td>BDI, DCU, Ireland</td>
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<tr>
<td>Bio-compatible platinum-cured silicone tubing</td>
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430
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## 2.22 Experimental equipment

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<th>Equipment</th>
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<td>Centoni GMbH, Karbussen, Germany</td>
</tr>
<tr>
<td>Sysmex-KX21N haematology analyser</td>
<td>Sysmex Corp., Kobe, Japan</td>
</tr>
<tr>
<td>Digital EM-CCD camera (Ixon, EM+)</td>
<td>Andor Technology, Belfast, Ireland</td>
</tr>
<tr>
<td>Zeiss Axiovert-200 epi-fluorescence</td>
<td>Carl Zeiss Microscopy, LLC, USA</td>
</tr>
<tr>
<td>BD FACS CANTO II</td>
<td>BD Biosciences, San Jose, CA 95121-1807, USA</td>
</tr>
</tbody>
</table>
2.23 Assembly of the parallel plate flow chambers

**Figure 2.21 Parallel plate flow chambers**

**Figure 2.21**: Parallel plate flow chambers consisted of three parts (Part A the top plate, Part B the microfluidic channel and Part C a microscope coverslip) and the chambers were assembled as follows: Part A consisted of a polymethymethacrylate (PMMA) top plate with an inbuilt 1/16 inch polypropylene inlet and outlet attached via epoxy glue. Part B consisted of a pressure sensitive adhesive (PSA) layer containing the microfluidic channel (50µM in depth, 2mm in width and 30 mm in length). Part C, the microscope class coverslip measured 24 x 50mm and the final assembled device.
2.24 Preparation of the parallel plate flow chambers

An up-to-date fresh batch of parallel plate flow chambers was prepared and available at the research site before sample analysis. The custom-designed microfluidic parallel plate flow was originally described by Kent et al (2010)\textsuperscript{283,284}, but with some recent modifications, the system easier to use enabling microliters of blood samples to be tested. The purpose of the parallel plate flow chamber is to ensure the sample is tested under uniform laminar flow rates. The chamber consists of three layers: (a) 25x55mm polymethylmethacrylate (PMMA) top plate (Ensinger Plastics, UK) fitted with inbuilt 1/16-inch polypropylene inlet and outlet connectors, a polyester gasket defining the flow path and coated on both sides with acrylic adhesive (Adhesives Research, Limerick, Ireland), and a microscope coverslip (24x55 mm, 160–190 lm thick, Bio-World, Dublin, OH, USA). The inbuilt inlet connector is designed in such a way as to swallow the air bubble separating the buffer and the blood in the silicon tube that guides the blood into the flow chamber at the given shear rate.

2.25 Addition of VWF

The assembled parallel plate flow chamber was coated with 100µg/ml VWF diluted in phosphate buffered saline (PBS) buffer overnight at 4 degrees. The channel was then washed 3 times with PBS, blocked with 1% BSA in PBS for 1 hour at room temperature. A final 3 washes with PBS prior to blood perfusion was undertaken. The chambers were then mounted on an inverted microscope (Zeiss Axiovert-200 epi-fluorescence) ready for sample perfusion.
Figure 2.22. The parallel plate flow channel is coated with VWF before blood samples are perfused at arterial shear rate across the chamber. (A) Final assembled flow chamber containing a blood sample that has been perfused through the channel. (B) Microscope image of VWF coating the channel (staining is via sheep-anti-human fluorescein isothiocyanate (FITC)-labelled anti-VWF antibody.)
2.26 Addition of dye to label the platelets

Blood samples were incubated with 1 μM DiOC₆ lipophilic dye (Invitrogen, Carlsbad, CA, USA) for 10 minutes at 37 °C, to fluorescently label the platelets for image acquisition. The dye is not specific for platelets but localises the endoplasmic reticulum and the mitochondria of platelets. This method has been described by Kent et al.²⁸³

2.27 Perfusion of sample and image acquisition

The parallel plate was mounted on an inverted microscope (Zeiss Anxiovert-200 epi-fluorescence). The blood sample was drawn through bio-compatible platinum-cured silicone tubing (Nalgene, 1/16 in internal diameter, Thermo Fisher Scientific, Denmark) into the flow channel of an assembled microfluidic device and over the VWF coated bottom-plate glass coverslip surface. A NEMESYS precision microfluidic syringe pump (Centoni GmbH, Korbussen, Germany) was used to draw the blood through the chamber device at a flow rate (Q) of 75 μL/min calculated to give an arterial shear rate (g) of 1500 s⁻¹ and corresponding shear stress (t) of 6 Pa (6 N/m²) at the bottom wall surface of the chamber, where interacting platelets are imaged.

The double-sided adhesive gasket is used to seal the assembled chamber and provide a uniform flow path 50 mm deep, 2 mm wide, and 35 mm long. The inbuilt inlet connector is designed in such a way as to swallow the air bubble separating the PBS buffer and the blood in the silicon tube that guides the blood into the flow chamber. The custom designed chamber is the second generation chamber designed by our team and allows the use of only 200μl of blood per run.
2.28 Image acquisition in parallel-plate blood flow assays

Fluorescently labelled platelets were imaged using a 60X plan-apochromat Lambda objective, final magnification 600X, with a 12 V 100W Halogen light source and a fluorescein isothiocyanate (FITC) filter set (Ex480/40x, EmS535/50m, Chroma Technology Corp, Vermont, USA). Images were acquired at a frame rate of 30 frames per second (fps) and an exposure time of 30 ms, using a vacuum-cooled (-80°C) digital EM-CCD camera (iXon Ultra 897 EM-CCD sensor, Andor Technology, Belfast, Ireland), with a viewing region of 512 x 512 pixels (8.2 x 8.2 mm) at 600x magnification. This objective enabled platelet interactions with VWF to be observed in real-time during the assay time-frame. Image acquisition begins immediately after blood is introduced to the chamber when initial platelet-surface interactions are observed (within 2-5 sec), and images are recorded up to 1000 frames. We have defined the endpoints, we use to characterise the platelet VWF interaction in more detail below. We have used a cut point of 500 frames to date as this covers a wide spectrum of platelet interactions with VWF from initial rolling to platelets adhering and covering the surface. This reflects the steps from initial binding, activation and recruitment of platelets to thrombus formation (figure 2.23)
Figure 2.23 Schematic representation of the dynamic platelet function assay (DPFA). (A) The experimental set-up consists of a microfluidic parallel plate flow chamber mounted on a microscope and connected to a syringe pump. The microscope is connected to an Andor camera connected to a PC with specialised software for image acquisition and analysis. Whole blood is perfused through the flow chamber at a pre-defined rate (set at arterial shear) via the syringe pump and the first 500 frames of platelet-surface interactions are recorded. Images are captured in real-time, where (B) demonstrates an example of a typical appearance of images from start to finish for a sample.332
2.29 Platelet tracking software

Specialised platelet tracking software was used to analyse the platelet parameters$^{283,332}$. In brief, fluorescently-labelled platelets were identified in each frame of an image sequence of 500 frames (corresponding to the first 16.7 seconds of image acquisition) using an auto-threshold method that reliably detects and records the $x,y$ centroid position and approximate size of each platelet in an image frame against a continuously changing background. Platelet tracks were constructed as each individual platelet’s movement was tracked from one frame to the next. A weighted distance matrix was generated between the platelet track’s current position and other platelets on the frame that gives preference to platelet movement in the direction of flow over cross-stream movement. Each track was extended by assigning the original platelet to a platelet positioned in the next frame, using a set of rules to the weighted distance matrix. Each platelet in the image sequence was linked using a list of tracks with $(x,y)$ positions and area that related to each individual platelet’s movement. The result was a list of platelet tracks corresponding to the associated positions over time for each platelet in an image sequence (figure 2.24). A number of advanced platelet behaviours are described (table 2.23). The tracked platelet movement is used to calculate a total of six well defined platelet behaviours that were used for the analysis in this thesis.
Figure 2.24: Worm plot of computer generated images of platelet function in healthy women in the third trimester of pregnancy (reproduced from Ralph et al)\textsuperscript{332}. Platelet movement is tracked as the platelet moves from one frame to the next over the course of the assay time-frame. A number of platelet tracks appear, which represent the behaviour of the platelets on VWF. (A) Stably adhered platelet which is defined as a platelet that has not moved more than 1.5 times its diameter over the image acquisition time of 500 frames. (B) A translocating platelet which is defined as a platelet that moves greater than 1.5 times its diameter over the image acquisition time of 500 frames. (C) Platelet Translocation Distance is the distance a translocating platelet travels over 500 imaged frames.
Table 2.23 Definitions of the key platelet parameters generated from the analysis of platelet behaviour on VWF using the microfluidic dynamic platelet function assay. A more comprehensive description of all of the behaviours tracked by the software is listed below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet tracks</td>
<td>The number of platelets that interact with VWF</td>
</tr>
<tr>
<td>Platelet translocation</td>
<td>The number of platelets that travel &gt; 1.5 times the average radius of the platelets</td>
</tr>
<tr>
<td>Velocity</td>
<td>Speed a translocating platelet moves in the direction of flow (µM/sec)</td>
</tr>
<tr>
<td>Static platelets</td>
<td>The number of platelets that travel &lt;1.5 times the average radius of the platelet</td>
</tr>
<tr>
<td>Unstable platelet interactions</td>
<td>Where a platelet interacts with VWF for a period of &gt;10 frames but &lt;490 frames.</td>
</tr>
<tr>
<td>% platelet surface coverage (end)</td>
<td>The percentage platelet surface coverage on the final frame (frame 500)</td>
</tr>
</tbody>
</table>
The key outputs

1. **Platelet tracks**

   The total numbers of platelets that interact with VWF over a period of 500 frames.

2. **Number of translocating platelets**

   This is the number of platelets that are translocating over 500 frames; our preliminary data suggests that this parameter is different between normal healthy volunteers and patients with cardiovascular disease on dual antiplatelet therapy.

3. **Velocity.**

   This is the average velocity of all the platelets in the direction of flow over 500 frames. This value tends to be relatively uniform as it is a function of the pump used in the assay in that the platelets translocate at the maximum flow rate set by the pump.

4. **The number of stably adhered platelets.**

   A stably adhered platelet is defined as a platelet that has not moved more than 1.5 times its diameter over the image acquisition time of 500 frames. This number is generated over 500 frames.

5. **Unstable platelet interactions**

   Platelets that interact with VWF for a period that is greater than 10 frames but less than 490 frames.

6. **Percentage surface coverage**

   At frame 500 this is defined as the percentage of the visible surface covered by adhered platelets in the imaged area relative to the background. This is a relatively standard measurement used by many groups.
2.291 Advanced Image Analysis Program

Platelets translocating on VWF do not behave in a uniform manner. Some stick, some roll and others roll for a little time and stick for a little time. The original program designed for quantitative assessment of platelet translocation behaviour has been refined by extracting information based on geometric and physical properties of the platelet. The initial parameters we were able to measure for platelets translocating on VWF included a number of measurements such as mean translocation distance and the number of stably adhered and translocating platelets. Since our previously published work, we have made significant modifications to the analysis programme that have enhanced the workflow, improved the reliability of the outputs and significantly reduced some potential sources of error in the analysis. The assay interpretation is largely automated in that we have now developed a single click software routine solution that allows analysis in 7 minutes. Thus this system is a near point-of-care diagnostic.

A unique feature of our analysis is reducing the effect of platelet fragmentation. Fragmentation occurs when platelets are not detected due to fluorescent bleaching or inter-collisions between interacting platelets. If left unchecked the reliability of the tracked platelets cannot be guaranteed. To address this issue, defragmentation needs to be performed. We have done this by stitching fragmented trajectories together to form single trajectory units. This reduces potential error in the analysis. Moreover, we can now measure additional outputs compared to those originally described. For example, one parameter we measured before was mean platelet translocation distance. As we have developed this assay we can now assay the frequency distribution profile of all the path lengths generated by a population of translocating platelets in an image sequence. Thus if a population of new platelets or platelets with altered functional characteristics are released from the marrow under the influence of pathways in diseased states, changes in frequency distribution profile would detect these changes in platelet behaviour.
2.292 The ideal data set

The ideal data set has been developed by software engineers at DCU, ICHEC and the RCSI research group. This data were validated in Transactions on Image Processing (Ralph et al 2015). The ideal data sets contained images with known values of platelet translocation behaviour, in addition to a range of different platelet tracking behaviours that were experimentally noted. Objects similar to the size of a platelet were placed on each image in the data set (figure 2.25). Each object had ‘no noise’ i.e. an intensity of one and a background of zero. On each start frame (frame 1) 25 objects were randomly placed on the image in the absence of overlapping objects. Each image set contained a number of ideal tracks (objects) that equalled the final number of objects on the last frame (frame 500). Objects were added at regular intervals on subsequent frames, care was taken to ensure by frame 500 that the expected number of tracks was achieved. For sets where there was no overlapping of objects a perfect tracking system would identify all object tracks and any deviations in this number would be down to miss-tracking. A fraction of objects were assigned as translocating objects (movement greater than 1.5 times the radius of the object). Each of the translocating objects had identical tracks that moved at 100 pixels at 1 pixel/frame before stopping. Static objects had no movement hence had a translocating distance of zero. The translocating objects moved in the direction of the flow and there was no restriction on merging and separating events of objects after frame 1.

For each of the measured platelet translocating behaviours (minus unstable platelet interactions parameter), the values were fixed: final object count (50, 100, 150, 200, 250, 300, 350), the fraction of static platelets (10%, 20%, 30%) and the translocating speed (0.5, 1.0, 1.5 pixels/frame). There were a total of 3 runs per experiment. In summary, a novel advanced platelet tracking software was designed and verified as accurate. The design was based on a weighted distance matrix using custom-developed idealized data sets, which determined the
percentage systematic error for key individual parameters of platelet translocation of biological relevance.

Figure 2.25 The ideal data set with objects mimicking platelet size (reproduced from Ralph et al)\textsuperscript{332}. An image of a frame generated for the ideal data set with the centroid position of each platelet masked by the platelet tracking software to enable the analysis of platelet translocation behaviour.
Altered Platelet Function in Intrauterine Growth Restriction: A Cause or a Consequence of Uteroplacental Disease?

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Abstract

Objective A limited number of platelet function studies in intrauterine growth restriction (IUGR) have yielded conflicting results. We sought to evaluate platelet reactivity in IUGR using a novel platelet aggregation assay.

Study Design Pregnancies with IUGR were recruited from 24 weeks’ gestation (estimated fetal weight < 10th centile) and had platelet function testing performed after diagnosis. A modification of light transmission aggregometry created dose–response curves of platelet reactivity in response to multiple agonists at differing concentrations. Findings were compared with healthy third trimester controls. IUGR cases with a subsequent normal birth weight were analyzed separately.

Results In this study, 33 pregnancies retained their IUGR diagnosis at birth, demonstrating significantly reduced platelet reactivity in IUGR using a novel platelet aggregation assay. When IUGR preceded preeclampsia or gestational hypertension, platelet function was significantly reduced compared with normotensive IUGR.

Conclusion Using this comprehensive platelet assay, we have demonstrated a functional impairment of platelets in IUGR. This may reflect platelet-derived placental growth factor release. Further evaluation of platelet function may aid in the development of future platelet-targeted therapies for uteroplacental disease.
Abnormal placental development by a process of altered vascular remodeling is the hallmark of intrauterine growth restriction (IUGR). Several observations suggest a link between platelet function and IUGR. There has been some success with the use of aspirin for the prevention of recurrent fetal growth restriction. Activated platelets have recently been isolated within maternal spiral arterioles in healthy pregnancies. The role of these activated platelets in the subsequent development of IUGR remains unclear, though it has been suggested that altered platelet function possibly contributes to the development of uteroplacental disease given the outcomes achieved with antiplatelet therapy.

There is also an established link between adverse pregnancy outcome, including IUGR, and later onset cardiovascular disease in both the infant and the mother. Abnormal platelet function, as evidenced by degree of platelet aggregation, has recently been implicated in predicting adverse events in patients with cardiovascular disease. Given this association, and the reduction in pregnancy complications observed with aspirin, researchers have undertaken several studies calling into question the role of platelet function in normal and complicated pregnancy.

Previous investigators have reported conflicting results of platelet function in pregnancy and preeclampsia (PET). and there have been limited approaches to the measurement of platelet function in the setting of IUGR. Overall, there have been reports of either decreased, increased, or no change in platelet function (normal pregnancy compared with non-pregnant controls, PET, and gestational hypertension (GH) compared with normotensive pregnancy controls, respectively). Most of the platelet functional assays used in these studies described either platelet reactivity (platelet aggregation in response to agonists either by whole blood impedance or light transmission platelet aggregometry) or platelet activation (where platelets are labeled with antibodies directed against surface membrane glycoproteins and their activation is analyzed by flow cytometry). In some previous studies, a limited number of agonists were studied at maximal concentrations; however, this does not represent a physiological effect. Although overall there have been several platelet function modalities described to date, these remain largely within the remit of clinical research settings, and platelet aggregometry methods using light transmission aggregometry (LTA) remain the gold standard.

Our research team recently reported increasing platelet reactivity proportional to advancing gestational age in normal pregnancy using a novel technique of LTA. As our next logical step, we sought to comprehensively investigate platelet behavior in pregnancies complicated by IUGR using this same methodology. Unique to this assay is the use of multiple agonists in addition to incremental concentrations of each agonist, such that platelet responses are measured as a rate of aggregation based on the amount of light transmitted (LTA). The addition of a wide panel of agonists to this assay is a better representation of in vivo platelet behavior compared with methods previously described.

Materials and Methods

Ethical approval was obtained from the Rotunda Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Participants were provided with written information before informed consent and blood draw, and each patient was given the option of retaining a copy of the patient information leaflet.

Patients with a singleton pregnancy and an estimated fetal weight (EFW) < 10th centile based on sonographic measurements of fetal biparietal diameter, head circumference, abdominal circumference, and femur length (Hadlock 4) were recruited from 24 weeks’ gestation and beyond at a single obstetric hospital. A blood sample was drawn at a single time point after IUGR diagnosis to measure platelet aggregation according to the protocol further described below, and results were compared with third trimester singleton controls. Exclusion criteria included patients with known platelet function disorders, diabetes mellitus, a diagnosis of fetal aneuploidy or structural fetal malformation, multiple pregnancy, or use of either aspirin or low-molecular-weight heparin within 7 days before blood sampling.

Smoking was not an exclusion criterion as it is thought to contribute significantly to placental disease, but smoking status was recorded for later evaluation as a potential confounding factor. To confirm that all study patients had a true diagnosis of pathological IUGR, patients were followed up at delivery to confirm that birth weight was < 10th centile, and placental histopathology was performed to confirm evidence of uteroplacental insufficiency in the IUGR group. Additional obstetric outcomes, such as the subsequent development of hypertensive disorders, were also recorded.

The definition of IUGR for the purpose of this analysis included all participants with a prenatal diagnosis of EFW < 10th centile and with subsequent confirmation of birth weight < 10th centile, together with histological evidence of uteroplacental insufficiency. Several subanalyses were subsequently performed within the IUGR group to determine the significance of severity of IUGR in relation to platelet aggregation. In those cases where an initial EFW < 10th centile was noted at time of antenatal enrolment, but where a birth weight > 10th centile was achieved in addition to normal placental histopathology, these were excluded from the IUGR cohort for the purpose of the primary analysis and were analyzed separately (IUGR, birth weight > 10th centile). All control subjects had a normal pregnancy outcome and birth weight confirmed.

After informed consent, patients were asked to fast from midnight and blood sampling was performed before 8 AM, with platelet analysis occurring within 60 minutes of blood draw to avoid spontaneous platelet aggregation. A single blood draw was obtained using a 19-gauge butterfly needle with an uncuffed technique to prevent platelet clumping, with the initial 5 mL analyzed to confirm a normal platelet count. A 30-mL syringe, pretreated with 3.2% sodium citrate, was then attached to the needle and 27 mL of blood was carefully collected with minimal agitation. The blood was then centrifuged for 10 minutes, following which platelet rich plasma was obtained and

American Journal of Perinatology
Platelet Aggregation Profile Model

Total Platelet Aggregation Model

Demographic data for study participants were compared using the chi-square test for categorical data and the two-sample t-test for continuous data. Continuous data were checked for nonnormality and for outliers. Fisher exact test was used to compare categorical data in the case of small category numbers. A rank sum test was used for nonnormality and for outliers. Fisher exact test was analyzed using the following three modeling approaches: one-parameter sigmoidal dose response (competition), two-parameter sigmoidal dose response (competition and interaction), and logistic dose response (competition). These allowed a free-varying dose term. These allowed a free-varying dose–response relationship.

Statistical Analysis

Platelet function was assessed using a novel modification of IITA which assesses platelet aggregation in response to a range of multiple agonists, where the percentage of light absorbance from baseline corresponds to the degree of platelet aggregation. The percentage aggregation response for each concentration of each agonist was calculated and plotted against the log values of the concentrations of agonists using GraphPad Prism software (GraphPad Prism, San Diego, CA). The T1/2 (or half-maximal effective concentration [Ec50]) values were further generated from the dose–response curves. Our group has described this assay in detail previously. 25,26

Results

A total of 51 patients with an EFW < 10th centile and 36 third trimester pregnancy controls were recruited from 24 weeks' gestation at a single center over the course of 2 years (2012–2014). Seven subjects were excluded for analysis (unsuitability of samples for testing [n = 3], thrombocytopenia [n = 2], aneuploidy [n = 1], and systemic lupus erythematosus [n = 1]) resulting in a total of 33 patients comprising the IUGR < 10th centile group, and with the remaining 11 in the IUGR birth weight > 10th centile group. The mean gestational age at enrollment to the study for the IUGR group was 34 weeks (+3 days) and the mean gestational age at delivery was 36 weeks (+3 days), resulting in a significantly lower birth weight compared with controls (p < 0.001). Congenital infection as a cause for IUGR was ruled out in all cases. Antenatal corticosteroids for fetal lung maturation were administered in 79 and 45% of the IUGR birth weight < 10th centile and birth weight > 10th centile groups, respectively. In IUGR cases where antenatal corticosteroids were administered, platelet function testing was generally delayed for greater than 48 hours from the time of corticosteroid administration to minimize any potential effect on platelet function, and in most cases, testing was undertaken on average a week from the administration of corticosteroids. None of the women had taken aspirin or were administered low-molecular-weight heparin at any point in the pregnancy. Table 1 outlines maternal demographic data and delivery outcomes.

Platelet Function

For platelet function analysis for the IUGR birth weight < 10th centile group (n = 33), platelet aggregation profile models generated for each agonist demonstrated statistically significant less platelet aggregation than normal pregnancy controls (Fig. 1). The overall mean platelet aggregation and AUC for each agonist were significantly reduced in IUGR (Table 2). The Ec50 (as a measure of platelet agonist potency) for each agonist in IUGR was significantly greater compared with normal controls. This implies that higher concentrations of each agonist in cases of IUGR were required to produce a response halfway from baseline to maximum aggregation to produce a similar response in controls. These findings imply a global “hyporeactivity” of platelets across all agonists at all concentrations in growth restricted pregnancies compared with pregnancies with an appropriately grown fetus. When analyzed separately, the IUGR birth weight > 10th centile group also demonstrated a similar effect, with significantly reduced or altered platelet function compared with normal pregnancy controls (Table 2).

To investigate whether the differences in platelet profiles described earlier correlate with severity of IUGR, a subgroup analysis was performed. The IUGR study group (n = 33) was divided into subgroups of pregnancies complicated by IUGR with either early-onset IUGR (< 34 weeks' gestation [n = 14]); abnormal umbilical artery (UA) Doppler absent end-diastolic flow (AEDF) (n = 13); EFW < 3rd centile (n = 15), or IUGR complicated with PET/GH (n = 12). The most prominent finding was that of statistically significant reduced platelet aggregation in response to all agonists when IUGR occurred in
the setting of subsequent development of PET/GH compared with normotensive IUGR, which is not surprising as this represents the severe end of the spectrum of uteroplacental disease (►Table 3). Early-onset IUGR < 34 weeks' gestation and IUGR with hypertensive disease were notably both associated with significantly reduced platelet responses to Col \( (p = 0.0019 \text{ and } p < 0.0001, \text{ respectively}) \). Within the abnormal UA Doppler group, there was significantly reduced platelet reactivity in response to the agonist ADP alone \( (p = 0.019) \). There were no differences in platelet aggregometry profiles in IUGR cases stratified according to EFW < 3rd centile when compared with IUGR > 3rd centile.

When evaluating the effects of maternal characteristics on platelet function in IUGR pregnancies, there were no differences in total platelet aggregation for smokers \( (n = 9) \), pregnancies with a BMI > 30 \( (n = 3) \), or concurrent antihypertensive medication use \( (n = 3) \) (AUC was analyzed because samples were too small for statistical analysis).

### Placental Findings in Relation to Platelet Function

For cases of IUGR < 10th centile, all had evidence of uteroplacental insufficiency as denoted by the presence of AVM, with all placentas weighing less than the 10th centile for gestational age \( (n = 33) \). For all cases with a prenatal diagnosis of IUGR < 10th centile, but with an eventual birth weight > 10th centile, a normal placental histopathology report was returned \( (n = 11) \). Of all cases of IUGR with AVM, in five cases, additional abnormalities were noted including three cases of fetal thrombotic vasculopathy; one case of fetal artery occlusion and infarction, and a single case of chronic histiocytic intervillositis.

### Comment

Despite the proposed link between platelets and uteroplacental insufficiency, platelet function has not been consistently characterized to date in pregnancies complicated by uteroplacental insufficiency.\(^9\)–\(^{23}\) We comprehensively

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**Table 1 Patient demographic data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IUGR birth weight &lt; 10th centile ( (n = 33) )</th>
<th>IUGR birth weight &gt; 10th centile(^a) ( (n = 11) )</th>
<th>Normal control(^b) ( (n = 36) )</th>
<th>( p )-Value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>29 ± 6</td>
<td>26 ± 4</td>
<td>32 ± 4</td>
<td>0.063 0.001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25 ± 6</td>
<td>27 ± 6</td>
<td>24 ± 4</td>
<td>0.527 0.141</td>
</tr>
<tr>
<td>Caucasian ethnicity</td>
<td>28 (85%)</td>
<td>7 (64%)</td>
<td>34 (94%)</td>
<td>0.247 0.021</td>
</tr>
<tr>
<td>History of PET</td>
<td>4 (12%)</td>
<td>0</td>
<td>0</td>
<td>0.034 –</td>
</tr>
<tr>
<td>History of IUGR</td>
<td>9 (27%)</td>
<td>1 (9%)</td>
<td>0</td>
<td>0.001 0.059</td>
</tr>
<tr>
<td>History of chronic hypertension</td>
<td>1 (3%)</td>
<td>0</td>
<td>0</td>
<td>0.485 –</td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>3 (9%)</td>
<td>1 (9%)</td>
<td>0</td>
<td>0.109 0.239</td>
</tr>
<tr>
<td>Antenatal steroids</td>
<td>26 (79%)</td>
<td>5 (45%)</td>
<td>0</td>
<td>&lt; 0.001 &lt; 0.001</td>
</tr>
<tr>
<td>Smoker</td>
<td>9 (27%)</td>
<td>1 (9%)</td>
<td>0</td>
<td>0.001 0.071</td>
</tr>
<tr>
<td>Gestation at blood draw (wk)</td>
<td>34 ± 3</td>
<td>32 ± 4</td>
<td>30 ± 6</td>
<td>0.009 0.546</td>
</tr>
<tr>
<td>Platelet count</td>
<td>252 ± 62</td>
<td>206 ± 35</td>
<td>252 ± 45</td>
<td>0.980 0.004</td>
</tr>
<tr>
<td>Gestation at delivery (wk)</td>
<td>36 ± 3</td>
<td>39 ± 1</td>
<td>40 ± 1</td>
<td>&lt; 0.001 0.002</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.0 ± 0.6</td>
<td>3.0 ± 0.4</td>
<td>3.7 ± 0.5</td>
<td>&lt; 0.001 &lt; 0.001</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; IUGR, intrauterine growth restriction; PET, preeclampsia.

\(^a\)IUGR birth weight > 10th centile: cases where an initial estimated fetal weight was < 10th centile were noted at the time of antenatal enrollment, but where actual birth weight was > 10th centile were excluded from the IUGR cohort for the purpose of the primary analysis, and were analyzed separately (IUGR, birth weight > 10th centile, \( n = 11 \)).

\(^b\)Normal control: pregnancy controls consisted of women in third trimester with a normally grown pregnancy with confirmed normal pregnancy outcome and birth weight.

\(^c\)\( p \)-Value comparisons: IUGR < 10th centile versus controls, IUGR > 10th centile versus controls.
evaluated platelet function in the setting of IUGR using a modification of LTA, where the addition of platelet-rich plasma to submaximal concentrations of multiple physiological agonists created significant optical effects and platelet aggregation results. This method is a better replication of actual in vivo platelet events. The most prominent finding of this study is significantly reduced platelet function in IUGR compared with normal pregnancy controls. We have demonstrated a highly statistically significant global “hyporeactivity” of platelets in response to all agonists and at all concentrations of each agonist. Similar findings were recorded in pregnancies where the fetus demonstrated a dynamic in utero growth spurt. When fetal growth restriction occurred in the setting of PET or GH, or occurred at an earlier gestation, platelet responses were significantly reduced, perhaps correlating with a more severe clinical entity on the spectrum of uteroplacental disease.

There have been limited previous studies of platelet aggregation in IUGR. Our findings are similar, in part, to a study by Norris et al, who investigated platelet function in pregnancies complicated by IUGR superimposed with hypertension compared with normotensive IUGR. The method for platelet function testing comprised whole blood aggregation in response to only two agonists: ADP and Col. In hypertensive IUGR pregnancies before delivery, platelet aggregation was found to be almost 50% lower compared with normal pregnancy controls; however, there were no differences reported in platelet aggregation between normotensive IUGR pregnancies and normal pregnancy controls. The authors concluded that the findings of reduced platelet function in hypertensive IUGR pregnancies may represent increased platelet activation at the uteroplacental interface, with resultant vasoactive substance release into the maternal circulation that may be

Fig. 1  Dose–response curves of platelet reactivity (aggregation) in response to each individual agonist (arachidonic acid, epinephrine, thrombin receptor–activating peptide, adenosine diphosphate, and collagen) at increasing concentrations of the agonist for IUGR birth weight < 10th centile compared with normal pregnancy controls (with a normal prenatal EFW and postnatal birth weight > 10th centile). EFW, estimated fetal weight; IUGR, intrauterine growth restriction. *TRAP, thrombin receptor–activating peptide. **ADP, adenosine diphosphate.
Table 2 Analysis of platelet function results for IUGR birth weight < 10th centile and IUGR birth weight > 10th centile versus normally grown third trimester pregnancy controls

<table>
<thead>
<tr>
<th>Platelet aggregation summary</th>
<th>IUGR birth weight &lt; 10th centile (n = 33)</th>
<th>IUGR birth weight &gt; 10th centile (n = 11)</th>
<th>Normal control (n = 36)</th>
<th>p-Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall difference in mean aggregation profile for each agonistb</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AUCc (mean)</td>
<td>23.5</td>
<td>23.5</td>
<td>59.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>E50d (estimate)</td>
<td>– 0.38</td>
<td>– 0.40</td>
<td>– 0.77</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>50.1</td>
<td>49.6</td>
<td>74.8</td>
<td>0.0364</td>
</tr>
<tr>
<td>E50 (estimate)</td>
<td>1.841</td>
<td>1.982</td>
<td>1.65</td>
<td>0.0191,1 0.00612</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>23.7</td>
<td>23.7</td>
<td>47.2</td>
<td>0.0285</td>
</tr>
<tr>
<td>E50 (estimate)</td>
<td>– 0.651</td>
<td>– 0.622</td>
<td>– 0.99</td>
<td>&lt; 0.0001,1 0.00052</td>
</tr>
<tr>
<td>Epinephrine</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>48.4</td>
<td>48.0</td>
<td>105.4</td>
<td>0.0027</td>
</tr>
<tr>
<td>E50 (estimate)</td>
<td>4.011</td>
<td>4.152</td>
<td>3.68</td>
<td>0.0020,1 0.00272</td>
</tr>
<tr>
<td>Thrombin receptor–activating peptide</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>30.0</td>
<td>30.3</td>
<td>69.1</td>
<td>0.0002</td>
</tr>
<tr>
<td>E50 (estimate)</td>
<td>1.981</td>
<td>2.132</td>
<td>1.52</td>
<td>&lt; 0.0001,1 &lt; 0.0012</td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; IUGR, intrauterine growth restriction.

*p-Values (see methods in the section Statistical Analysis) present are any overall difference in the mean platelet aggregation profile for all five platelet agonists (arachidonic acid, adenosine diphosphate, collagen, epinephrine, and thrombin receptor–activating peptide) between groups using ANOVA.

bDifference in EC50 (half maximal concentration) between groups using sigmoidal growth curve models.

cDifference in AUC between groups using two-sample t-tests.

dDifference in E50 (half maximal concentration) between groups using sigmoidal growth curve models.

responsible for the more severe clinical syndrome that is typical of combined preeclampsia and IUGR.

Ahmed at al investigated IUGR with platelet-activating factor (PAF) and noted higher concentrations of PAF were required to achieve maximal aggregation compared with appropriately grown fetuses, also indicating reduced platelet reactivity in IUGR; however, there were only three subjects in this study.20 In a further study, changes in uterine artery Doppler waveform assessed at 23 weeks’ gestation in pregnancies that subsequently developed IUGR were correlated with platelet function, with the finding of increased mean platelet volume (MPV) and ADP-induced platelet aggregation for cases that subsequently developed IUGR.21 Lees et al undertook a similar study of pregnancies with bilateral uterine artery notches; however, logistic regression analysis did not identify platelet volumetric changes at the time of Doppler assessment to be predictive of adverse pregnancy outcome.22 Piazze et al concluded that increased hematomal parameters (platelet count and MPV) were associated with abnormal Doppler velocimetry for pregnancies complicated by IUGR or PET compared with normal pregnancy controls.23 Platelet count and volume do not accurately reflect platelet function.

In this study, we use a standardized platelet assay to comprehensively describe platelet function in uteroplacental disease. The studies of platelet function in pregnancy to date have used either single agonists or limited concentrations of agonists, whereas in this study, we report on incremental concentrations of five different agonists which represent a more physiological assessment. The methodology adopted included strict inclusion and exclusion criteria, with blood draw occurring at a specific time point in the morning after a period of fasting to avoid circadian influence.27 Pregnancies with diabetes mellitus were excluded in this study, as this condition is associated with platelet hyperreactivity. Some previous studies of platelet function in pregnancy have included diabetic patients, which may explain, in part, their findings of increased platelet reactivity.17,28,29 The protocol for blood draw was specifically designed to eliminate factors known to influence platelet reactivity, with a single operator carrying out each blood draw in a controlled fashion as detailed above. Our study group comprised a well-defined IUGR cohort, with analysis occurring after delivery outcomes and placental histopathology results were available. These data collection afforded us the opportunity to confirm the antenatal diagnosis and to investigate platelet behavior on not only “pathologically small” but also on infants that demonstrated increasing in utero growth trajectories with a normal delivery outcome, which has not been previously reported to our knowledge to date.

Subgroup analyses based on the severity of IUGR were performed and revealed that when fetal growth restriction occurred for the most part in the setting of subsequent development of hypertensive disease, platelet responses
were most significantly reduced compared with normotensive IUGR. Although the number of patients within this subgroup was small, the finding of reduced platelet function in response to all agonists and all concentrations in IUGR for the most part before the development of later hypertensive disease suggests a possible differing and more severe pattern of vascular disease compared with normotensive IUGR. These findings may also suggest that when IUGR occurs as the apparent primary condition, platelet function may be a useful predictor of subsequent development of superimposed hypertensive disease. Although IUGR subjects were recruited at a later gestational age than normal pregnancy controls (34 ± 3 vs. 30 ± 6 weeks’ gestation, respectively), our research group previously validated the method of platelet function comprising this study longitudinally in each trimester of normal pregnancy, reporting increasing platelet reactivity with increasing gestation. Therefore, had we assayed our IUGR cases at an earlier gestational age, we would have expected an even more significant degree of platelet hyporeactivity results for this group.25

A weakness of our study is the lack of longitudinal data particularly from the first trimester onward, and therefore, we missed an opportunity to study platelet changes during a subclinical period before established disease. Information on platelet function early in complicated pregnancies might afford the opportunity for a predictive test of adverse pregnancy outcome and enable individualized aspirin dosing based on platelet function in high-risk pregnancies to optimize outcomes.30 The method of blood sampling, and the large blood volume required (30 mL) for testing, would make serial sampling by the method described in this study impractical within the setting of a large prospective study in pregnancy throughout all trimesters.

The question remains as to whether our findings of significant platelet hyporeactivity represent a cause or consequence of IUGR? Exaggerated platelet activation has long been thought to accompany early preclinical uteroplacental disease.10,31 The rationale for aspirin use in current pregnancies with a history of early-onset IUGR implies an indirect pathogenic role of platelets early in the development of this condition. Platelets have recently been shown to promote normal placental development, where activated platelets have been localized within maternal spiral arterioles.32 Despite the constant remodeling and exposure of the highly thrombogenic Col lining these vessels, it is surprising that platelets, though activated, uniquely do not appear to aggregate or promote thrombus in the normally developing placenta.4 Our research group recently observed the most significant reduction of platelet reactivity to the agonist Col in healthy first trimester pregnancies.25 Therefore, it is possible that defective platelet behavior or activation with unwanted thrombus formation in response to endovascular subendothelial Col is occurring early during the establishment of the uteroplacental unit in IUGR pregnancies, and that

### Table 3 Subgroup analysis of Platelet function results for IUGR with PET/GH versus normotensive IUGR

<table>
<thead>
<tr>
<th>Platelet aggregation summary per agonist</th>
<th>PET/GH with IUGR (n = 12)</th>
<th>Normotensive IUGR (n = 21)</th>
<th>p-Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arachidonic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall difference in mean aggregation profile for each agonist&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sup&gt;b&lt;/sup&gt; (mean)</td>
<td>18.7</td>
<td>29.1</td>
<td>0.1158</td>
</tr>
<tr>
<td>EC50&lt;sup&gt;c&lt;/sup&gt; (estimate)</td>
<td>– 0.19</td>
<td>– 0.48</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>Adenosine diphosphate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>33.9</td>
<td>75.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>EC50 (estimate)</td>
<td>2.24</td>
<td>1.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>7.7</td>
<td>33.1</td>
<td>0.0033</td>
</tr>
<tr>
<td>EC50 (estimate)</td>
<td>– 0.55</td>
<td>– 0.82</td>
<td>0.0482</td>
</tr>
<tr>
<td><strong>Epinephrine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>42.7</td>
<td>77.5</td>
<td>0.0538</td>
</tr>
<tr>
<td>EC50 (estimate)</td>
<td>4.26</td>
<td>3.85</td>
<td>0.0015</td>
</tr>
<tr>
<td><strong>Thrombin receptor-activating peptide</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (mean)</td>
<td>21.0</td>
<td>49.2</td>
<td>0.0113</td>
</tr>
<tr>
<td>EC50 (estimate)</td>
<td>2.19</td>
<td>1.81</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; GH, gestational hypertension; IUGR, intrauterine growth restriction; PET, preeclampsia.

<sup>a</sup>p-Values (see methods in the section Statistical Analysis) present are any overall difference in the mean aggregation profile for all five platelet agonists (arachidonic acid, adenosine diphosphate, collagen, epinephrine, and thrombin receptor–activating peptide) between groups using ANOVA.

<sup>b</sup>Difference in AUC between groups using two-sample t-tests.

<sup>c</sup>Difference in EC50 between groups using sigmoidal growth curve models.
this may well explain the mechanism by which aspirin counteracts. Our findings in this study of reduced reactivity of platelets in IUGR diagnosed in third trimester may be reflective of an adaptation or reversion back to first trimester conditions, in an attempt to promote angiogenesis and improve uteroplacental perfusion.

Another hypothesis is that this finding of platelet hyporeactivity may represent a function of the "platelet release reaction," whereby platelets have been shown to have the ability to preferentially release either pro- or antiangiogenic material within a given microenvironment. There have been consistent lines of evidence showing that soluble fms-like tyrosine kinase-1 (sFlt-1) levels are increased in PET and IUGR. As sFlt-1 is the soluble protein receptor for growth factors vascular endothelial growth factor (VEGF) and placental growth factor, the upregulation of sFlt therefore explains the observation of reduced levels of these factors in these conditions. The proangiogenic VEGF is predominantly stored in platelets, and it is possible that the global hyporeactivity of platelets in this study is mirroring preferential platelet release of VEGF. The method of platelet function that we used in this study (platelet aggregation) possibly represents an "end" result of a potentially large number of platelet growth factor release mechanisms. Although our study is unable to biologically support this concept, we observed an interesting finding of reduced platelet reactivity in response to the agonist ADP alone when IUGR was complicated by abnormal UA Doppler waveform. ADP has been shown to promote the preferential release of VEGF from platelets, perhaps suggesting a therapeutic role for ADP-driven platelet growth factor release in severe IUGR.

Animal studies have supported a role for VEGF therapy as a growth stimulant in IUGR. An ongoing randomized controlled EVERREST (does vascular endothelial growth factor gene therapy safely improve outcome in severe early-onset fetal growth restriction) trial of VEGF gene therapy for severe early-onset fetal growth restriction diagnosed between 20 and 26–26 weeks' gestation is currently recruiting. It has been proposed that delivery of adenovirus containing VEGF gene to the uteroplacental circulation leads to local overexpression of VEGF, alteration of uterine artery vascular tone and improved angiogenesis and uterine blood flow. Given that most of the VEGF in our circulation is stored and released by platelets, it is plausible that platelets may play a role in the reconciliation of placental-mediated IUGR and the promotion of fetal growth through platelet growth factor release.

A unique finding in this study is that of significantly reduced platelet reactivity in those fetuses with a prenatal diagnosis of IUGR, but with a subsequent normal birth weight and outcome, which may lend further support to a possible proangiogenic role of platelets in pregnancy. In unpublished data from the PORTO (prospective observational trial to optimize pediatric health in IUGR) study, dynamic in utero growth spurts were described in as many as 17% of IUGR fetuses. Neither single nor combinations of growth factors have emerged as useful screening tools for PET or IUGR. Future research may focus on extrapolating further possible platelet-derived growth factors as an effective intervention in early-onset IUGR. In addition, future point-of-care tests of platelet function may evolve to identify novel modifiable markers of uteroplacental disease. Such measures may also provide a platform for enabling individualized dosing of aspirin in pregnancies deemed at high risk for developing uteroplacental disease to optimize outcomes.

Conclusion

In women with a prenatal diagnosis of IUGR, this study demonstrates that platelet function, as assessed by in vitro platelet reactivity in response to multiple concentrations of five physiological agonists is altered compared with women with a normally grown fetus. Future point-of-care tests of platelet function may evolve as novel bedside markers of uteroplacental disease that may enable more individualized platelet-targeted interventions early in pregnancy to maximize outcomes.

Acknowledgment

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References

1. Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response—a review. Placenta 2003;24(Suppl A):S21–S27
3. Sato Y, Higuchi T, Yoshioka S, Tatsumi K, Fujihara H, Fuji S. Trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype. Development 2003;130(22):5519–5532
11. Morrison R, Crawford J, MacPherson M, Hepinstall S. Platelet behaviour in normal pregnancy, pregnancy complicated by...