A Novel Assay of Platelet Function reveals Altered Platelet Activity during Healthy Pregnancy

A thesis submitted to the National University of Ireland for the degree of Master in Science

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Abstract

It is well established that markers of platelet activation are elevated during normal pregnancy. For example, levels of platelet-derived thromboxane are persistently elevated in serum and urine of pregnant women, to levels equivalent to those observed in acute cardiovascular disease. In addition, other markers of platelet activation, such as elevated plasma levels of platelet derived P-Selectin and CD40Ligand, are elevated in pregnancy. However, the clinical significance of platelet activation during pregnancy is not understood. Furthermore, systematic studies of platelet activation throughout pregnancy are not widely available.

In this study I aimed therefore to characterize changes in platelet function in women during normal healthy pregnancy. With our collaborators in the Coombe Women's and Infants University Hospital in Dublin, 20 healthy pregnant women were recruited at their first-trimester hospital visit (T1; 9-14 weeks gestational age). They donated a 12 ml blood sample for analysis of platelet function and plasma thromboxane levels at this first visit and again in the second trimester (T2; 14-27 weeks), the third trimester (T3; 27-37 weeks) and within 8 weeks of delivery of their baby (post-partum; PP). Platelets were prepared from the whole blood samples and analysed as follows: (1) Light Transmission Platelet Aggregation, a gold standard test of platelet function. Platelet aggregation responses are measured in response to a thrombin-derived activator peptide (TRAP), a thromboxane mimic (U46619), a collagen related agonist (CRP), and Arachidonic acid (AA). (2) Platelet ATP/ADP secretion (PAS) assays were used to measure the sensitivity of platelets to secrete their granular contents in response to dose-ranges of agonists (TRAP, U46619, CRP and AA), in order to quantify relative changes in platelet sensitivity during and after pregnancy. Finally, the levels of thromboxane, a prothrombotic prostaglandin, were assessed in the plasma of the women, to gain an insight into levels of platelet activation that might be present in circulating platelets in the women.

Although there have been previous studies that have assessed various aspects of platelet activation and prostaglandin levels during pregnancy, no study has covered all aspects over the full range of gestation from T1 to PP. Thus, this is a novel study that established baseline levels of multiple parameters throughout pregnancy in healthy women. The results of this study will be critical for the design of a follow-on study that will attempt to determine if platelet function differs significantly in mothers at risk of pre-eclampsia or intrauterine growth retardation (IUGR) compared to mothers with normal healthy pregnancies.

The results of this study show that there is substantial evidence for modulation of platelet activation during normal healthy pregnancy. Platelet aggregation in response to standard doses of soluble platelet agonists (TRAP, U46619 and AA) are suppressed in T1 compared to other time-points. In contrast, the responses to CRP, a collagen-related peptide are increased. In PAS assays, differences in response between soluble agonists (TRAP, U46619 and AA) and the collagen-related peptide are also observed. Most strikingly, there is an increase in potency for the collagen related peptide that suggests that this mechanism of platelet activation acquires a new importance in pregnancy. In parallel, responsiveness to other soluble platelet agonists is down regulated. I hypothesize that this subtle regulation of platelet responsiveness during pregnancy reflects a differential regulation of platelet function that is required during a normal healthy pregnancy.
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Masters of Science (M.Sc.), 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

Student Number 07211040

Date May 10th, 2014
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INTRODUCTION

1.1 Haemostasis and Platelet Signaling Pathways

Platelets are small cell fragments produced by bone marrow megakaryocytes. Their fundamental role is involved in maintaining hemostasis, and facilitating thrombosis in response to vessel injury when there is damage to the endothelium of blood vessel. The life span of a platelet is 7-10 days \[^1\]\. Platelet activation is inhibited by Nitric Oxide \[^2\]\, PGI\(_2\) \[^3\]\ and endothelial-ADPase \[^4\]\.

When the endothelium of blood vessel gets damaged, the sub-endothelial collagen gets exposed. Von Willebrand factor (vWF) glycoprotein facilitates platelet adherence to the exposed collagen and the formation of fibrin-rich thrombi at the site of injury. In parallel, tissue factor (TF), released from damaged endothelial cells, initiates the coagulation cascade in the blood plasma. The end result of the coagulation cascade is the activation of plasma prothrombin (Factor II) to thrombin, and the conversion of plasma fibrinogen to fibrin. This fibrin forms a polymer that is the basis for a blood clot. Thrombin is also a potent activator of platelets \[^5\]\. It cleaves and activates the PAR-1 protease activated receptor on the platelet surface. This 7-transmembrane domain receptor is linked to G protein coupled pathways that promote platelet activation events.

In response to platelet activation by thrombin, activation of cytoplasmic Phospholipase C (PLC) is observed. PLC uses membrane phospholipids as a substrate to generate two second messengers in the platelet cytoplasm: inositol triphosphate (IP\(_3\)) and Diacylglycerol (DAG) \[^54\]\. Raised cytoplasmic levels of IP\(_3\) facilitate release of calcium from intracellular stores, while elevated DAG levels promotes activation of many cellular kinases such as protein kinase C. Calcium promotes granule secretion from platelets and, in parallel, activates another enzyme known as Phospholipase A\(_2\) (PLA\(_2\)) \[^55\]\. PLA\(_2\) initiates a cascade of responses within platelets that ultimately leads to the synthesis of the potent prothrombotic prostaglandin, Thromboxane A\(_2\) (TxA\(_2\)) via the aspirin-sensitive enzyme Cyclooxygenase (COX) \[^55\]\. In parallel, DAG-activated PKC initiates a cascade of events in the platelet that finally results in activation of the platelet surface glycoprotein GpIIb/IIa. When activated, GpIIb/IIa acts as a high affinity receptor for Fibrinogen. Fibrinogen binding to activated platelets acts like a bridge to cross-link and aggregate platelets (see figure 1.1) \[^53\]\.
Figure 1.1 Demonstrating platelet activation \cite{50} illustrates platelet hemostasis state. The resting platelet has several surface glycoprotein complexes, including GPIb-IX-V, GPIIb-IIIa, and GP1a-IIa/VI. During vascular injury, the extracellular matrix is exposed, and platelets are temporarily attached to collagen-bound von Willebrand factor (vWF) via GPIb-IX-V. At this reaction activates platelets, thereby resulting in (1) conversion of the platelet surface GPIIb-IIIa complex into its active conformation (GPIIb-IIIa\(^*\)) and causing irreversible binding of platelets to vWF, (2) secretion of different factors from the platelet granules, including ADP and factor V, pursuing to coagulation reactions and (3) recruitment of additional platelets to the site of injury via fibrinogen bridging between the activated GPIIb-IIIa complexes on adjacent platelets to form a platelet plug. Other tissue factor released from the site of injury activates the production of thrombin and the formation of fibrin.

Once the platelets are activated, the contents of their alpha, dense/delta and lysosomes granules are released into the extracellular medium. Alpha granules contain large adhesive proteins whilst the dense granules contain small non-protein molecules (including ATP, ADP and 5-HT-serotonin) that are secreted to recruit other platelets to a site of damage, whereas lysosomes contain hydrolases that lyse the fibrin in platelet clots \cite{6}.
Wound repair is one of the secondary roles of platelets. Platelets release bioactive agents that include growth factors (PDGF, VEGF and TGFβ) from the alpha granules\textsuperscript{[7-9]} that promote the migration of fibroblasts from connective tissues into the wounded site to form a scar\textsuperscript{[10]}.

1.2 Assessing platelet function in clinical samples

Platelets circulate in normal healthy people in an inactivated state in the blood. Their major function is believed to be to survey the vasculature for signs of damage or injury. Measurement of platelet function ex-vivo can give useful insights into a patients' clinical status\textsuperscript{[11]}. While numerous types of platelet function tests are available, we will restrict our analysis to 3 tests of platelet function: (1) the gold-standard test (platelet aggregometry); (2) plasma thromboxane analysis (TxA\textsubscript{2}), which is commonly used for platelet assessment and (3) a novel assay of platelet secretion termed the Platelet ADP/ATP secretion or PAS assay.

1.2.1 Aggregometry

Light transmittance aggregometry (LTA) is a historic laboratory method introduced by haematologists and researchers more than 40 years ago\textsuperscript{[12]}, since the 1960s. It assesses the rate of platelet activation by measuring the ability of platelets to aggregate and form thrombi. Blood samples are centrifuged to remove red and white blood cells in order to prepare Platelet Rich Plasma (PRP). PRP is then transferred into the aggregometry
cuvette. Platelet aggregation is induced by the addition of agonists. A light beam passes through the PRP sample where the platelets aggregate in response to the agonist. As they aggregate they fall to the bottom of the cuvette allowing more light to pass through the sample and hence the percentage light-transmission is calculated as shown in Figure 1.3. Due to the restrictions of the aggregometry, we explored a second assay that is described below.

Figure 1.3 Schematic representation of platelet aggregation as per the Born principle (52)

1.2.ii Limitations to Gold Standard platelet Aggregometry

Although Born Aggregometry has been used in the clinical assessment of platelet function since the 1960's, it is far from ideal. It measures platelet aggregate formation by simple light transmission through a glass cuvette. It depends on the formation of large aggregates of platelets that then fall to the bottom of the glass vial, permitting increased light transmission. This Gold-standard assay has certain downfalls such as the fact that it is labor intensive, time consuming, measures responses to few dose of agonist, requires a large volume of blood sample and most importantly, is insensitive to the aggregation caused by low doses of agonists that induce aggregates that were not dense enough to fall to the bottom of the cuvette. Although it is fully capable of assessing platelet aggregation to strong agonists, it has limitations at low agonist concentrations or with weak agonists. It is non-linear in its response to increasing doses of agonists, since it depends on the ability of platelets to form large aggregates. However, with low doses of strong agonists (thrombin, TRAP, Collagen etc.) or with weak agonists (adrenaline, 5-HT), only small platelet aggregates form, that do not fall to the bottom of the vial, resulting in no observed change in light transmission.
Thus, assessment of the dose-response relationship of platelets to agonists is not accurate using platelet aggregation techniques. Because of this, the RCSI platelet group developed an assay of platelet function that could more accurately assess platelet responsiveness and sensitivity to a range of common platelet agonists. This assay is the Platelet ADP/ATP secretion (PAS) assay.
1.2.iii Platelet ADP/ATP section (PAS) assay

The PAS assay is an assay developed in-house at RCSI that measures both the maximal amount of ADP/ATP secreted from activated platelets by a range of activators (efficacy) and the potency of each activator (EC₅₀). It is a simple technique that uses a commercially available kit to quantify the amount of ATP/ADP released in response to increasing doses of agonists. It quantifies ATP release from low volumes of Platelet Rich Plasma (PRP) using ChronoLume™, a commercial kit that uses firefly luciferase enzyme analogue to detect ATP as shown in Fig 1.4. It is a 96-well plate assay that is rapid, easy and is capable of simultaneously analyzing platelet responses to multiple doses of a variety of platelet agonists in a short period of time and requires relatively small volumes of blood.

![Diagram of PAS assay](image)

**Figure 1.4:** Schematic representation of assay for determining ADP/ATP release from platelets using a commercially available ATP-detection kit from ChronoLume™, as described in Shanley et al, 2011[13].

1.2.iv Platelet Agonists

**Thrombin Receptor Activating Peptide (TRAP)** is a peptide derived from the N-terminus of the thrombin protease-activated receptor (PAR). It corresponds to the new N-terminus of the receptor exposed after receptor activation by thrombin. Thrombin is a key enzymatic intermediate in the coagulation cascade and, in parallel, a strong activator of
platelet aggregation. Thrombin activates platelets by enzymatically cleaving the PAR receptor, revealing the peptide sequence SFLLRN, a tethered agonist for the PAR receptor. A synthetic version of this peptide sequence is termed TRAP-Thrombin receptor activating peptide. TRAP is a peptide mimic of thrombin. It is readily available, cheap, stable and potent, in contrast to the enzymatic thrombin. Its key benefit is that it specifically activated platelet PAR-1 receptors without activating the coagulation cascade in PRP samples.

**Arachidonic Acid** It can be released from its membrane storage site by the action of the PLA$_2$ enzyme. It is then rapidly metabolised by the Cyclooxygenase enzyme that incorporates oxygen into the lipid to form endoperoxide prostaglandin G$_2$ (PGG$_2$). PGG$_2$ is then converted to prostaglandin H$_2$ (PGH$_2$). In platelets, thromboxane synthase then converts this unstable endoperoxide into Thromboxane A$_2$. Thromboxane A$_2$ is well established to be a potent platelet activator.

U46619 is a potent and stable thromboxane A$_2$ (TP) receptor agonist. It potently stimulates platelet TP receptor-mediated, but not other prostaglandin receptor-mediated responses in platelets.

**Collagen** is a structural protein that is ubiquitous throughout the human body. During primary haemostasis, following blood vessel injury, the adhesion of platelets to exposed collagen will cause platelets to change their shape, release endogenous ADP/ATP and aggregate. This platelet adhesion plays a key role in the arrest of bleeding from this site. Any interference with the ability of platelets to interact with exposed collagen is therefore a possible cause of an unexplained bleeding tendency. Collagen binds to platelets via two different receptor proteins: integrin receptors $\alpha_2\beta_1$ and Glycoprotein VI (GPVI). As a laboratory reagent, collagen, like thrombin, can be difficult to work with. It can vary in its potency from batch to batch. Also it is expensive and unstable. It cannot be frozen and thawed and so it has a short shelf life. In contrast, the group of Professor Richard Farndale, University of Cambridge developed a peptide mimic of collagen that acts as a direct and potent agonist at GPVI receptors. This peptide is known as Collagen Related Peptide (CRP) and is potent, stable, reliable and easy to use. Therefore we opted to use this peptide as a collagen-mimic in our studies.

1.2. v Thromboxane assay

Thromboxane A$_2$ (TxA$_2$) a lipid mediator synthesized from Arachidonic acid in platelets as described above. Released TxA$_2$ then enhances platelet aggregation. In addition to enhancing platelet aggregation caused by other agonists, it also causes vasoconstriction. It then rapidly gets hydrolyzed to inactive Thromboxane B$_2$ (TxB$_2$). The presence of TxB$_2$ in plasma is an indicator of recent platelet activation events.
This assay is a quantitative analysis of TxB₂ levels in the sample. It operates on the basis of competition between the enzyme conjugate and the TxB₂ in the sample for a limited number of binding sites on the antibody coated plate. The bound enzyme conjugate is detected by the addition of substrate that generates a coloured product.

Measuring and comparing the absorbance reading of the wells of the samples against the standards using a microplate reader may obtain quantitative test results. The extent of color development is inversely proportional to the amount of TxB₂ in the sample.

### 1.3 Platelet activation in Pregnancy

Multiple possible roles for platelets and the haemostatic system have been proposed during normal healthy pregnancy. While changes in the coagulation are well documented [14], the role of platelets is more controversial.

Platelets play a critical role in trophoblast infiltration into maternal spiral arteries [15]. The activation of maternal platelets within spiral arteries could assist trophoblastic arterial infiltration, corresponding to physiological vascular remodeling [16]. In addition, it has been strongly suggested that platelets may contribute to protective mechanisms against excessive bleeding during childbirth [17].
1.3.i Hemostatic changes during Pregnancy

Many studies have explored the hemostatic changes that occur during pregnancy. Pronounced alterations in coagulability and hemostatic parameters have been shown to occur in a systematic manner in normal pregnancy. In addition, abnormalities of these parameters are observed in complications of pregnancy such as preeclampsia, gestational hypertension and Intrauterine Growth Restriction (IUGR) that have been correlated to fetal death, preterm delivery and severe neonatal morbidity and mortality, due to generalized vasoconstriction, endothelial activation \[^{[18]}\], fibrin deposition \[^{[19]}\] and inflammation. Studies to determine the cause of altered hemostasis in pregnancy have mainly focused on coagulation proteins, tissue factors and secreted bioactive components that contribute to coagulation.

Preeclampsia affects up to 5% of pregnancies, and severe cases develop in about 1-2% of pregnancies. It is characterized by an increased vasoconstriction causing elevated life-threatening blood pressure. It is frequently associated with increased platelet aggregation, reduced uteroplacental blood flow, and the need for premature delivery \[^{[20]}\].

Another common pregnancy complication thought to be associated with altered platelet activity is Intrauterine growth retardation (IUGR) which is mainly due to a pathologic slowdown in the fetal growth pace, resulting in a fetus that is unable to reach its growth potential. The incidence of IUGR in newborns is between 3% and 7% of the total population \[^{[21]}\]. One theory is that excessive platelet aggregation causes microthrombi in the placental circulation that restricts the delivery of nutrients to the growing foetus. However, there is very little experimental support for this hypothesis.

1.3.ii Markers of platelet activation during Pregnancy

During pregnancy, there are many documented changes in platelet responsiveness. Upon activation, the α-granules secrete a higher quantity of adhesive proteins (Platelet Factor 4 and β-thromboglobulin) during pregnancy, suggesting increased platelet turnover, clotting, and fibrinolysis \[^{[22]}\]. Other markers of platelet activation, such as the expression of P-Selectin\[^{[23]}\] are elevated in normal healthy pregnancy. P-Selectin can mediate endothelial activation resulting in a more prothrombotic state \[^{[14]}\]. Similarly CD40 ligand and platelet-monoocyte aggregates \[^{[24]}\] are elevated in normotensive pregnant women. Finally, spontaneous aggregation is observed in blood samples collected from pregnant women. These indications of platelet activation during normal pregnancy are believed to suggest an enhanced need for control of thrombotic function during pregnancy \[^{[25]}\]. Thus in preeclamptic pregnant women, it appears that a lack of this level
of control could lead to the development or the maintenance of pro-inflammatory and prothrombotic state resulting in an excessive coagulability [26].

In addition, levels of platelet-derived thromboxane are persistently elevated in serum and urine of pregnant women to levels equivalent to those observed in acute cardiovascular disease [27]. It is therefore well established that markers of platelet activation are elevated during normal pregnancy [28]. However, the clinical significance of platelet activation during pregnancy is not yet understood.

1.3.iii Altered Endothelial-derived platelet inhibitors during Pregnancy

Nitric Oxide (NO) is involved in various stages of pregnancy including implantation, maintenance of uterine acquiescence during pregnancy, control of uterine contractions and relaxation for successful gestation and regulation of blood pressure [29]. NO is released from endothelial cells in normal vasculature and is believed to suppress platelet activation in intact blood vessels. Elevated levels of Nitric Oxide are observed in normotensive pregnancy, meanwhile a drop in NO levels was observed in preeclamptic pregnancies [30]. This may contribute to hypertension, endothelial dysfunction and coagulation activation. This may contribute to hypertension, endothelial dysfunction and coagulation activation, commonly seen in PE [30].

Nitric oxide also plays a critical role in regulation of platelet function. In normal non-pregnant humans, endothelial cells tonically produce NO, to prevent unwanted, or inappropriate, thrombus formation in the region of an intact endothelial layer. NO diffuses into platelets, activates guanylate cyclase and modulates agonist-induced platelet activation. In contrast, damaged endothelial cells do not produce NO, permitting platelet activation. In pregnancy, NO synthesis is down regulated, thus facilitating platelet activation [31].

Like NO, PGI₂ is a synthesized tonically in the vascular endothelium. It has been observed that the PGF1α, a metabolite of PGI₂, is dramatically elevated after the first trimester of pregnancy as well as later in pregnancy. However, in preeclamptic pregnancies, it is observed that the PGF1 α is in decline throughout the pregnancy [38]. Thus altered prostacyclin function is associated with preeclampsia.

Prostacyclin (PGI₂) mainly prevents formation of the platelet plug involved in primary hemostasis by inhibiting platelet activation. It also plays a secondary role in haemostasis by inducing vasodilatation. The actions of Prostacyclin contrasts to that of thromboxane (TxA₂) resulting in inhibition of thrombosis and vasodilation and many studies have assessed metabolites of both PGI₂ and TxA₂ during pregnancy. TxA₂
biosynthesis is increased during pregnancy whereas Pgl₂ is reduced

Thus normal healthy pregnancy is accompanied by enhanced platelet activation (as evidenced by increased levels of plasma or urinary TxA₂ [27], increased soluble P-selectin [32]), and decreased platelet inhibition (as evidenced by decreased vascular production of NO [31] and decreased production of Pgl₂. The assumption is that platelets are therefore providing a pro-thrombotic function. However, the need for thrombosis in pregnancy is not apparent.

1.3.iv Platelet Aggregation during Pregnancy

A recent study by Kim and Lee (2013) of healthy pregnant women showed a significant decrease in platelet function compared to non-pregnant controls using the PFA-100 platelet function device [33]. The PFA-100 platelet function analyser is a bedside devise that monitors platelet aggregation under conditions of high shear equivalent to arterial blood flow. A reduction in platelet responsiveness in this assay suggests that pregnant women have a reduced level of activation compared non-pregnant controls. This data initially appears to contradict the large body of literature summarized above. However, further scrutiny of the data on platelet hyper-responsiveness during pregnancy reveals that most of the supportive evidence is derived from studies analyzing release or synthesis of bioactive agents from platelets [34]. These are surrogate markers of platelet activation. Assays that measure platelet function directly are less common. A reasonable explanation of the apparently contradictory data found in the literature may be that platelets are hyper-secretory during pregnancy but that control mechanisms are in place to prevent an accompanying risk of thrombotic events.

1.3.v Non-Thrombotic role for Platelets during Pregnancy

Recently in the literature, it has also become apparent that platelets play unique non-thrombotic roles in the inflammatory system [35] and also in cancer metastasis [36]. In these situations, platelets appear to be able to mobilize bespoke secretions from their dense and alpha granules that mobilize the inflammatory system, promote vascular permeability, enhance angiogenesis and facilitate metastasis; all independent of their thrombotic role. It seems reasonable to suggest that platelets may play a similar role in pregnancy; releasing bioactive agents from their granule stores to support local needs for enhanced angiogenesis or tissue remodeling.
Also, recently, our group has demonstrated that a pregnancy specific glycoprotein, PSG1 \cite{37}, which is elevated in normal healthy pregnancy, plays a strong role in the suppression of the thrombotic role of platelets \cite{13}. However, the platelet secretion from alpha and dense granules is unaffected by PSG1. Thus platelet activation and secretion may be preserved in pregnancy but thrombus formation is inhibited. This may suggest a unique role for platelet secreted bioactive agents in pregnancy. In this scenario, it could be hypothesized that bioactive agents are released from platelets in a systematic way during pregnancy to enable required hemostatic changes. However, platelet aggregation and thrombus formation is not required, or could be contraindicated during pregnancy and so is suppressed by PSG1 or similar soluble proteins. To test this hypothesis, systematic studies of platelet function during pregnancy would be required. To date, no such studies are available in the literature. While many research groups have focused on markers of late-stage pregnancy and comparisons with preeclampsia or IUGR, no systematic review of platelet function during normal healthy pregnancy could be identified. Therefore, we embarked on this study to evaluate changes in platelet activation during pregnancy to enable a definitive record of platelet response throughout the trimesters to be obtained.

Thus, we believed that a study is needed to assess the platelet function throughout a healthy pregnancy that could later be used as a baseline data for a study assessing platelet function during complicated pregnancies such as Preeclampsia and Intrauterine Growth Restriction (IUGR). Many authors suggest that enhanced platelet activation may be a cause of microthrombi that can adversely affect pregnancy outcomes \cite{32}. Altered platelet interaction with the endothelial layer of blood vessels may also be evident. Finally, release of platelet stores of bioactive agents may contribute to the health of the fetus.

\textbf{1.4. Study Design}

The study strategy is to assess 20 pregnant females in collaboration with The Coombe Women and Infant’s University Hospital, by sequentially collecting 12 ml of their blood in the three trimesters of pregnancy and an additional sample in the postpartum period. Correlation between platelet aggregation, platelet secretion, platelet count and plasma Thromboxane levels will be evaluated in these samples in order to determine if changes in platelet function (secretion vs aggregation) can be identified during a normotensive pregnancy.
Also, recently, our group has demonstrated that a pregnancy specific glycoprotein, PSG1 [37], which is elevated in normal healthy pregnancy, plays a strong role in the suppression of the thrombotic role of platelets [13]. However, the platelet secretion from alpha and dense granules is unaffected by PSG1. Thus platelet activation and secretion may be preserved in pregnancy but thrombus formation is inhibited. This may suggest a unique role for platelet secreted bioactive agents in pregnancy. In this scenario, it could be hypothesized that bioactive agents are released from platelets in a systematic way during pregnancy to enable required hemostatic changes. However, platelet aggregation and thrombus formation is not required, or could be contraindicated during pregnancy and so is suppressed by PSG1 or similar soluble proteins. To test this hypothesis, systematic studies of platelet function during pregnancy would be required. To date, no such studies are available in the literature. While many research groups have focused on markers of late-stage pregnancy and comparisons with preeclampsia or IUGR, no systematic review of platelet function during normal healthy pregnancy could be identified. Therefore, we embarked on this study to evaluate changes in platelet activation during pregnancy to enable a definitive record of platelet response throughout the trimesters to be obtained.

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Materials and Methods

2.1 Materials

All reagents were purchased from Sigma Aldrich (Dublin, Ireland) unless otherwise indicated. For blood collection, 3ml Sodium Citrate vacutainers were obtained from Sarstedt (Wexford, Ireland). Thrombin receptor activating peptide (TRAP6-amide) was obtained from Bachem (Bubendorf, Switzerland). U46619- a thromboxane mimetic was obtained from Tocris Bioscience, (Bristol, UK); Arachidonic Acid was obtained from Bio/Data Corporation,(Pennsylvania, USA) and CRP-XL- Collagen related peptide was purchased from the Department of Biochemistry, University of Cambridge, UK. For the detection of ADP/ATP released from platelets, Chromolume kits were purchased from Chrono-log Corporation (Labmedics Ltd, Manchester, UK). Finally, Nunc white flat bottom Fluoronorunc® 96 well plate with lid were obtained from Bio-sciences Ltd., (Dublin, Ireland).

2.2 Methods

2.2.1 Sample preparation

Ethical approval was obtained from the Coombe Women and Infants University Hospital, Dublin, Ireland for the collection of a 12 ml blood sample from pregnant women at 3 separate times during pregnancy and on one occasion between 4 and 8 weeks post partum. The participating pregnant patients signed a consent form of their willingness to be recruited in the study. The study was briefly explained to all patients. Separate ethical approval was obtained to assess platelet function in healthy human donors. This approval was obtained from the RCSI Research Ethics committee (REC).

Blood was drawn from the antecubital vein into four 3 ml Sodium Citrate vacutainers. The sodium citrate is an anticoagulant that prevents the coagulation of plasma proteins. The blood was transported by taxi to the RCSI labs, where platelet counts in the whole blood samples were assessed utilising the Sysmex blood analyzer (Sysmex Corporation, Kobe, Japan). The blood was then transferred into four 15 ml tubes and centrifuged at 150 (g) rcf (*) for 10 minutes (acceleration 5, brake 5) in an Eppendorf Centrifuge (Eppendorf AG, Hamburg, Germany). Using a 1 ml pipette, platelet rich plasma (PRP) from each centrifuged tube was transferred to a new 15 ml tube without disrupting the buffy coat. Platelet counts in the PRP samples were assessed utilising the Sysmex blood analyzer as above.
The remaining blood was centrifuged at 720 (g) rcf (*) for 10 minutes (acceleration 5, brake 5) to obtain the platelet poor plasma (PPP), which was used to set the baseline aggregometer readings.

A minimum of 3400 µl of PRP was required for complete analysis of platelet function: 1200 µl sample of PRP was transferred in another tube and reserved for aggregation assay and 2200 µl sample of the PRP was reserved for ADP/ATP secretion assay. Activated PRP samples and the PPP from aggregometry were frozen and then used estimation of the capacity of platelets to generate TxA2 in a TxA2 ELISA assay.

2.2.ii Preparation of Reagents

All agonists were prepared fresh from frozen stocks by dilution in a modified Tyrode's buffer, which we call JNL Buffer. It is isotonic with blood and has corresponding physiological concentrations of glucose, salts and cations. It contains 6 mM dextrose, 130 mM NaCl, 9 mM NaHCO3, 10 mM sodium citrate, 10 mM Tris base, 3mM KCl, 0.81 mM KH2PO4 and 0.9 mM MgCl26H2O, pH 7.35). This buffer is prepared from 4 separate stock solutions as detailed in appendix.

2.2.iii Platelet ADP/ATP secretion assay

The assay of platelet function using this novel platelet assay requires careful preparation of all reagents in advance.

2.2.iii.1. Preparation of Agonist Stocks

Thrombin Receptor Activating Peptide (TRAP):
TRAP was weighed out using a micro-balance to give 1.5 mg of powder. This was then transferred to a 2 ml eppendorf and 2 ml of de-ionised water was added before vortexing. Aliquots of the 1mM stock were made and stored at – 20 °C.

Thromboxane mimetic (U46619):
A volume of 20 µl of U46619 in the acetic acid solvent from the supplier was centrifuged in a rotovap for 7 minutes until the liquid evaporated off. Subsequently, 20 µl of pure ethanol was then added and vortexed in order to form a homogenous solution. Afterwards, 265.3 µl of the ethanol was added and vortexed repeatedly. This yielded a
2mM stock solution. The agonist was then stored at – 20 °C. The final assay concentrations ranged from 0-20 μM and were freshly prepared each day as 10X assay stocks. Thus, 10μl of the 2mM stock was diluted with 90 μl JNL buffer to create a 200 μM assay-stock. This was serially diluted 1:2 with JNL to create, respectively, stocks of 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.13 μM and 1.56 μM. Each agonist dose (10 μl) was dispensed into the appropriate wells of a 96 well assay plate prior to the addition of 70 μl PRP and 10 μl Chronolume. Thus the final assay concentrations of agonist were 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.31 μM and 0.16 μM.

**Arachidonic Acid:**
The Arachidonic Acid reagent obtained from the supplier was allowed to reach room temperature before the vial was reconstituted with 0.5 ml of de-ionised water yielding a final concentration of 5 mg/ml concentration. Half of the total volume was transferred to Eppendorfs and stored at – 20 °C.
The remaining volume in the vial received 0.25 ml of JNL resulting in a concentration of 2.5 mg/ml. Aliquots were of this were then stored at – 20 °C.

**Collagen Related Peptide (CRP):**
CRP was obtained from University of Cambridge as an aqueous solution at 9.1 mg/ml. Aliquots were stored at + 4 °C. On the day of each assay, a fresh dilution of this stock was made by dispensing 2.2 μl of the CRP into 197.8 μl JNL buffer to create a 100μg /ml assay-stock. This was serially diluted 1:3 with JNL to create, respectively, assay-stocks of 330 μg/ml, 110 μg/ml, 37.0μg/ml, 12.3 μg/ml, 4.0 μg/ml, 1.4 μg/ml and 0.46 μg/ml. Each agonist dose (10 μl) was dispensed into the appropriate wells of a 96 well assay plate prior to the addition of 70 μl PRP and 10 μl Chronolume®. Thus the final assay concentrations of agonist were 100 μg/ml, 37 μg/ml, 11 μg/ml, 3.7μg/ml, 1.23 μg/ml, 0.4 μg/ml, 0.14 μg/ml and 0.046 μg/ml.

2.2.iii.2. Serial Dilution of agonists

Stock solutions of the following agonists were stored at – 20 °C in Eppendorfs: (1) 1mM Thrombin Receptor Activating Peptide (TRAP), (2) 2mM U46619 (a thromboxane mimetic) and concentrations of 5 mg/ml and 2.5 mg/ml of Arachidonic acid.

All agonists were prepared freshly on the days of experiments by serial (1:2) for TRAP and U46619 and (1:3) for CRP dilutions to achieve 10X assay concentrations as follows using JNL:
• TRAP (μM): 320, 160, 80, 40, 20, 10, 5, 2.5, 0
• U46619 (μM): 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0
• CRP (μg): 100, 33.33, 11.11, 3.704, 1.235, 0.412, 0.137, 0.0457, 0

2.2.iii.3. ATP Standard Preparation

The lyophilized reagent obtained in the Chronolume® kit was reconstituted with 5ml of JNL buffer yielding 2μM standard. The reconstituted reagent was stored in 100μL aliquots at – 20°C.

2.2.iii.4. Chrono-lume® preparation

The Chronolume® vial was reconstituted using 1250μl of de-ionised water which was then allowed to stand for 10 minutes with occasional inversion before use, according to the manufacturer's instructions. This reagent is photo-sensitive and therefore, was protected from light by covering with tin foil.

2.2.iii.5. Assay procedure
The Chronolume®, ATP Standard and agonist stock solution were taken out of storage and thawed in an ice-bath. As the reagents are in the ice-bath, 10 μl of JNL buffer was pipetted into the wells were the agonist would be used in. Into the assigned wells 10 μl of the agonist were dispensed. (Figure 2.1)

<table>
<thead>
<tr>
<th>Well number</th>
<th>PRP</th>
<th>JNL</th>
<th>ATP Standard</th>
<th>Agonist (TRAP)</th>
<th>Chronolume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>70μl</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>3,4</td>
<td>&quot;</td>
<td>10</td>
<td>-</td>
<td>1μl of 320μM</td>
<td>&quot;</td>
</tr>
<tr>
<td>5,6</td>
<td>&quot;</td>
<td>10</td>
<td>-</td>
<td>1μl of 160μM</td>
<td>&quot;</td>
</tr>
<tr>
<td>7,8</td>
<td>&quot;</td>
<td>10</td>
<td>-</td>
<td>1μl of 80μM</td>
<td>&quot;</td>
</tr>
<tr>
<td>etc</td>
<td>&quot;</td>
<td>10</td>
<td>-</td>
<td>etc</td>
<td>&quot;</td>
</tr>
<tr>
<td>X,Y</td>
<td>&quot;</td>
<td>88</td>
<td>2μl</td>
<td>-</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Figure 2.1
ATP standard (2 µL) was pipetted into 2 other wells. In addition, 88µL of JNL buffer was transferred into one of the ATP standard. PPP (88µL) was transferred into the other ATP standard wells. The remaining ATP standard was then discarded.

Making sure that the plate reader Wallac 1420 Multilabel Counter (PerkinElmer Life and Analytical science, Turku, Finland) is at 37 ºC, 70 µl of PRP was pipetted into the agonist wells. Subsequently the plate was incubated in the plate reader at 37°C for 3 minutes with fast, orbital shaking to allow the components to mix together properly and to imitate the circular stirring achieved in a platelet aggregometer. Once this cycle was over, 10 µl of Chronolume® was pipetted into each well. The plate was incubated again in plate reader at 37°C for 10 seconds on 96 well plate with fast, double orbital shaking. The Luminescence protocol was then selected and values were measured from the selected wells.

The left over Chronolume® was stored again at – 20 ºC. Usually a bottle of Chronolume was only freeze-thawed once.

2.2.iii.6. Statistical Analysis for Platelet ADP/ATP secretion Assay

The raw data was composed using Excel Microsoft, once calculations were converted to nmoles ATP. The composed data was analysed in SPSS using ANOVA 1-way statistic programs.

2.2.iv Platelet Light Transmittance Aggregometry assay

Platelet aggregation assays measure dynamic changes in light transmission though a Platelet Rich Plasma (PRP) sample treated with an agonist, compared to light transmission though a platelet poor plasma sample (PPP). Using Aggregometer PAP-8E (BIO/DATA Corporation, Pennsylvania, USA) temperature was adjusted to 37 ºC and 250 µl of PPP was transferred in a cuvette and used as a reference. Five small stirring magnets were placed into 5 empty cuvettes each containing 225 µl of PRP from the total 1200 µl sample of PRP that was reserved. The 5 cuvettes were allowed to equilibrate for 1 minute. Once the 1 minute mark was passed, using a gel loading tip, 25 µl of a stock solution of 4 µM TRAP was transferred into the first PRP cuvette, 25 µl of 25 µM U46619 was transferred into the second PRP cuvette, 25 µl of 37.04 µg/ml CRP was transferred into the third PRP cuvette, 25 µl of 25 mg/ml Arachidonic acid was transferred into the fourth PRP cuvette and 25 µl of JNL was transferred into the fifth PRP cuvette. The PRP samples were stirred at 1100 rpm and platelet aggregation was recorded for 10 minutes.
After the aggregation results were obtained, the aggregated samples in the cuvettes were transferred into 0.5 ml Eppendorfs individually, labeled and stored at -20 °C for the Thromboxane assay.

2.2.iv.1 Statistical Light Transmittance Aggregometry Assay

The raw data that was obtained from the Aggregometers was in percentage aggregation values. The composed data was then transferred via Excel to SPSS, a programme able to analyse differences by 1-way ANOVA statistical analysis

2.2.v Assessment of plasma Thromboxane by ELISA kit

2.2.v.1. Wash Buffer Reagent Preparation

A 1:20 dilution of the wash buffer concentrate was carried out by diluting 5 ml of supplied wash buffer concentrate with 95 ml of deionized water and stored at room temperature. The wash buffer can be stored until kit expiration or for 3 months.

2.2.v.2 Assay Buffer Reagent Preparation

Diluting 10 ml of supplied assay buffer concentrate with 90 ml of deionized water carried out a 1:10 dilution of the assay buffer concentrate. This was stored at +4 °C with the addition of 0.09% (w/v) Sodium azide.

2.2.v.3 TxB₂ Standard Preparation

The generation of the logarithmic standard curve involves the addition of 900 µl of Assay Buffer to tube 1 and 666 µl to tubes 2 to 6. Then 100 µl of TxB₂ Standard solution was added to tube 1 and vortexed extremely well. Subsequently, 333 µl of the solution in tube 1 was added to tube 2 and 333 µl of tube 2 to tube 3 and so on until tube 7, all standards were vortexed extremely well. This produced serially-diluted decreasing concentrations of TxB₂ in tubes 1 to 7 (Table 2.2). The diluted standards were used within 1 hour.
<table>
<thead>
<tr>
<th>Eppendorf Tube</th>
<th>Concentration of TxB₂ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10,000</td>
</tr>
<tr>
<td>2</td>
<td>3,3333</td>
</tr>
<tr>
<td>3</td>
<td>1,111</td>
</tr>
<tr>
<td>4</td>
<td>370</td>
</tr>
<tr>
<td>5</td>
<td>123</td>
</tr>
<tr>
<td>6</td>
<td>41.1</td>
</tr>
<tr>
<td>7</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Table 2.2 Concentrations of standard TxB₂ values used in the ELISA assay

2.2.v.4 Sample Preparation

PPP samples were taken out of the -20 °C freezer and thawed by an ice bath. Samples (6 µl) were dispensed into 294 µl of Buffer. This corresponds to a 1:50 dilution. Upon analysis of the data from the first run, it was decided that a 1:5 dilution was more appropriate (i.e. 60 µl of PPP was added to 240 µl of buffer) for the second and third run of the assay. After each dilution was carried out the sample was vortexed well to ensure complete mixing of the sample. Samples were analysed in duplicate.

2.2.v.5 Assay Procedure

A layout of the 96-well plate is created to ensure all the correct reagents are added to the plate wells and this layout was followed throughout the experiment. Gloves were worn at all times to minimize contamination by exogenous alkaline phosphatase, the goat anti-rabbit IgG coated 96 well microliter plate is removed from the fridge and
appropriate numbers of wells are removed and placed in frame provided, with remaining unused wells being returned to the fridge (+4 °C) in a sealed bag.

The first step in the assay procedure was performed by adding 100 μl of Assay Buffer, in duplicate, to the non-specific binding (NSB) wells. Following that, 100 μl of the previously prepared standards (1-7) and patient samples were added in duplicate to the appropriate wells. This is followed by the addition of 50 μl of assay buffer into the NSB wells. Subsequently, 50 μl of TxB2 EIA alkaline phosphatase conjugate (blue solution) was added into every well, followed by the addition of 50 μl of TxB2 EIA rabbit polyclonal antibody to TxB2 (yellow solution) into every well except the duplicate NSB wells. At this point, every well was green except the NSB wells that were blue. The plate was covered with a plate sealer and placed on a plate shaker and incubated for 2 hours at room temperature at 500 rpm.

After 2 hours incubation, the wells contents were emptied onto a paper towel and the plate was washed by the addition of 300 μl of wash buffer. This was then decanted and the wash step was repeated twice more, ensuring that, after the third wash, the excess wash buffer was removed by firmly tapping the plate on a paper towel.

After the washing step, 200 μl of pNpp substrate solution was added to every well and the plate was covered by tinfoil during the incubation period due to the light sensitivity of the pNpp substrate and incubated at room temperature for 45 minutes. Following the 45 minutes incubation, 50 μl of Stop solution was added to each well and the optical density at 405nm was read immediately using a Wallac 1420 (PerkinElmer Life and Analytical science, Turku, Finland).

2.2.v.6 Statistical Analysis for Thromboxane Assay

In order to analyse the data on Graphpad Prism certain calculations were carried out. Firstly, the average of the standard and the NSB absorbance readings were obtained by using Microsoft Office Excel 2007. Secondly the average optical density (OD) for each standard was obtained by using the equation; Average Net OD = Average of the standard – NSB OD. The NSB OD was also subtracted from each patient sample absorbance reading. This data was inputted into Graphpad PRISM to obtain the extrapolated values for the TxB2 concentration in the patient samples using the standard curve. The dilution factors were taken into account before all 3 datasets were used to calculate mean +/- standard error on the mean (SEM).
Results

It is well established that markers of platelet activation are elevated during normal pregnancy. For example, levels of platelet-derived thromboxane are persistently elevated, in serum and urine of pregnant women, to levels equivalent to those observed in acute cardiovascular disease. In addition, other markers of platelet activation such as P-Selectin are elevated in pregnancy. However, the clinical significance of platelet activation during pregnancy is not understood. Furthermore, systematic studies of platelet function throughout pregnancy are not widely available.

Before embarking on a study of platelet function in pregnant women, it was important to establish the assays and to determine normal responses in non-pregnant women. Figures 3.1 to 3.7 below show data obtained from platelets of non-pregnant females donors. These values were determined in order to establish control values for comparison with the values obtained in pregnant women.

3.1 Assessment of Platelet Function in Non-pregnant healthy female donors

3.1.i Light transmittance Aggregometry (LTA) assay

Platelet aggregation was assessed to 4 separate agonists: a thrombin receptor activating peptide (TRAP 4 μM), a collagen related peptide (CRP 3.7 μg), a stable thromboxane mimetic (U46619 2.5 μM) and Arachidonic acid (AA 2.5 mg/ml). Platelet aggregation was assessed by light transmission aggregometry (LTA), a process described by Born et al., as explained in section 2.2.iv above.

For platelet aggregation studies, the dose of agonist to be used was selected from prior laboratory analysis of volunteer platelet samples. The data is shown in Figure 3.1. Adequate responses were obtained to TRAP, U46619 and AA. Minimal or absent aggregation was recorded in response to treatment of the platelets with an equal volume of buffer instead of an agonist. This demonstrates that the process of preparing the PRP does not activate the platelet samples. The choice of the dose of the agonist is important, as it will serve as a baseline figure for comparison of responses in platelets from pregnant women. Ideally, we aimed to select a dose that gave a submaximal response, as this will allow us to determine if responses increase or decrease during pregnancy. For CRP, our initial study had selected a higher agonist dose (11.1 μg). This
yielded an average response of 80% in non-pregnant donors. However, when we assessed our first clinical samples from 1st Trimester patients, this dose of CRP always yielded a 100% LTA response. Because this was not going to allow accurate assessment of function in the pregnant donors, the dose that was routinely assessed in non-healthy donors was reduced to 3.7 µg/ml. We therefore adjusted the dose of CRP to be used in our study to 3.7 µg/ml. In the non-pregnant donors, this dose of CRP routinely caused an aggregation response of 35 +/- 9%.

![Agonist Aggregation Responses](image)

**Figure 3.1: Platelet aggregation in normal healthy female donors.**

Aggregation was assessed in PRP obtained from non-pregnant female donors of child bearing age. Aggregation was monitored for 10 minutes at 37 °C in PAP-8 aggregometer in response to TRAP (4 uM), U46619 (2.5 uM), CRP (3.7 ug), AA (2.5 mg/ml). In addition, baseline, or spontaneous aggregation was monitored by assessing platelet responses following the addition of an equal volume of buffer. The maximal aggregation response was recorded. Data is presented as mean ± sem (standard error of the mean) for 32 volunteers.
3.1.ii Platelet ADP/ATP secretion (PAS) assay

Next, I determined a protocol for the platelet ATP/ADP secretion (PAS) assay. This assay had been established in the RCSI labs prior to my project. However, it had never been used on clinical samples. In addition, the assay in the lab was mainly used to assess platelet function in samples of washed platelets. My intention was to assess platelet function in PRP obtained from patient blood donations. It was important to assess activity in PRP, because difference in manipulation of samples or time taken to prepare clinical samples could have profound impact on the measured response. It was important in this assay to attempt to restrict any source of variation in samples. I therefore decided to assess PAS in PRP samples in a cohort of 32 non-pregnant female donors. The data are illustrated in Figures 3.2, 3.3 and 3.4 for TRAP, U46619 and CRP respectively. Fig 3.5 shows the response to AA. Because AA is not a strong inducer of PAS, multiple agonists’ doses were not assessed. Instead, only the responses to 2 separate dose of AA were assessed.
PRP samples from non-pregnant women were treated with TRAP (0.25 uM to 32 uM), U46619 (0.156 uM to 20 uM), CRP (0.046 ug to 100 ug) and Arachidonic acid (2.5 and 5 mg/ml) for 10 minutes. Data was analysed by Graphpad Prism software and the dose response curve yielded maximal response values for each donor. Data is presented as nmoles ADP/ATP released per 1000 platelets and is presented as mean ± sem (standard error of the mean) for 32 volunteers.

Because the PAS assay measures responses to 7 different doses of each agonist (except AA), this assay can measure both the maximal amount of adenine nucleotides released by a range of platelet activators and the potency of each activator. AA is a very poor agonist in this assay. Data was obtained by entering the raw data observed in the 96-well plate assays into GraphPad Prism version 5.0 software for calculation of ligand parameters. Using a curve-fitting program, the dose response curve was analysed to give values for EC₅₀ and Maximal response. Figures 3.2 and 3.3 show the mean maximal responses and mean EC₅₀ values, respectively, observed to the stated agonists as determined from the software analysis. Maximal response data are expressed as nmoles ADP/ATP released per 1000 platelets and EC₅₀ values are expressed as the dose required to give a half-maximal agonist response. Their units are
the same as those used for the agonist concentration. These values will be used as comparators for the results from the clinical arm of this study.

Figure 3.3: Potency of platelet ADP/ATP release for all agonists used.

ADP/ATP release was assessed in PRP obtained from non-pregnant female donors of child bearing age in response to increasing doses of TRAP (0.25 uM to 32 uM), U46619 (0.156 uM to 20 uM), CRP (0.046 ug to 100 ug) and Arachidonic acid (2.5 and 5 mg/ml). The EC50 value for each donor were determined from the dose-response curves using Graphpad Prism software. Data is presented μM for TRAP and U46619 or as μg/ml for CRP. Data represents mean ± sem (standard error of the mean) for 32 volunteers.
Figures 3.4-3.6 show the profile of the agonist-responses averaged over all donors. The mean data for all agonists in all 32 healthy non-pregnant female donors is summarized in table 3.1 below.

![CONTROL_TRAP](image)

**Figure 3.4:** Platelet ADP/ATP secretion in non-pregnant women in response to TRAP.

ADP/ATP release was assessed in PRP obtained from non-pregnant female donors of child bearing age in response to increasing doses of TRAP (0.25 uM to 32 uM). Samples were incubated for 10 minutes at 37 °C in a 96-well assay plate. Released ADP/ATP was assessed using a commercial Chronolume kit. Data represents mean ADP/ATP secretion and is presented as mean ± sem (standard error of the mean) for 32 volunteers using GraphPad Prism software.
Figure 3.5: Platelet ADP/ATP secretion in non-pregnant women in response to U46619

ADP/ATP release was assessed in PRP obtained from non-pregnant female donors of child bearing age in response to increasing doses of U46619 (0.156 uM to 20 uM). Samples were incubated for 10 minutes at 37 °C in a 96-well assay plate. Released ADP/ATP was assessed using a commercial Chronolene kit. Data represents mean ADP/ATP secretion and is presented as mean ± sem (standard error of the mean) for 32 volunteers using GraphPad Prism software.
Figure 3.6: Platelet ADP/ATP secretion in non-pregnant women in response to CRP

ADP/ATP release was assessed in PRP obtained from non-pregnant female donors of child bearing age in response to increasing doses of CRP (0.046 μg/ml to 100 μg/ml). Samples were incubated for 10 minutes at 37 °C in a 96-well assay plate. Released ADP/ATP was assessed using a commercial Chronolume kit. Data represents mean ADP/ATP secretion and is presented as mean ± sem (standard error of the mean) for 13 volunteers using GraphPad Prism software.
Figure 3.7: Platelet ADP/ATP secretion in non-pregnant women in response to AA
ADP/ATP release was assessed in PRP obtained from non-pregnant female donors of child
bearing age in response to two doses of Arachidonic acid (AA; 2.5 and 5 mg/ml). Samples were
incubated for 10 minutes at 37 °C in a 96-well assay plate. Released ADP/ATP was assessed
using a commercial Chronolume kit. Data represents mean ADP/ATP secretion and is
presented as mean ± sem (standard error of the mean) for 32 volunteers using GraphPad Prism
software.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Maximal response (nmoles ADP/ATP released per 1000 platelets)</th>
<th>EC$_{50}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP (μM)</td>
<td>5.93±1.9</td>
<td>4.89 ± 0.12</td>
</tr>
<tr>
<td>U46619 (μM)</td>
<td>3.06 ± 0.43</td>
<td>1.9 ± 0.14</td>
</tr>
<tr>
<td>CRP (μg)</td>
<td>3.73 ± 0.6</td>
<td>3.84 ± 0.2</td>
</tr>
<tr>
<td>AA (mg/ml)</td>
<td>0.07 ± 0.02</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of PAS parameters in normal healthy donors.

Analysis of the dose-response curves for ADP/ATP release from platelets was performed using Graphpad Prism. Data represent mean ± sem for 32 non-pregnant female donors.
Since the volumes required for the PAS assay are low, (only 70 ul of PRP used in every well) using 96-well plates, I was able to analyze the responses to multiple doses of agonist and multiple agonists (TRAP, U44619, CRP, AA) simultaneously. This assay required 1.6 mls of PRP in total for all 4 agonists.

In order to complete the assessment of all responses in the platelet aggregation assay, an additional 2ml of PRP was required. A further 0.5 ml of PRP was frozen at -80°C to allow determination of plasma levels of the thromboxane metabolite (TxB₂). In total therefore, it was determined that 4.5 or 5 ml of PRP was required. To obtain this, I calculated that a blood donation of 12 mls of whole blood was routinely required.

During the process of applying for Ethics approval for the clinical part of this study (Ethical approval obtained from The Coombe Women’s Hospital, Dublin), it was made clear that a minimal volume of blood would be approved. On the basis of the calculations above, we determined that this minimal volume would be 12 mls. In some cases, this proved to be insufficient, as the yield of PRP was not always predictable. In those cases, the aggregation response to buffer alone was not determined, yielding an additional 250μl of PRP for use in the other assays.

As Arachidonic acid is not very effective at causing secretion, I did not examine responses to low doses of this agonist. Instead, I looked only at responses to two separate high doses of AA. In part the decision to only perform a limited investigation into the response to this agonist was driven by the need to preserve PRP for other assays. In addition, AA is an expensive agonist and funding for this study was limited. Finally, AA is a lipid moiety and doses greater than those used induce a toxic response in platelets. Consequently the choice of dose and the number of assay points for these agonists was a balance between resources, cost and scientific limitations.

Overall, the data demonstrated that non-pregnant females donors display unique consistent responses in standard platelet assays. Although there can be considerable variation in the magnitude of the response between donors and this is reflected in the relatively large sem values. However, despite this variation, there is a consistency in the shape of the response and in the dose of the agonist that yields a half maximal response. This value, the EC₅₀, represents an estimate of the potency of the agonist in each donor. As can be seen in Table 3.1, this value is consistent for each agonist. I therefore propose to use these values as baseline parameters for comparison of the results in the pregnant women to be analysed later in this study. In addition, we can conclude that the dose of agonist that causes a half-maximal response is an index of platelet responsiveness.
3.2 Assessment of Platelet Function in Healthy Pregnant donors

During the process of applying for Ethics approval for the clinical part of this study (Ethical approval obtained from The Coombe Women’s Hospital, Dublin), it was made clear that approval would only be granted for a minimal volume of blood to be collected. On the basis of the calculations above, we determined that this minimal volume would be 12 mls per visit per donor. In most cases, and in all healthy volunteers, this volume was sufficient to yield at least 5ml of PRP. However, in 2 cases, this proved to be insufficient in the first trimester sample, as the yield of PRP was not always predictable. In those cases, the aggregation response to buffer alone was not determined, yielding an additional 250μl of PRP for use in the other assays.

3.2.i Patient Demographics

We aimed to recruit 20 normal healthy pregnant patients for this study. Ethical approval was obtained from Coombe Women and Infant University Hospital. In the first 9 weeks, 3 patients dropped out due to change-of-mind (2) and miscarriage (1). We sought and obtained permission to recruit an additional 3 patients to complete the numbers as originally planned. However, we lost an additional 3 patients throughout the study despite our best attempts. One patient left the country for good after her T2 sample, another patient simply failed to turn up for repeated scheduled visits and so we never obtained a T2 sample from her. This patient and two others failed to return for their post-partum sample. In the end, we had complete compliance from 16 patients. One patient gave 3 samples but was missing a T2 sample. We did our analysis on 20 patients for the T1 time point. However, we only had 16 samples for T2, 17 samples for T3 and 17 samples for post-partum. The final patient details are summarized in Table 3.2. The Mean Age of the pregnant female volunteers is 28.4 years (range 20-43) and there are 3 smokers taking part in this study.

Whilst for the control patients weight was not taken but by observing the physical appearance of the volunteers they ranged normal weight- borderline over weight, excluding bring underweight and obese categories and we were not permitted to ask if the volunteers are smokers resembling the rest of population.
<table>
<thead>
<tr>
<th></th>
<th>Normal Control donors</th>
<th>Pregnant Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age</td>
<td>28 +/- 5.3</td>
<td>29 +/- 6.22</td>
</tr>
<tr>
<td>BMI</td>
<td>n/a</td>
<td>24.4 +/- 5.3</td>
</tr>
<tr>
<td>Smokers</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Median platelet count at first</td>
<td>288 +/- 65.3</td>
<td>178 +/- 15.3</td>
</tr>
<tr>
<td>donation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Demographic information available for patients and non pregnant donors.
<table>
<thead>
<tr>
<th>Trimester</th>
<th>Gestational weeks</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9-14</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>14-27</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>27-37</td>
<td>17</td>
</tr>
<tr>
<td>Post Partum</td>
<td>2-8 weeks post delivery</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 3.3: Classification of trimesters according to the weeks of gestation.

The study design was adjusted to match the normal visits that the patients attend in the hospital and to have sufficient and equal intervals between each of the collected samples. Therefore, the time periods for the study were selected as follows: Trimester 1 (T1) sample: average 11 weeks (range 9-14); Trimester 2 (T2) sample: average 20 weeks (range 14-27); Trimester 3 (T3) sample: average 32 weeks (range 27-37) and Post Partum (PP) sample: average 4 weeks post delivery (range 2-8 weeks post-delivery). Some patients were non-compliant and did not show up for all of their scheduled visits. For that reason, the numbers (n) used in our study vary as indicated.
3.2.ii Patient Blood counts

**Figure 3.8: Platelet count throughout pregnancy and post partum period**

Platelet count was estimated using a Sysmex automated blood counter in either whole blood samples or in PRP samples, as indicated. Data is presented as mean ± sem (standard error of the mean) for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post-partum sample.

A total of 12 ml blood sample was taken from volunteers on the day of their scheduled hospital visits. The blood was taken by taxi to the laboratories in RCSI. The time taken for transport was always less than 20 minutes. The platelet count was determined for whole blood and PRP was prepared as described in Materials and Methods section. A platelet count in each PRP sample was also estimated. Figure 3.8 Shows that the mean platelet count decreases throughout pregnancy in both whole-blood and PRP samples and then recovers to normal ranges in Post-partum sample. This is due to a known phenomenon of haemodilution of blood during pregnancy.
3.2.iii Light Transmission Aggregometry (LTA) assay

I then explored platelet function in PRP obtained from these healthy pregnant volunteers using the LTA aggregation assay. Figures 3.9 to 3.13 shows the aggregation responses observed in PRP taken from pregnant donors during the first (T1) trimester, second (T2) trimester, third (T3) trimester or in the Post-partum (PP) period. Because only 16 patients has complete data for all 4 timepoints; only this subsection of patients were analysed. This all the data presented represents mean +/- sem for the 16 patients who successfully gave samples for all 4 timepoints. The data for responses to all agonists are summarized in Figure 3.14. For all aggregation responses, the Post partum sample aligns with the other non-pregnant sample. There is no significant difference in the aggregation responses to TRAP throughout all the samples obtained from the pregnant volunteers. However, the aggregation response to U46619 was significantly reduced in T1 compared to the responses in all other trimesters and in the post-partum samples (Figure 3.10). In contrast, responses to CRP (Figure 3.11) demonstrate an increased responsiveness to this agonist at T1 and T2 trimesters. This hyper-responsiveness is reducing in T3, and at the time of the PP post partum samples, the responses are returning towards the values that were recorded for the non-pregnant volunteers as shown in Figure 3.1.
Figure 3.9: Changes in platelet aggregation in response to TRAP during pregnancy.

Aggregation was assessed in PRP obtained from pregnant patients in Trimester 1, 2, 3 and post partum. Aggregation was monitored for 10 minutes at 37 °C in PAP-8 aggregometer. The maximal aggregation response to 4 μM TRAP was recorded. Data is presented as mean ± sem (standard error of the mean) for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post partum sample. There is no significant difference between groups.
Figure 3.10: Changes in platelet aggregation in response to U46619 during pregnancy.

Aggregation was assessed as described in the legend to figure 3.9. The maximal aggregation response to 2.5 μM U46619 was recorded. Statistical analysis was performed using 1-way ANOVA using SPSS and compared all responses to those obtained in T1. * indicates P<0.05, T1 vs all groups.
Figure 3.11: Changes in platelet aggregation in response to CRP during pregnancy.

Aggregation was assessed as described in the legend to figure 3.9. The maximal aggregation response to 3.7 μg/ml CRP was recorded. Statistical analysis was performed using 1-way ANOVA using SPSS. * indicates P<0.001 compared to PP.
Figure 3.12: Changes in platelet aggregation in response to Arachidonic acid during pregnancy.

Aggregation was assessed as described in the legend to figure 3.9. The maximal aggregation response to 2.5 mg/ml AA was recorded. Statistical analysis was performed using 1-way ANOVA using SPSS. * indicates P<0.001 compared to T1.
Figure 3.13: Changes in platelet spontaneous aggregation in during pregnancy
Aggregation was assessed as described in the legend to figure 3.9. The maximal aggregation response was recorded over 10 minutes in the absence of any agonist. Statistical analysis was performed using 1-way ANOVA using SPSS. * indicates P<0.001 compared to PP, *P<0.05
The doses of agonists selected for assessing platelet aggregation that normally would give submaximal response in aggregation was discussed above. The doses selected were 4 μM of TRAP (Figure 3.19), 2.5 μM U46619 (Figure 3.10), 3.7 μg CRP (Figure 3.12) and 2.5 mg/ml Arachidonic acid (Figure 3.13). As discussed above, the initial choice of 10 μM CRP was subsequently shown to be unsuited for assessment of response in early pregnancy since all recorded responses from T1 patients were >100%. We therefore reduced the concentration of CRP to be used to 3.7 μM. This lower dose of CRP yielded an average platelet aggregation response of 35% (Figure 3.1) in non-pregnant donors. As can be seen in Figure 3.11, this dose of CRP induced a maximal response on LTA in pregnant T1 and T2 donors. This strongly suggests that platelets are hyperresponsive to collagen related agonist in the early stages of pregnancy. In contrast, responses to prostaglandin-related platelet agonists (U46619 and AA) are lowest in the first trimester and recover thereafter. The responses recorded to these agonists in the first and second trimester are low compared to the responses observed in non-pregnant donors (Figure 3.1). However, as pregnancy progresses, these responses return towards non-pregnant values. Platelet aggregation responses to TRAP are largely unchanged throughout pregnancy and do not differ from non-pregnant controls. Finally, it is worth noting that a low level of spontaneous platelet aggregation is observed when buffer-alone, in the absence of any agonists, is added to the assay tubes. This is particularly obvious in T2 and T3 samples. Compared to healthy donors (see Figure 3.1), this is a significant response. This is consistent with reports from other groups on platelet hyper-responsiveness during pregnancy.
Figure 3.14: Summary of platelet aggregation responses throughout pregnancy.
Platelet aggregation responses from figures 3.9-3.13 at the 4 different time points (Trimester 1, 2, 3 and post partum) to the various agonists tested. In T1 CRP vs. TRAP, *P<0.05, whilst in T1 CRP vs. U46619 and AA **P<0.001
3.2.iv Platelet ADP/ATP secretion (PAS) assay

After the LTA assay was completed, I then examined platelet ADP/ATP secretion in samples obtained from this cohort of pregnant women. I analyzed responses to increasing doses of TRAP, U46619 and CRP and Arachidonic acid in this assay as described above for the non-pregnant female PRP samples.

It is immediately apparent that the dose-response curves for all agonists differ significantly across trimesters. In figure 3.15, the responses to TRAP are shown. There is considerable variation in the magnitude of the response between donors and for this reason, all values were normalized to values obtained for T1 samples. The maximal value obtained in the T1 sample for each patient was assigned a nominal value of 100%. All other values for that patient were normalized to this value. When we assessed the absolute raw data from the patients, it was also apparent that the values obtained from the post partum (PP) samples most closely aligned with the values for non-pregnant females. We therefore examined if statistical differences could be determined between PP samples and samples from other trimesters. Using TRAP as an agonist, we can show strong statistical differences in the maximal PAS response at T2, and increases in EC\textsubscript{50} values in T1 and T3 samples. It is noteworthy that an increase in the EC\textsubscript{50} values presents a decrease in the potency of TRAP, as more TRAP is now required to induce a half-maximal response in these platelets. Thus platelets from pregnant patients show a decrease in responsiveness to TRAP as early as week 9 of gestation. This decrease in responsiveness is manifested as a decrease in potency while the maximal response is unchanged in T1. Later in gestation, there is a compensatory increase in the maximal response, while the potency of the TRAP receptors remains lower than in non pregnant females or in PP samples.
Figure 3.15: ATP/ADP secretion response to TRAP in pregnant women.

(A) Platelet secretion was assessed in PRP samples from pregnant women in response to increasing dose of TRAP (0.25 uM to 32 uM). All data is normalized to data obtained from trimester 1 (T1). Normalized data from T1 are shown in blue, data from T2 is in red, data from T3 is in green and post partum samples are plotted in purple. Data represents mean ± sem for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post partum sample. Data was analysed by SPSS software. (B) Maximal responses and (C) EC50 values were extracted from the dose-response curves using GraphPad Prism software analysis and plotted separately. The colour code is the same as for section (A) above. Statistical analysis (1-way ANOVA) was performed using IBM SPSS statistical and compares all response to PP. * indicates *P<0.01 and **P<0.001.
For the thromboxane analog U46619, the EC$_{50}$ value is increased in T1 (Figure 3.16). This means that the potency of the agonist is lower in T1 than in PP samples, since a higher dose of agonist is required to yield a half-maximal response. However, this effect is no longer apparent in T2 and T3. Similar to TRAP responses, the platelets seem to be able to mobilize a bigger response, resulting in significant increases in the amount of ADP/ATP released in T2 and T3. Thus platelets from pregnant patients show a decrease in responsiveness to U46619 as early as week 9 of gestation. This decrease in responsiveness is manifested as a decrease in potency (or an increase in the EC$_{50}$ value). Later in gestation, there is an increase in the maximal response, which reverts to baseline levels non-pregnant levels in PP samples.

For the Collagen Related Peptide, CRP, the responses are strikingly different (Figure 3.17). Here the potency is dramatically increased (as evident from the lower EC$_{50}$ values) in T1, T2 and T3. This suggests that platelets from pregnant women will be highly responsive to low exposure to collagen especially in T1. The magnitude of this effect gets progressively less in T2 and T3. Similar to observations for TRAP and U46616, the absolute amount of ADP/ATP released by the platelets increases as pregnancy progresses and falls back to baseline levels in the post-partum period.
Figure 3.16: ATP/ADP secretion in response to U46619 in pregnant women.

(A) platelet ATP secretion was assessed in PRP samples from pregnant women in response to increasing dose of U46619 (0.156 μM to 20 μM). All data is normalized to data obtained from trimester 1 (T1). Normalized data from samples obtained in trimester 1 (T1) are shown in blue, data from T2 is in red, data from T3 is in green and post partum samples are plotted in purple. Data represents mean ± sem for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post partum sample. (B) Maximal responses and (C) EC50 values were extracted from the dose-response curves using GraphPad Prism software analysis and plotted separately. The colour code is the same as for section (A) above. Statistical analysis (1-way ANOVA) was performed using IBM SPSS statistical and compares all response to PP. * indicates *P<0.01 and **P<0.001. * indicates *P<0.05 and **P<0.001.
Figure 3.17: ATP/ADP secretion in response to CRP in pregnant women.

(A) platelet ATP secretion was assessed in PRP samples from pregnant women in response to increasing dose of CRP (0.046 µg/ml to 100 µg/ml). All data is normalized to data obtained from trimester 1 (T1). Normalized data from samples obtained in trimester 1 (T1) are shown in blue, data from T2 is in red, data from T3 is in green and post partum samples are plotted in purple. Data represents mean ± sem for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post partum sample. Data was analysed using Graphpad Prism. (B) Maximal responses and (C) EC50 values were extracted from the dose-response curves and plotted separately. The colour code is the same as for section (A) above. Statistical analysis was performed using IBM SPSS statistical and compares all response to PP. * indicates *P<0.05 and **P<0.001.
For AA, it is difficult to draw conclusions (Figure 3.18). Because no dose response relationship was assessed for this agonist, we are restricted to examining responses to two selected doses of this agonist. Although platelet response with a robust aggregation response to 2.5mg/ml of this agent, only very low levels of ADP/ATP secretion are observed. In the PAS assay, different responses are observed to 2.5 and 5 mg/ml of AA. If we therefore dismiss the responses to 5mg/ml AA, we can then say that they observed results on platelet ADP secretion in response to AA are similar to those observed for the other agonists; namely that the capacity of the platelets to secrete ADP/ATP in response to agonists appears to up regulated from T1 to T3 and to revert to baseline levels during the post partum period.

![Arachidonic Acid Max Effect](image)

**Figure 3.18: ATP/ADP secretion in response to AA in pregnant women.**

Platelet ATP secretion was assessed in PRP samples from pregnant women in response to 2 doses of AA (2.5 mg/ml & 5 mg/ml). Data from samples obtained in trimester 1 (T1) are shown in blue, data from T2 is in red, data from T3 is in green and post partum samples are plotted in purple. Data represents mean ± sem for 20 pregnant volunteers at T1, 16 pregnant volunteers at T2, 17 pregnant volunteers T3 and 17 volunteers for the Post partum sample. Data was analysed by 1-way ANOVA using IBM SPSS statistical and compares all response to PP. * indicates *P<0.05.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Maximal response (nmoles ADP/ATP released per 1000 platelets)</th>
<th>Non-pregnant donors</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>PostPartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP</td>
<td>5.93 ± 0.78</td>
<td>4.34 ±0.51*</td>
<td>6.63±0.84</td>
<td>8.01 ±0.82*</td>
<td>4.94 ±0.63</td>
<td></td>
</tr>
<tr>
<td>U46619</td>
<td>3.06 ± 0.43</td>
<td>1.70 ±0.32*</td>
<td>2.78±0.48</td>
<td>4.22 ±0.11*</td>
<td>1.97 ±0.30*</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>3.73 ± 0.61</td>
<td>3.96 ±0.40*</td>
<td>6.09±0.78</td>
<td>7.41 ±0.71</td>
<td>5.14 ±0.57</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.07 ± 0.02</td>
<td>0.018±0.002*</td>
<td>0.022±0.003*</td>
<td>0.037 ±0.006*</td>
<td>0.015 ±0.002*</td>
<td></td>
</tr>
</tbody>
</table>

**EC50 value**

| TRAP (µM) | 4.89 ±0.12 | 5.83±0.37* | 5.02±0.53* | 5.36 ±0.38* | 3.66±0.26 |
| U46619 (µM) | 1.9 ± 0.14 | 3.91±0.32* | 2.73±0.21 | 2.98±0.052 | 2.90±0.28 |
| CRP(µg/ml) | 3.84 ± 0.2 | 0.34±0.05** | 0.97±0.26** | 2.56±0.46* | 2.96±0.67* |
| AA (mg/ml) | ND         |           |           |           |           |

Table 3.4 Summary of the PAS parameters on all donors.
Maximal ATP/ADP secretion response and EC<sub>50</sub> values obtained from PAS analysis of from platelets from non-pregnant donors and from pregnant donors at the indicated stage of pregnancy. Significance was determined by comparison of PP samples with T1, T2 or T3 samples as indicated. *P<0.05 Unpaired Student T-Test vs PP sample, **P<0.01 Unpaired Student T-Test vs PP sample.
3.3.v TxB_2 ELISA

Seventeen Post Partum samples were obtained in the study of the twenty volunteers of the healthy pregnant women were analyzed for TxB_2. TxB_2 is the stable metabolite of TxA_2 (half life 37 seconds). Therefore analysis of maternal plasma TxA_2 was performed on only the samples for which we had complete samples for all trimesters. The assay to assess plasma levels of TxB_2 is by a commercially available competitive TxB_2 ELISA.

The thromboxane levels for each patient at the different time points throughout pregnancy were obtained by extrapolation of values from a standard curve (inverted standard curve) shown in Figure 3.19.

![Transform of Raw Data](image)

Figure 3.19: Standard curve for TxB_2 ELISA.

To obtain a correct dilution suitable for assessment of plasma levels of TxB_2, three dilutions of plasma from 3 pregnant donors were initially assessed; 1:10, 1:50, 1:100. In all cases the plasma was diluted using a modified Tyrode's buffer (JNL). This assay was
repeated on four different days, for each sample to ensure consistency and accuracy. These dilutions were tested on 3 pregnant patient samples (other than the twenty volunteers in this study) that dropped out after obtaining trimester 1 samples. These initial patient samples were therefore not included in our final study but were available for set-up of the ELISA assay. Upon analysis of the data it was initially decided a 1:50 dilution was the optimal dilution. Following the first analysis of the patient samples, it was noted that a number of the samples had very low levels of plasma thromboxane that did not fall on the standard curve. Consequently, all subsequent experiments used a 1:5 dilution of platelet plasma samples. Results of maternal plasma TxB₂ (pg/ml) for all 17 healthy pregnant women that were assessed at all trimesters and post pregnancy are shown in Figure 3.20 and 3.21.

![Pattern of TxB₂ during pregnancy](image)
Figure 3.20: Plasma thromboxane levels during and after pregnancy.

The levels of TxB₂ in plasma samples were assessed as described in material and methods. All plasma samples were assessed in duplicate on 3 separate occasions using a commercial ELISA assay. The data was separated into trimester 1, 2, 3 and post pregnancy assignments. Data represents mean +/- standard error on the mean.

![Graph showing plasma TxB₂ levels over gestation weeks](image)

\[ y = -162.41x + 8287.5 \]
\[ R^2 = 0.2974 \]

- \( \text{week of sample}^* \)
- Linear (week of sample*)

Figure 3.21: Time-dependent changes in plasma TxB₂ during pregnancy

This plot illustrates the data plotted on a time continuum; the x-axis shows the gestational weeks for trimester 1, 2, 3 and post pregnancy. The data was expressed as a time continuum showing individual plasma TxB₂ levels for each individual patient at the different gestational time points. It was found that although individual fluctuations in plasma TxB₂ occurred for each patient, an overall decrease in TxB₂ was observed. As well as that, this version of the data shows variation in the samples and that not all samples exhibit substantially high plasma TxB₂ levels in early pregnancy.
The Thromboxane level peaks in the first trimester and then gradually decreases to the post partum levels. Levels vary from patient to patient and not all patients display high levels of Thromboxane in the T1 samples that we obtained. Similarly, there was considerable variation in the platelet count in our samples. We wondered if the samples with the low platelet count might be responsible for the samples with low TxB2 levels. Figures 3.22 to 3.25 illustrate the correlation between the Thromboxane levels and the platelet (PRP) count in each trimester. In contrast to what was expected, it is apparent that thromboxane levels are highest in the patients with the lowest total platelet count. Since platelets are assumed to be the principle source of high thromboxane levels, and only activated platelets produce thromboxane, this suggests that the activated platelets must be removed from the circulation by the spleen. However, plasma TxB2 will be removed from the circulation by filtration in the kidney. In parallel, platelets are constantly being regenerated from the megakaryocytic process for platelet generation in the long bones. Thus, we must consider if an alternate source of plasma thromboxane can exist. In other words, if the platelet count is low, then what can be the source of the elevated plasma thromboxane levels? Sources of elevated plasma thromboxane other than the platelet could be the placenta or umbilical cord. Our data cannot resolve this question but do highlight that an alternate source of plasma thromboxane may exist in pregnant women.
Figure 3.22: Plasma TxB₂ (pg/ml) levels in Trimester 1.
Plasma TxB₂ levels were measured by ELISA assay and compared to platelet count (PRP) for each individual patient sample.

Figure 3.23: Plasma TxB₂ levels (pg/ml) in Trimester 2.
Plasma TxB₂ levels were measured by ELISA assay and compared to platelet count (PRP) for each individual patient sample.
Figure 3.24: Plasma TxB2 levels (pg/ml) in Trimester 3.
Plasma TxB2 levels were measured by ELISA assay and compared to platelet count (PRP) for each individual patient sample.

Figure 3.25: Plasma TxB2 levels (pg/ml) in post partum plasma samples.
Plasma TxB2 levels were measured by ELISA assay and compared to platelet count (PRP) for each individual patient sample.
Discussion

This study was carried out in order to determine if platelet function was altered during a normal healthy pregnancy. It is anticipated that this study will yield baseline data for future studies on complications of pregnancy, such as intrauterine growth retardation, preeclampsia or pregnancy induced hypertension. Here, the main focus of this study was on the analysis of maternal platelet function and plasma thromboxane levels in healthy pregnant women, in all trimesters and post-partum, in order to establish normal baseline values in a healthy pregnant population.

In this study of 20 normal healthy pregnant women we observed alterations in platelet function associated with gestational age. A significant decline in platelet count occurs as the pregnancy progresses in all volunteers, whilst platelet levels returns to normal in post delivery. This has previously been noted by other researchers and may be partly explained by haemodilution as blood volume increased in pregnancy [39].

Haemodilution is a condition of expansion of the plasma volume and an increase in red blood cell mass that is observed from early weeks of pregnancy, peaks at 28 to 34 weeks of gestation, and then plateau until child birth [40]. Plasma volume expansion is accompanied by a lesser increase in red cell volume [41]. As a result, there is a modest reduction in hematocrit, with peak haemodilution occurring at 24 to 26 weeks. Such effects are also observed in athletics that drink fluid excessively during prolonged exertion.

Platelet responsiveness was assessed in 2 separate assays in response to 4 separate physiologically-relevant platelet agonists. In particular, platelet aggregation is assessed by light transmission aggregation (LTA) and platelet secretion is assessed by ATD/ADP secretion (PAS) assay. Responses are assessed independently in response to thrombin receptor activating peptide (TRAP), collagen related peptide (CRP), a thromboxane-mimetic (U46619) and Arachidonic acid (AA). In addition plasma thromboxane levels were assessed throughout the study. Samples were analyzed in each trimester, in the first trimester 9-14 weeks, second trimester 14-27 weeks, third trimester 27-37 weeks and 2-8 weeks after delivery for Post-partum sample. In addition, responsiveness was assessed in a cohort of 32 non-pregnant female blood doors. Initial analysis of data from the non-pregnant female donors, compared to post-partum samples, showed that these values are similar but not identical to each other (Table 3.4). This posed a dilemma for us in terms of how to analyse the data. We expected that all parameters would have returned to baseline at the time of the postpartum samples (4-8 weeks after birth). However, although the PP samples were similar to the non-pregnant donors, there remained a significant difference in response to U46619 and AA between non-
pregnant and PP platelet. However, since all the data in the volunteers was paired, it was deemed appropriate to do the final statistical analysis only using the patents from the pregnancy study. Thus all significance was assessed by comparing responses in PP samples to responses in T1, T2 or T3 samples. It is likely that a return of platelet functionality to pre-pregnant levels takes longer than the 2-8 weeks allowed in our study design.

A further dilemma was posed regarding the assessment of the data in the patient samples. We had recruited 20 patients but only 16 patients provided all 4 required timed samples corresponding to T1, T2, T3 and PP. The main reason for their non-compliance was a reluctance to attend for clinics. Thus 2 patients failed to turn up for post-partum clinics, one patient failed to show for a T2 clinic and one further patient failed to attend a T3 clinic. We had to decide on where to analyse all the data or only the data obtained from the 16 fully compliant patients. The former would comprise an 'intention to treat' (ITT) analysis.

ITT analysis includes every subject who is recruited according to the study protocol. This type of analysis ignores missing samples, protocol deviations, withdrawal, and anything that happens after recruitment. In ITT analysis, estimate of significant effects is generally conservative. A better statistical analysis is possible if complete data are available for all subjects. ‘Per-protocol population’ is defined as a subset of the ITT population who completed the study without any protocol violations or sample omissions. We therefore decided to restrict our analysis to the per-protocol population. Thus 16 patients were analysed for differences in platelet function and plasma thromboxane levels during normal healthy pregnancy.

A decreased efficacy to all agonists was observed in the PAS assay in the first trimester compared to non-pregnant control donors. Efficacy, or maximal response, elicited by each agonist was then elevated in the second and third trimesters. In contrast, in samples taken 4-6 weeks after delivery of the baby, values had returned to levels similar to those found in non-pregnant healthy female donors. There was considerable variance in the absolute value of the maximal response. This varied from donor to donor. In order to compare trends in data amongst donors, the dose-response curves of the data from the PAS assays are expressed as percentage of the first trimester values. However, the Maximal response values (shown in the corresponding bar charts and in Table 3.4) represent absolute values (+/- sem)

The potency of each agonist was also measured in the PAS assay by analysis of the parameters of the dose-response curve. The dose of the agonist that caused a half-maximal response (Effective concentration of 50%; EC_{50}) is inversely related to the agonist potency and can inform on the biological status of the relevant receptor. This value has not been measured previously in any assessment of platelet function. Here, I
show dramatic differences in the agonist potency for each of the agonists tested throughout the pregnancy. The potency of platelet response to TRAP and U46619 is greatly reduced in T1. This is reflected in an increase in the EC$_{50}$ value. In contrast, the potency of the collagen receptors is greatly enhanced at T1 (observed as a decrease in the EC$_{50}$ value).

In platelet aggregation assays, a similar pattern of activity is observed. The capacity of platelets to aggregate is reduced in T1 for all the soluble agonists (TRAP, U46619 and AA). In contrast, aggregation to the collagen-derived agonist (CRP) is heightened in T1 and reduces in T2 and T3.

The difference in the pattern of activity for platelet secretion in response to agonists normally available as soluble platelet activators (Thrombin, thromboxane and AA) compared to responses normally activated by insoluble agonists (Collagen; CRP) may reflect the changing need of the haemostatic system during pregnancy. Responsiveness to the soluble agonists is suppressed in the first trimester. This may reflect a need to suppress platelet activation during the early stages of pregnancy when other factors are tending to be prothrombotic. Spontaneous platelet aggregation was seen in samples from T2 and T3 patients, whereas this value was negligible in Post-partum and non-pregnant donors. This suggests that regulation of thrombosis is an issue in pregnancy. For all the values of platelet responsiveness measured, we observed a recovery to baseline values in the Post-partum sample.

A decreased aggregation response is observed in T1 samples for doses of the soluble agonists TRAP, U46619 and AA that normally induce maximal responses. However, responses to the collagen agonist, CRP, are heightened at this time-point. Doses of CRP (3.7 µg/ml) that can cause only 35% aggregation in non-pregnant donors, are able to induce 100% aggregation in platelets obtained from T1 and T2 pregnant donors. In both platelet aggregation and ADP/ATP secretion assays, sensitivity to collagen is dramatically increased in first trimester samples compared to the Post-partum or normal healthy donors. I hypothesize that this subtle regulation of platelet responsiveness during pregnancy reflects a differential regulation of platelet function that is required during a normal healthy pregnancy.

Platelets contain many bioactive agents in their $\alpha$-granules$^{[42]}$. It is likely that regulated release of $\alpha$-granule contents plays a role in the regulation of angiogenesis and placentation in early pregnancy. It is assumed that during pregnancy, platelets can help localize and or deliver such bioactive agents to the required location (placenta/ uterus) to facilitate placental implantation and blood vessel development. Release of these factors will require platelet activation, while thrombosis might be a consequence. Therefore separate regulation of platelet aggregation and platelet secretion would be critical in pregnancy.
Recently our group at the RCSI has demonstrated that a pregnancy specific glycoprotein, PSG1, which is elevated in normal healthy pregnancy, plays a strong role in the suppression of the thrombotic role of platelets\cite{13}. Elevated levels of PSG-1 are observed in early in pregnancy \cite{43}. It is possible that PSG-1 may serve to modulate thrombotic/aggregation responses of platelets while preserving the ability of platelets to secrete the contents of their granules. Thus the enhanced responsiveness to CRP (increased potency, increased maximal response in secretion assays and enhanced aggregation) would facilitate localized release of granule contents on demand at sites of collagen exposure. In parallel, the down-regulation of platelet responsiveness to soluble agonists may be an adaption to prevent unwanted thrombotic events in a prothrombotic environment. The existence of a prothrombotic environment is attested to by our observations of elevated thromboxane levels in the first trimester of pregnancy and the observations of spontaneous aggregation of platelets during pregnancy. In addition, there are many other reports of heightened thrombotic risk during pregnancy, such as increased levels of pro-coagulant plasma markers \cite{44} or decreased levels of inhibitors of thrombosis \cite{29,45}. Finally, the heightened response to CRP (collagen mimetic) throughout the pregnancy may reflect a need to allow full platelet activation for thrombosis in response to an injury that might occur or to prevent excessive blood loss, particularly during childbirth.

Next, I focused on analysis of maternal plasma thromboxane levels in healthy pregnant women, in all trimesters and post pregnancy, in order to establish normal baseline values in a healthy pregnant population. Elevated plasma thromboxane may activate platelets and induce small thrombi in the placenta, leading to lack of nutrients to the developing fetus, causing intrauterine growth retardation. Therefore the knowledge of normal ranges of maternal plasma thromboxane levels may help further delineate the reasons for complications in pregnancy \cite{46}. Although previous studies have been carried out on analysis of thromboxane levels, no study has covered all trimesters and post pregnancy. This may be due to the fact that complications arise later in pregnancy \cite{46-48}. Therefore, many studies that analyze thromboxane levels do so after IUGR or preeclampsia has already been established.

Seventeen Post-Partum (PP) samples were obtained in the study of the twenty volunteers initially enrolled. Platelet-poor plasma (PPP) was obtained from these samples and was analyzed for TxB2. TxB2 is the stable metabolite of TxA2 (half life 37 seconds). Therefore competitive TxB2 ELISA was performed, analyzing maternal plasma TxA2, through the analysis of TxB2. The thromboxane levels for each patient at the different time-points throughout pregnancy were obtained by the production of logarithmic standard curve (inverted standard curve) shown in figure 3.19.

The data was separated into trimester 1, 2, 3 and post pregnancy assignments and a bar graph with mean +/- standard error on the mean was produced. It is clear that
plasma thromboxane levels are high in trimester 1 patients and a clear reduction occurs thereafter. TxB2 levels observed in the post-partum samples recover to mostly reflect levels in normal healthy non-pregnant individuals (Figure 3.21). Thus, in our cohort of patients, a very high average level of TxB2 was observed in T1. To our surprise, this level decreased incrementally in T2 and again in T3. Of note, we also demonstrate that there is a large variation from patient to patient in the recorded TxB2 levels.

In order to determine if maternal plasma thromboxane, during pregnancy, is produced by the placenta/umbilical cord or by activated platelets, a correlation between platelet count and the maternal plasma thromboxane levels was performed. We argue that if the platelets were producing the thromboxane, then when the platelet count was high, the thromboxane levels should also be elevated, and when the platelet count was reduced the thromboxane levels should be reduced simultaneously. However the opposite outcome was observed; when the maternal platelet count was elevated the corresponding thromboxane levels were reduced, and when the maternal platelet count was reduced this correlated with an increased thromboxane level (Figures 3.22, 3.23, 3.24 and 3.25). This may suggest that the origin of the plasma thromboxane is probably not from the platelets. We would speculate that instead, it must come from placental sources. Thus, I propose that the inverse correlation between thromboxane levels and platelet count suggests that activated platelets are not producing the elevated thromboxane. Instead a different source, such as the placenta is somehow regulating production of thromboxane.

In summary, this study has characterized aspects of platelet activation in normal healthy pregnancy by assessing platelet aggregation, platelet secretion and plasma thromboxane levels. I show that large changes in functional parameters are observed. Most strikingly, there is an increase in potency for the collagen related peptide that suggests that this mechanism of platelet activation acquires a new importance in pregnancy. In parallel, responsiveness to other soluble platelet agonists is down regulated. Although there have been studies that have assessed various aspects of platelet activation, prostaglandin levels and pregnancy, no study has covered all aspects, such as platelet aggregation, platelet ADP secretion and plasma thromboxane levels. Thus, this study is a novel study that aimed to establish baseline levels of all these parameters throughout pregnancy in healthy women. Further work would need to done with a bigger population of patients to confirm these results. The designed tools with selected concentration of agonist could be made and marketed for an easy use for laboratories in hospitals to be used, along with other laboratory blood data of pregnant patients, to indication early detection and diagnosis of impaired platelet activation. Ultimately, this can affect treatment and prophylaxis for mothers at risk of preeclampsia, reduced fetal growth and rarely fetal death, to decrease any thrombotic risk for the mother or the fetus.
Abbreviation

AA: Arachidonic Acid
ACD: Acid Citrate Dextrose
ADP: Adenosine diphosphate
ATP: Adenosine triphosphate
COX: Cyclooxygenase
CRP: Collagen related peptide
LTA: Light Transmission Aggregometry
PAS: Platelet ATP/ADP Secretion
PGG₂: Prostaglandin G₂
PGH₂: Prostaglandin H₂
PGI₂: Prostaglandin 1₂
PLA₂: Phospholipase A₂
PP: Post-partum
PPP: Platelet Poor Plasma
PRP: Platelet Rich Plasma
SFFLRN: Serine- Phenylalanine- Leucine- Leucine- Arginine- Asparagine = TRAP
T1: Trimester 1 (9-14 weeks of gestation)
T2: Trimester 2 (14-27 weeks of gestation)
T3: Trimester 1 (27-37 weeks of gestation)
TRAP: Thrombin Receptor Activating Peptide
TxA₂: Thromboxane A₂
TxB₂: Thromboxane B₂
U46619: a stable mimetic of thromboxane A₂
Appendix
6.1 Consent Form for healthy pregnant patients volunteering

COOMBE WOMEN'S HOSPITAL CONSENT FORM

Title of research study: A Novel Assay of Platelet Function reveals Altered Platelet Function during Healthy Pregnancy:

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

Name of sponsor:

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date:

Date on which the participant was first furnished with this form:

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.
6.2 Information leaflet for healthy pregnant patients volunteering

COOMBE WOMEN’S HOSPITAL Patient Information

Title of study: A Novel Assay of Platelet Function reveals Altered Platelet Function during Healthy Pregnancy:

We have designed a new laboratory assay (or test) for platelet function. Platelets are a blood component, which is important in clotting and prevention of bleeding. This test is superior to other laboratory methods for determining how platelets work in non-pregnant patients. We believe that this assay should improve our understanding of how platelets work in pregnancy.

We are asking patients over the age of eighteen with an uncomplicated pregnancy carrying a singleton fetus are considered for enrollment. You will be asked to give a small sample of blood (12 mls) in addition to your booking bloods at the time of booking and at three time-points thereafter (2\textsuperscript{nd} trimester, 3\textsuperscript{rd} trimester and postnatally).

This study will not have any direct benefit on you or your pregnancy but may enhance our understanding of pregnancy and may guide management in pregnancy in the future.

Apart from the discomfort of blood sampling there are no risks to engaging in this study.

If you have a history of bleeding in the pregnancy, a known history of bleeding or a family history of bleeding you will not be able to participate in this study. If you have a medical condition or are on prescription medication you will be unable to participate also.

Your identity will remain confidential. Neither you nor your baby's name will be published or disclosed to anyone outside the hospital.

You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you decide to leave the study, you will not be penalised and will not give up any benefits, which you had before entering the study. You understand that your doctor or the sponsoring company may stop your participation in the study at any time without your consent.

This trial has hospital Research Committee approval. You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Dr. Regan who can be telephoned at 01 4085200. If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.
6.3 Basic buffers required for platelet preparation

**JNL buffer**

<table>
<thead>
<tr>
<th>JNL</th>
<th>Content</th>
<th>Weight (g) : Deionised water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNL A</td>
<td>6 mM Dextrose</td>
<td>5.94 : 500</td>
</tr>
<tr>
<td>JNL B</td>
<td>9 mM Na Bicarbonate</td>
<td>3.7 : 500</td>
</tr>
<tr>
<td></td>
<td>130 mM NaCl</td>
<td>37.9 : 500</td>
</tr>
<tr>
<td></td>
<td>10 mM Na Citrate</td>
<td>3.78 : 500</td>
</tr>
<tr>
<td></td>
<td>3 mM KCl</td>
<td>1.12 : 500</td>
</tr>
<tr>
<td></td>
<td>10 mM Tris Base</td>
<td>6.07 : 500</td>
</tr>
<tr>
<td>JNL D</td>
<td>0.8 mM KH2PO4</td>
<td>0.55 : 500</td>
</tr>
<tr>
<td>JNL E</td>
<td>0.9 Mm MgCl2.6H2O</td>
<td>1.9 : 500</td>
</tr>
</tbody>
</table>

Table 6.1 shows the contents and measurements of each ingredients used in the JNL buffer.

**ACD buffer**

<table>
<thead>
<tr>
<th>Content</th>
<th>Weight (g) : Deionised water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid anhydrous</td>
<td>1.464 : 200</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>4.4 : 200</td>
</tr>
<tr>
<td>Dextrose</td>
<td>4.90 : 200</td>
</tr>
</tbody>
</table>

Table 6.2 shows the contents and measurements of each ingredient used in the ACD (Acid Citrate Dextrose) buffer.
References


Acknowledgements

I would like to acknowledge a number of people who supported and enabled the completion of this thesis. Work was performed in the Royal College of Surgeons in Ireland under the supervision of Professor Niamh Moran. The study was performed in association with the Coombe Women’s Hospital with the support of Dr Carmen Regan. Firstly I am grateful to Dr. Carmen Regan, a consultant obstetrician at the Coombe Women’s hospital in Dublin. It was Dr. Regan who designed the study and applied for, and obtained, ethical approval allowing us to obtain blood samples from pregnant women. In addition, Dr. Samar Gamal, a registrar in the Coombe Hospital, was essential for the recruitment of all the patients. She also motivated the patients to stay involved and was an invaluable help until she finished her rotation and left for the UK. As well as the Phlebotomy in Coombe Women’s hospital operated by Aramio Arganio.

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I would also like to thank sincerely all the members of the platelet laboratory in RCSI who taught me all the essential assays to complete this study. In particular, my PI, Professor Niamh Moran who believed in me and encouraged me in my study. Furthermore I would like to thank Tadhg McGivern who taught me the PAS assay and Kalyan Golla, who had originally developed this assay. Both of these scientists provided me with all the support I needed to learn, implement and trouble-shoot this novel assay. In addition, they, along with others in the Molecular and Cellular Therapeutics department in the Royal College of Surgeons in Ireland especially Lab 4 and 5, including Seamus Allen, Annachiara Mitroghno, Emily Reddy, Naadiya Carrim, Rand Al-Hashmi, along with Amro Widaa, Fahad Buskandar and Ruth Connolly that have moral support for the completion of this study and thesis.

Finally, one does not achieve great things alone: a sincere thanks to my family, friends, and PI. This accomplishment is a reflection of their love, encouragement, guidance and their constant support and belief in me during the completion of my education. Words seem inadequate to portray my deepest sincerity.