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Platelet Reactivity and Pregnancy Loss

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a MD, 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 Karen Flood

RCSI Student Number 95043

Date 13.10.11

For John, Alex and Rory.

CONTENTS

SUMMARY:

Although many advances in reproductive medicine have been made during the past quarter century, miscarriage remains the most common complication of pregnancy. Recurrent Miscarriage (RM) is traditionally defined as three or more consecutive losses before 20 weeks post menstruation. A causal factor can be identified in less than half of affected couples, leading to the term idiopathic RM in the remainder. The empiric use of the antiplatelet agent aspirin is widespread in this population as there is a longstanding hypothesis that women affected by RM are already in a prothrombotic state before pregnancy begins. However, there are few studies to date that efficiently evaluate platelet function in this cohort.

Platelet reactivity refers to the ex vivo measurement of platelet responses to various agonists, providing an index of platelet functional capacity. To date, the role of platelet reactivity has not been clarified in the RM population. The aim of this research was to determine the thrombotic risk of this cohort of patients by assessing *in vivo* platelet function. This was made possible by the development of a novel platelet assay by Dr. Aaron Peace and Professor Dermot Kenny of the Cardiovascular Biology Group at the Royal College of Surgeons in Ireland. This assay uses multiple concentrations of different agonists and thus reflects the complexity of agonist-induced platelet aggregation in vivo. The results revealed that patients with a history of unexplained recurrent first trimester pregnancy loss have significantly greater platelet aggregation to submaximal doses of Arachidonic Acid (AA) compared to an appropriate control group. The heightened response to AA in particular is interesting due to controversy surrounding the therapeutic role of aspirin in RM management. The results of this research study lend further support to re-evaluating the benefit of low dose aspirin in a clearly defined cohort.

Proposed further research involves assessment of the platelet aggregatory response in the RM group at different stages of achieved pregnancy. However examination of platelet function in normal healthy pregnancy needs to be known to allow comparison. This novel assay provided the opportunity to characterize platelet function throughout the three trimesters of normal pregnancy. This yielded interesting results including the changes in platelet reactivity at different stages of pregnancy. By characterizing platelet function in uncomplicated pregnancy we now have the opportunity to provide comparisons to pregnancies complicated not only by RM but other pathologies such as intra-uterine growth restriction (IUGR) and pre-eclampsia (PET).

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Abstracts

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Platelet function in the first trimester *American Journal of Obstetrics & Gynaecology*, 2009; 201(6):S89-90.

1. INTRODUCTION

1.1 Platelets

1.1.1 Platelet biology and activation

Platelets were described by several scientists dating back to the last quarter of the 19th century but ultimate credit for the detailed anatomical identification of platelets is attributed to the work of Giulio Bizzozero (1846-1901) in 1882. Bizzozero described their disc-like shape and called them '*piastrine*' i.e. small plates (later platelets). He also demonstrated their role in haemostasis and thrombosis (1,2). In his seminal paper he prophesised that '*in the future, one will have to consider this new blood constituent in studies of pathological events as well'*.

Platelets are the smallest of all circulating blood cells measuring 2 to 5 μm in diameter, 0.5μm in depth and have a mean cell volume of 6 to 10 femtolitres (3). These uniquely anucleate cells are derived from the cytoplasm of polypoid megakaryoctes and have a lifespan of 7 to 10 days culminating in the reticuloendothelial system. Under normal conditions, the circulating platelet is in a functionally passive state unless it becomes involved in its fundamental role of maintaining blood circulation. Recent studies of platelets at the genetic and proteomics level have highlighted the complexity of this seemingly simple cell as shown in figure 1. For example, platelets have over 40 surface receptors and approximately 3000 transcripts (4, 5).

The platelet surface is a dynamic structure, which through a host of membrane receptors, reacts to changes in the vascular endothelium and mediates response of the platelet to a number of activating agents such as agonists and adhesive proteins.

Some of the most important receptors involved in physiological and pathological haemostasis include the glycoprotein (GP) Ib-IX-V complex, the integrin $\alpha_{11b}\beta_3$ (GP IIb-IIIa complex), GP VI receptor, thrombin receptors, adenosine diphosphate (ADP) receptors ($P2Y_{12}$, $P2Y_1$, $P2X_1$), thromboxane receptors, P-selectin, platelet-endothelial cell adhesion molecule 1 (PECAM-1) and receptors for coagulation factors.

As well as normal cellular organelles such as mitochondria and lysosomes, the platelet cytoplasm contains two types of storage granule: α and dense. The α granules store matrix adhesive proteins including P-selectin, fibrinogen, fibronectin, thrombospondin, vitronectin and von Willebrand factor (vWF). The dense granules are smaller and contain soluble platelet activating agents such as ADP and serotonin. The makeup of the platelet permits rapid morphological and biochemical changes, in order to prevent haemorrhage following blood vessel injury. When the vascular endothelium is damaged, circulating platelets undergo a highly regulated set of functional responses beginning with initial adhesion, activation and aggregation, all leading to the formation of a hemostatic platelet plug.

This activation cascade is fundamentally protective and is controlled by intrinsic cellular constraints of the resting platelet and extrinsic factors secreted by the vascular endothelium, such as prostacyclin (PGI2) and nitric oxide (6, 7). The cascade may be triggered under pathological conditions resulting in arterial thrombosis and in the aetiology of conditions such as acute myocardial infarction and ischaemic stroke.

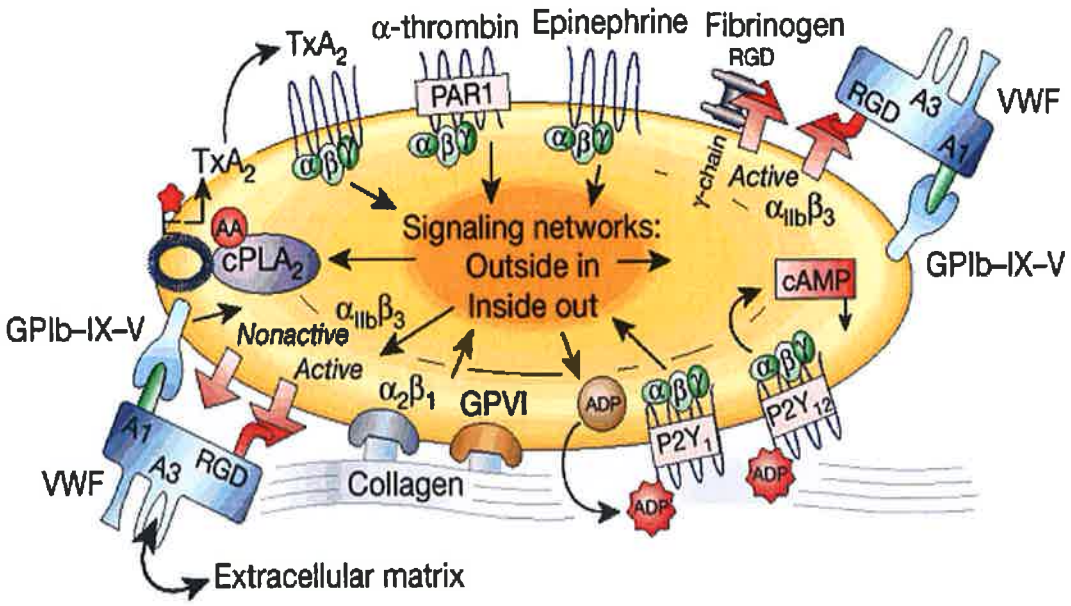


Figure 1. This simplified schematic shows the complexity of the platelet.

1.1.2 The platelet and haemostasis

When vessel injury occurs, a highly thrombogenic subendothelial matrix containing a number of adhesive molecules such as laminin, fibrinogen, collagen and vWF, is exposed to the blood. When platelets encounter the subendothelial matrix, they tether and begin to roll along its surface, mainly due to a shear dependent interaction with collagen and vWF and their corresponding platelet receptors; GP VI and α2β1 integrin for collagen and GP Ib-IX-V complex for vWF. The platelets gradually decelerate until they are captured by the matrix, and once stationary, the platelets spread to form a monolayer. Intracellular signalling enhances the affinity state of the platelet integrins and induces exocytosis of the storage granules releasing mediators ADP and thromboxane, all whilst thrombin is being generated on the platelet surface. ADP, thromboxane and thrombin are soluble agonists that activate platelets via G protein- coupled receptors (G_q, G_{12}/G_{13}, G_i) which in turn induces full platelet activation. Secretion of granular contents is further enhanced

and triggers the binding of plasma fibrinogen to the platelet's $\alpha_{11b}\beta_3$ integrin, which fastens the platelets into a platelet plug. The soluble agonists extend haemostatic platelet plug formation by recruiting circulating platelets to the growing plug. The developing thrombus is then stabilized by additional forces, such as the cross-linking of fibrin by factor XIII and $\alpha_{IIb}\beta_3$-dependent outside-in signalling.

1.1.3 Platelet agonists

As outlined, platelets are simultaneously activated by a variety of different agonists at the site of vascular endothelial disruption. Along with the description of the platelet activation process for each individual pathway, where relevant, I have included its targeted antiplatelet therapy.

1.1.3.1 Agonists that mediate platelet signalling via G protein coupled receptors

1.1.3.1.1 Arachidonic Acid (AA)

AA is an important element of the platelet membrane because of its role as a precursor for the eicosanoid thromboxane A_2 (TxA_2). When phospholipase A_2 is activated, it cleaves AA from membrane phospholipids. The sequential action of prostaglandin G/H synthase and thromboxane synthase leads to the production of TxA_2, a potent platelet agonist. The production of TxA_2 is rapid and leads to the amplification of the activation response and the recruitment of additional platelets to the site of injury. TxA_2 binds a specific G protein-coupled transmembrane thromboxane receptor, which is coupled to G_q and G_{13} subunits. TxA_2 induces phospholipase C-β (PLC-β), which results in a rise intracellular

calcium concentration, and facilitates dense granule secretion and integrin $\alpha_{11b}\beta_3$ (GP IIb-IIIa complex) activation (8).

Antiplatelet therapy: Ingestion of aspirin or aspirin-containing compounds inhibits the production of TxA_2 and resultant activation of the thromboxane receptor.

1.1.3.1.2 Adenosine Diphosphate (ADP)

ADP is stored in platelet dense granules and is secreted by platelets upon activation or by red blood cells at the site of vessel injury. It produces primary and secondary waves of aggregation particularly in response to lower concentrations of ADP. The primary wave of this biphasic response results from the engagement of ADP to its receptor leading to downstream signalling and the secondary wave is triggered by TxA_2, leading to release of various molecules from the dense granules including ADP, ATP and serotonin. Despite the fact that ADP has been used to stimulate platelets to aggregate since 1961 (9), it was only relatively recently that the receptors for ADP were characterised (10,11). The receptors for ADP belong to the family of G protein-coupled receptors and are known as the purinergic receptors: $P2Y_1$ and $P2Y_{12}$. The binding of ADP to these two receptors has different effects. $P2Y_1$ has a G_q protein subunit and when ADP engages the $P2Y_1$ receptor, PLC-β is activated and results in the release of diacylglycerol (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol-(4,5)-bisphosphate (PIP_2). DAG and IP_3 in turn activate protein kinase C (PKC), leading to the release of calcium from the dense tubular system. The binding of ADP to $P2Y_1$ is felt to cause shape change and reversible aggregation. $P2Y_{12}$ has a G_{i2} protein subunit and when ADP binds to this receptor, adenylate cyclase is inhibited, reducing the amount of cyclic adenosine monophosphate (cAMP) which normally inhibits platelet

aggregation. The dampening of platelet responses by cAMP becomes particularly relevant when platelets are exposed to certain prostaglandins, which act to minimise platelet activation by increasing cAMP levels. Therefore ADP activation of $P2Y_{12}$ is responsible for stabilisation of platelet aggregation (12-15).

Antiplatelet therapy: The thienopyridines, clopidogrel, ticlopidine and prasugrel, selectively inhibit the $P2Y_{12}$ receptor. They prevent ADP induced platelet aggregation and blunt platelet response to several other stimuli which induce platelet degranulation and secretion of prothrombotic and inflammatory mediators.

1.1.3.1.3 Epinephrine

In times of stress, epinephrine is released from the adrenal medulla and elevated levels are associated with hypertension and coronary artery disease (16). Epinephrine induced aggregation has been extensively investigated *in vitro* but the *in vivo* role of epinephrine in thrombus formation remains to be fully determined. The platelet expresses both α and β adrenergic receptors (17). The most abundant adrenergic receptor on platelets with a copy number of approximately 300 is the α_{2A} receptor (18) which like the purinergic receptors for ADP, is a G protein-coupled receptor. Once epinephrine binds to the α_{2A} receptor the G_z protein subunit, (a member of the inhibitory G_i family) leads to inhibition of adenylate cyclase in a similar manner to that of ADP and $P2Y_{12}$. In addition, platelets exposed to epinephrine will undergo increased turnover of phosphatidylinositol 4, 5-bisphosphate (PIP_2) with a slight increase in intracellular Ca2+ from phospholipase C (PLC) production (19). The PIP_2 is then hydrolysed by the PLC, resulting in diacylglycerol (DAG) and inositol triphosphate (IP3) production which then activates protein kinase C, promoting

phosphorylation of various proteins (20). Epinephrine also has the ability to activate the Na+/H+ exchange across the platelet membrane, thus activating phospholipase A_2 (PLA_2) and initiating AA production.

1.1.3.1.4 Thrombin receptor activating peptide (TRAP)

Thrombin is a potent stimulator of platelet aggregation via a protease activated receptor (PAR) (21). It is a coagulation protein produced from prothrombin (by Factor X) and it then converts fibrinogen to fibrin (22). It triggers a unique proteolytic cleavage of the PAR's N terminus, which in turn leads to activation of the PAR itself (23). Synthetic peptides have been developed based on these cleaved portions to allow investigation of each of the PAR receptors in isolation. TRAP is one of these synthetic peptides (SFLLRN) and is derived from the N-terminal amino acid sequence of the PAR-1 receptor (24). The addition of TRAP to platelets elicits the strong activation response seen with thrombin without the complications of fibrinogen cleavage and clot formation. The PAR-1 receptor is one of many G protein-coupled receptors on the platelet surface. TRAP binds to the PAR-1 receptor which is coupled to two G protein subunits G_q and G_{13}. Receptors that are coupled to G_q subunits stimulate phospholipase C-β which catalyses the creation of DAG and IP3 from PIP_2. DAG and IP3 then activate PKC which increases cytosolic Ca2+ leading the platelet activation. Receptors coupled to G_{13} subunits activate GTPase RhoA, which leads to Rho A and Rho kinase which reorganises the actin cytoskeleton resulting in shape change (25).

1.1.3.2.1 Agonists that do not mediate platelet signalling via G protein coupled receptors

1.1.3.2.2 Collagen

When the integrity of the vascular endothelium is disrupted platelets come into contact with collagen, which is one of the major elements of the subendothelial matrix and a potent agonist. Collagen induces rapid platelet adhesion, shape change, activation and aggregation. There are 2 main receptors that bind directly to collagen; GP VI, a member of the immunoglobulin superfamily, and the integrin receptor $\alpha_2\beta_1$; and two receptors that bind indirectly to collagen; integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa complex) and GP Ib via vWF (26). Once GP VI is engaged by collagen, clustering of the receptor occurs and phosphorylation of FcR by tyrosine kinases takes place (27). The resultant GPVI/ chain complex activates tyrosine kinase Syk, leading to phosphorylation and activation of phospholipase C (PLC 2). PLC 2 then converts PIP_2 into DAG and IP_3 through hydrolysis which opens Ca^{2+} channels, increasing Ca^{2+} influx and stimulating platelet activation. The integrin receptor $\alpha_2\beta_1$ is also believed to play an important role in collagen induced activation. The $\alpha_2\beta_1$ receptor is felt to be primarily involved in platelet adhesion following vessel injury although more recent work suggests that it may also be involved in platelet signalling.

Antiplatelet therapy: Abciximab, eptifibatide and tirofan inhibit the GPIIb/IIIa receptor.

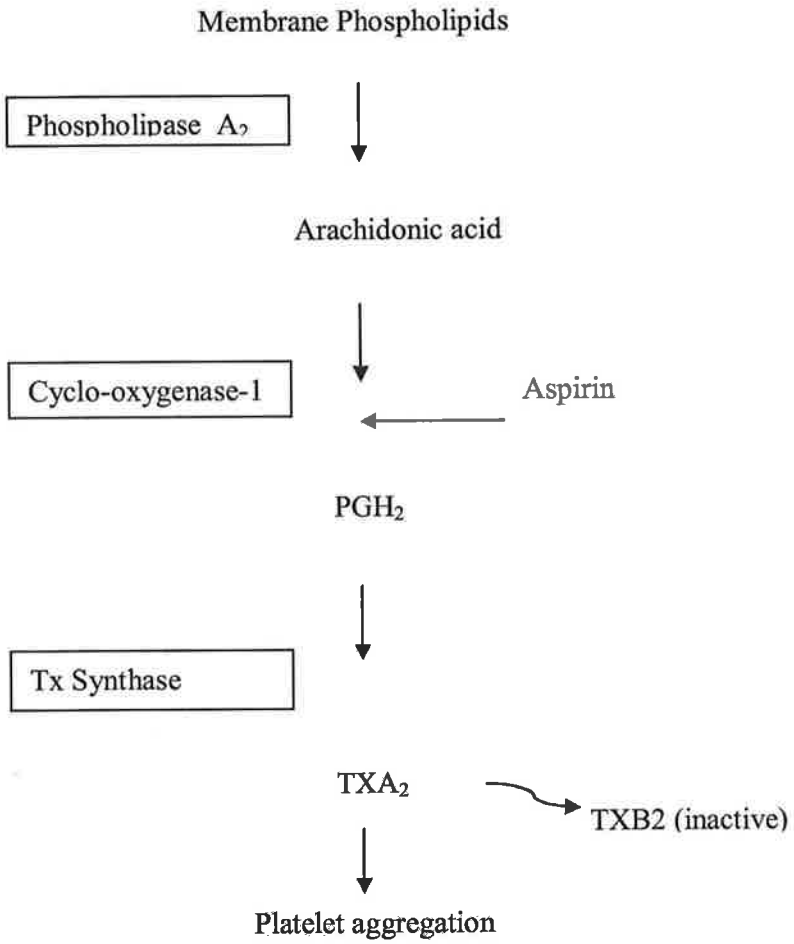
1.1.3.2.2 Shear induced aggregation

Shear force can be thought of as the force felt by the platelet when caught between the fast flowing blood and the stationary vessel wall. In normal circumstances

platelets pass through the circulation in a quiescent state despite experiencing a wide range of mechanical shear forces, which vary depending on the calibre of the vessel in which they are flowing. In general terms, the smaller the diameter of the blood vessel the greater the shear force applied to the platelet. As the amount of shear stress that is applied to platelets increases, the degree of platelet activation increases. Anything above 50 dynes/cm^2 has been shown to be pathological with measurable changes in platelet response (28). In stenotic coronary arteries the platelet encounters extremely high shear stresses such as 380 dynes/cm^2. Shear modulates platelet function through the VWF receptor, glycoprotein Ib/IX/V complex and through GP IIb/IIIa (29, 30). At high shear stresses, VWF binds to both of these receptors leading to an increase in cytosolic Ca_{2+} which causes platelet aggregation (31). The precise signalling events by which shear induced platelet aggregation occurs are not fully appreciated as yet but it is known that it is independent of the arachidonic acid-cyclooxygenase (COX) pathway and is not inhibited by aspirin (32).

1.1.4 Antiplatelet Therapy:Aspirin

As outlined above, aspirin is an antiplatelet therapy which targets the arachidonic acid activation pathway. Aspirin is a century-old drug. Since the discovery of its mode of action, the classic analgesic, anti-inflammatory and anti-pyretic uses of aspirin have evolved into vascular and anti-platelet effects. Aspirin irreversibly inactivates the cyclooxygenase (COX) activity of prostaglandin H (PGH) synthase 1 and 2, also known as COX-1 and COX-2, and prevents PGH_2 production. As shown in Figure 2, this results in the inhibition of TXA_2 generation which in turn reduces platelet aggregation.



PGH2 = prostaglandin H2

Figure 2. The role of cyclooxygenase-1 in thromboxane (TX) synthesis and the inhibitory action of aspirin

The use of aspirin has been very successful in the cardiology setting and is widely used for secondary prevention of cardiovascular disease. Aspirin has been assessed across a spectrum of cardiovascular risk populations, ranging from low risk to those presenting with acute ischaemic events. A number of landmark trials demonstrated that the efficacy of aspirin in the setting of unstable angina, the reduction of mortality associated with myocardial infarction, the reduction of stroke in high risk

patients and more recently the prevention of ischaemic complications associated with percutaneous coronary intervention (33-40).

In contrast, the role of aspirin in obstetric practice has experienced very limited success. Results of large trials on secondary prevention of pre-eclampsia have shown that aspirin is only beneficial in patients at a very high risk of developing severe, early onset disease (41). Aspirin has been widely used in attempts to treat women with Recurrent Miscarriage (RM) both in the setting of antiphospholipid syndrome and unexplained cases. Despite numerous clinical trials to evaluate its efficacy, the role of aspirin in RM continues to be a topical and controversial issue.

1.2 Recurrent Miscarriage

1.2.1 Definition and Prevalence

Although many advances in reproductive medicine have been made during the past quarter century, miscarriage remains the most common serious complication of pregnancy. Miscarriage is the spontaneous loss of a pregnancy before the fetus has reached viability. The term therefore includes all pregnancy losses from the time of conception until 24 weeks of gestation. There are two types of miscarriage: sporadic and recurrent. Recurrent Miscarriage (RM) is traditionally defined as three or more consecutive losses before 20 weeks post menstruation (42, 43). From a research setting RM is often defined as at least three first trimester losses or at least two second trimester losses. The recognised important features of the definition include the consecutive nature of the miscarriages and that the losses were clinically detectable pregnancies that failed to progress. Primary RM as compared to secondary RM refers to recurrent losses that have not been preceded by a livebirth.

This distressing problem may affect as many as 0.5% to 3% of fertile couples of reproductive age (44). This incidence is greater than that expected by chance alone since 10-15% of all clinically recognised pregnancies end in miscarriage and the theoretical risk of three consecutive losses is $(15\%)^3$ or 0.34% (45). Delayed childbirth has become a common phenomenon in the developed world as a result of social, educational, and economic factors (46). The UK census data showed that the number of babies born to mothers aged 35 years or more doubled between 1985 and 2001, from 8% to 16% of total births (47). As maternal age is a strong risk factor for both sporadic and RM (48), increasing maternal age will in turn result in an increased prevalence of RM in the overall population.

1.2.2 Known causes of RM

RM is a heterogeneous condition. Historically, the condition has been attributed to genetic, anatomical, thrombophilic-(acquired or inherited), immune, endocrine and unexplained causes. This in turn makes the investigation and management of RM difficult for both the clinician and patient. A brief summary of each of the accepted causes evaluated in current practice is outlined below.

1.2.2.1 Genetic

In approximately 3-5% couples with RM, one member of a couple carries a balanced structural chromosomal anomaly (47). If one member of a couple is a carrier of such a chromosomal abnormality the risk of miscarriage is doubled. Karyotyping of both partners is a standard test in the investigation of RM with referral to a clinical geneticist indicated when chromosomal abnormalities are detected. Other factors such as low maternal age at second miscarriage and significant miscarriage history

in a first degree relative are concerning indicators of a possible genetic cause. The commonest type of parental chromosomal abnormality is balanced reciprocal or Robertsonian translocations (49).

The other possible genetic cause to consider is sporadic recurrence of cytogenetically abnormal pregnancies. Miscarriage in itself can be viewed as a natural process of quality control. In the first trimester 90% of karyotypically abnormal pregnancies miscarry and 93% of normal pregnancies continue (50). The embryonic period is between 5-9 weeks gestation, (3-7 weeks post-conception). The fetal period is greater than 10 weeks gestation. Branch and Silver suggested that fetal deaths are more likely associated with a maternal aetiology such as APS for example, whereas embryonic losses are more likely due to embryonic anomalies (51). Bricker *et al* looked at miscarriage patterns in their RM clinic and found that embryonic losses outnumbered fetal losses by a factor of 6:1 (52).

An interesting study was performed by Coulam *et al* where karyotypic analysis over 200 abortuses from women with unexplained RM and same partner showed 55% of losses were chromosomally abnormal, with a rate of 35% in those with secondary RM (53). Skinner *et al* demonstrated a chromosomal abnormality rate of 84% in a series of 50 sporadic miscarriages (54).However, as cytogenetic testing of each pregnancy is not a recommended part of routine practice the exact percentage of chromosomal abnormality in patients without a history of RM, is unknown. Nevertheless, it is widely accepted that as the number of miscarriages increases, the prevalence of chromosomal abnormality decreases and the chance of a recurring maternal cause increases (55, 56).

1.2.2.2 Anatomical

It is difficult to assess the exact contribution of congenital uterine anomalies to RM as the prevalence and reproductive implications of uterine anomalies have not been clearly established. The reported incidence of uterine anomalies in the general population ranges between 1.8% and 37.6% (57). This prevalence seems to be higher in women with late miscarriages compared with early and this is assumed to be related to the cervical weakness that is frequently associated with uterine malformation (58). In the past invasive testing of the uterus in women with RM was recommended however it is now accepted that hysterosalpingogram or hysteroscopy is no more sensitive than the non-invasive 2-D pelvic ultrasound assessment of the uterine cavity when performed by skilled and experienced personnel (49).

1.2.2.3 Thrombophilia

The term *'thrombophilia'* is most often used to describe a laboratory phenomenon that is associated with an increased tendency to venous thromboembolism, either acquired or inherited. The presence of antiphospholipid antibodies (APA) (directed against anticardiolipin and β-glycoprotein 1) and/or lupus anticoagulants (LAC) are the laboratory criteria for the acquired antiphospholipid syndrome (APS). Primary antiphospholipid syndrome (APS) first described in the 1980s, refers to the association between APA and adverse pregnancy outcome or vascular thrombosis (59). Adverse pregnancy outcome includes RM, second trimester loss, preterm delivery associated with severe pre-eclampsia (PET) or placental insufficiency and intra-uterine growth restriction (IUGR).

International classification criteria for definition of APS

The presence of at least one clinical and one laboratory criterion are required for the diagnosis of APS (60).

Pregnancy complications

- Three or more consecutive unexplained miscarriages before 10 weeks' gestation.
- One or more unexplained deaths of morphologically normal fetuses after 10 weeks' gestation.
- One or more premature births of a morphologically normal fetus at 34 weeks' gestation or earlier, associated with severe PET or placental insufficiency (includes placental abruption).

Vascular Thrombosis

- One or more clinical episodes of arterial, venous or small vessel thrombosis in any tissue group or organ.

Laboratory Criteria

- Anticardiolipin antibody ACA (IgG or IgM) of medium or high titre on two or more occasions at least 6 weeks apart.
- Lupus anticoagulant LAC present in plasma on two or more occasions at least 6 weeks apart.

Antiphopholipid antibodies are present persistently in 15% of women with RM (61) compared with less than 2% in the normal population (62). Women with APS have a rate of fetal loss of 90% when no specific treatment is given during pregnancy (63). Miscarriage related to APS has been traditionally attributed to thrombosis of the uteroplacental vasculature and placental infarction (64, 65). More recently, Sebire *et al* demonstrated that defective decidual endovascular trophoblast invasion rather

than excessive intervillous thrombosis was the most frequent histological abnormality in APS-associated early pregnancy loss (66).

A randomised controlled trial performed by Rai and colleagues showed that the livebirth rate for women with RM-associated APS treated with low dose aspirin (LDA) rises from 10% to 40% and that this is improved further to 70% with the addition of low molecular weight heparin LMWH (67). Another RCT however reported a high success rate with LDA alone with no additional benefit being found with the addition of LMWH (68). This study was compromised however by the late randomisation of some pregnancies, at up to 12 weeks' gestation in some instances. Empson *et al* performed a systematic review of the various trials exploring interventions to improve pregnancy outcome in women with APS. This group again endorsed the combination of LMWH and LDA but cited the need for a large RCT to evaluate the benefit further (69).

The term 'inherited thrombophilias' refer to defiencies of the natural anticoagulants antithrombin, protein C and S, as well as the gain of function mutations, factor V Leiden (that causes resistance of FVa inactivation by activated protein C, APC resistance) and prothrombin 20210A. Initially it was believed that these inherited 'thrombophila' were associated with fetal loss (70), however subsequent studies have been inconsistent. Some studies have even reported a decreased risk of RM in these groups (71, 72). Coulam and colleagues believe the risk of RM is related to the presence of multiple thrombophilic mutations rather than specific singular gene defects involved (73). Jivraj *et al* reported that the prevalence of inherited thromophilia was similar in RM couples when compared to controls but that multiple genetic thrombophilic mutations in either partner significantly increases the risk of miscarriage in subsequent pregnancies (74). Larger epidemiological studies

are required before testing couples with RM for inherited thrombophilia can be recommended in routine clinical practice (75). Until further data on this issue are available the European Society for Human Reproduction and Embryology (ESHRE) suggests that the incorporation of detailed inherited thrombophilia screening investigations for RM patients should only be included in the setting of research trials (76).

1.2.2.4 Immunological

From a traditional immunological perspective, survival of the semiallogenic fetus is dependent on suppression of the maternal immune response. At the time of implantation, natural killer (NK) cells are the predominant lymphocyte population in the decidua. The NK cells are in close contact with the invading trophoblast cells. Their exact function remains unclear, although their role is most likely to regulate the development of the placenta to ensure sufficient fetal blood supply. The hypothesis is that this process is impaired in pregnancies complicated by miscarriage and also in PET and IUGR. Also, returning to the hypothesis that RM pregnancies have a higher degree of karotypic abnormality there is another theory that patients with RM are immunologically more receptive and therefore are less selective against abnormal pregnancies. This theory initially offered a potential breakthrough regarding treatment of unexplained RM. However, a Cochrane systematic review of 18 RCTs has concluded that the various forms of immunotherapy, including paternal cell immunisation, third-party donor leucocytes, trophoblast membranes and intravenous immunoglobulin (IVIG), in women with previous unexplained RM provides no significant benefit over placebo in improving the live birth rate (77).

1.2.2.5 Endocrine

The prevalence of polycystic ovaries, defined according to pelvic ultrasound criteria, is significantly higher amongst women with RM (41%) when compared to the general population (22%) (78). However, the presence of this ovarian morphology does not predict an increased risk of future pregnancy loss amongst ovulatory women with RM who spontaneously conceive. On the other hand, the presence of an ovulatory disorder, the commonest cause of subfertility in the 25-30% of women with concomitant RM, confers a poor prognosis for future pregnancy outcome (49). With respect to systemic maternal endocrine disorders such as diabetes mellitus and thyroid disease, there is no increased risk for RM when these conditions are treated and well-controlled. Indeed the prevalence of diabetes mellitus and thyroid dysfunction in women who suffer RM is similar to that reported in the general population (79).Two recently published studies have linked an increased risk of RM amongst women at the extremes of the BMI range: obese ($\geq 30 kg/m^2$) and underweight ($\leq 19.0\ kg/m^2$) (80,81).

1.2.2.6 Unexplained

Despite complete investigation, a significant proportion of cases of RM remain unexplained, i.e. no identifiable cause in either partner. A causal factor can be identified in less than half of affected couples (50). This has led to the term idiopathic RM. With the possible element of chance, it is important to acknowledge that amongst the idiopathic group, a significant proportion of couples presenting with recurrent miscarriage will not have a persistent underlying cause found. The prognosis for successful future pregnancy using supportive care alone has been reported as being up to 75% (58). Nevertheless, failure to find a specific treatable

cause for RM adds further to the emotional distress associated with this condition (82).

1.2.3 Routine Investigations

Recommendations for the testing of the couple presenting with recurrent miscarriage (≥3 miscarriages), as agreed by the **ESHRE Special Interest Group for Early Pregnancy (SIGEP) 2006** (76), are outlined below in Table 1.

Table 1: Recommended Investigations for RM

Basic investigations

- Full blood count (blood sugar level and thyroid function tests can be added if additional risk factors for diabetes mellitus and thyroid disease are present)
- Antiphospholipid antibodies (LAC and ACA)
- Parental karyotypes
- Pelvic ultrasound and/or hysterosalpingogram, followed by hysteroscopy with laparoscopy in cases with inconclusive or abnormal findings

Additional investigations which may be considered within the context of a research trial

- Feto-placental karyotypes
- Testing of uterine and/or peripheral blood NK cells
- Mannan-binding lectin (MBL) level
- Luteal phase endometrial biopsy
- Homocysteine/folic acid level
- Thrombophilia screening

1.2.4 Reproductive characteristics and long-term risks

There is a positive correlation between the number of previous miscarriages and the miscarriage rate in the next pregnancy (83). The prognosis is not better for couples with previous livebirth i.e. secondary RM (48, 84). In patients with a history of RM, subsequent ongoing pregnancies have been shown to have an increased risk of certain obstetric complications, such as PET and placental insufficiency. Rai *et al*

performed a detailed review of RM patients and identified specific reproductive characteristics associated with subsequent poor pregnancy prognosis, which are shown in Table 2.

Table 2: Reproductive characteristics associated with poor pregnancy prognosis and RM (47)

	Number of women	
	N=500	%
>3 miscarriages	500	100
Conception delays	160	32
Late miscarriage (2nd trimester)	109	22
Stillbirth or neonatal death	31	6
Ectopic pregnancy	23	5
Abortion or fetal abnormality	8	2
Previous livebirth	223	45
Pregnancy complicated by prematurity / IUGR	98	20

Recently Trogstad and colleagues performed a large population-based study of RM and found a trend towards an increased risk of PET when there was also a history of RM. This association became significant with the additive effect of a history of fertility treatment, producing an adjusted odds ratio for PET of 2.40 (95% CI – 1.11-5.18) (85). Jivraj *et al* looked at 162 RM-associated pregnancies that progressed beyond 24 weeks' gestation and found a significantly higher percentage of pre-term labour (PTL), small for gestational age (SGA), perinatal loss and caesarean section

rate (86). They advised that RM-associated pregnancies should be treated according to high risk obstetric management protocols with close antenatal surveillance. Beyond the pregnancy risks there are also implications for the health of these women both during and beyond their reproductive years. Smith *et al* published a large population study in 2001 which linked complications such as IUGR, PTL and PET to the increased risk of subsequent ischaemic heart disease (IHD) in the mother (87).

The same research group then went on to look at the association with miscarriage and found an increased risk of IHD that rose with the number of previous losses (88). They proposed that this may reflect occult prothrombotic tendency resulting in pregnancy complications during the reproductive years and overt cardiovascular disease in later life. Quenby *et al* also demonstrated an increased long-term risk of thrombosis in patients with a history of unexplained RM. They reported a risk of thrombosis of 2 per 1000 women years in this population which was significantly higher than the reported value of 0.27 per 1000 women years in the general population (89). This raises the question as to whether RM patients should be counselled regarding appropriate lifestyle modifications such as avoidance of the oral contraceptive pill, smoking, weight gain and monitoring for high blood pressure, high cholesterol or other cardiovascular risks. In fact, there may be benefit to the prophylactic use of low dose aspirin in this cohort of women to decrease the risk of cardiovascular disease. Further studies are warranted to explore this issue.

1.2.5 Aspirin and Recurrent Miscarriage

The empiric use of aspirin therapy in the treatment of idiopathic RM is a widespread clinical practice in the community. One of the reasons for its widespread use is the

assumed similarities of pathogenesis between RM associated with APS and unexplained RM. This is based on the extrapolation of the benefit seen with aspirin use in RM caused by APS. The other main reason is the historical hypothesis that women affected with RM are already in a prothrombotic state before pregnancy begins. Evidence has been accumulating over the years that some cases of RM are the result of exaggerated haemostatic response as shown by investigations of placental thrombosis (90,91), thrombin-antithrombin complexes (92), circulating microparticles (93,94) and performance of thromboelastography in the unexplained RM population (95). This is further reinforced by the poor reproductive characteristic and long-term prothrombotic risks previously outlined. As a result, it is often concluded that there is a subgroup of RM patients who are in a prothrombotic state and would likely benefit from aspirin treatment to improve outcome. However, the clinical trials exploring this benefit have shown inconsistent findings.

Rai *et al* performed a large observational study of 805 women with idiopathic RM. They commenced 75mg nu-seal aspirin (NSA) five weeks after amenorrhoea and found a livebirth outcome of 68% in the aspirin cohort versus 63.5% in the non-aspirin cohort. The main benefit found was in the subgroup with previous late miscarriages. This cohort had an increase in livebirth rate from 49.3% to 64.6% when aspirin was prescribed (96). However this trial was limited by the lack of randomisaton. A Cochrane review of the use of aspirin in idiopathic RM performed in 2005 concluded that there was no benefit even though they only included 2 small randomised control trials with a total of only 54 women (97). Kaandrop *et al* recently addressed these issues further and performed a large randomized trial with a study population of 364 women (98). They were unable to show any improvement in

livebirth rate with aspirin among women with unexplained miscarriages. However, there was a major flaw in their study cohort as they recruited women with only two consecutive miscarriages and therefore, did not meet the correct definition for RM. To date, the role of aspirin in idiopathic RM remains unanswered.

2 PLATELET FUNCTION TESTING

2.1 Introduction

Laboratory evaluation of platelets has traditionally been limited to a quantitative assessment of platelet number, with testing of platelet function being rarely performed. Platelets have an established role in the regulation of both haemostasis and thrombosis (99). Therefore it is surprising that platelet function testing is only used in the rare situation of diagnosis and management of bleeding disorders and specific platelet conditions such as Bernard Soulier syndrome (100). However there are several lines of evidence to suggest that platelet function testing may have a useful clinical role in cardiovascular disease (101,102). Moreover, the finding that high platelet reactivity may be clinically important and useful for predicting the risk of recurrent cardiovascular events in patients taking antiplatelet therapy has also generated considerable interest in the area of platelet function testing (103,104).

Platelet reactivity refers to the ex vivo measurement of platelet responses to various agonists providing an index of platelet functional capacity. The laboratory investigation of platelet function usually involves measuring platelet aggregation and/or granule content/release. There are a number of issues that have impeded the use of platelet function testing in routine clinical practice. A major limitation, is the lack of standardisation which makes it difficult to compare results from different laboratories. Also, traditional platelet function tests have limited clinical

applications as they require onerous or impractical blood sample processing, which is time consuming and labour intensive and results can be difficult to interpret. There are over 30 commercially available tests of platelet function which share many common features and underlying principles. The aim of this section is to briefly discuss the principles of well characterised platelet function assays, to describe a novel microtitre platelet aggregation (MPA) assay which has been developed and to explore platelet function testing in the setting of idiopathic RM.

2.2 Platelet Function Tests

2.2.1 Platelet Aggregation

Light transmission aggregometry (LTA) has been instrumental in providing us with a greater understanding of thrombosis and haemostasis since it was first described by Born in 1962. It is probably the most widely used platelet function test and considered by many to be the gold standard of assessing platelet function (105-107). The basic principle is simple; light is passed through a stirred suspension of platelets (platelet rich plasma (PRP) generated from centrifugation of citrated whole blood). As the light travels through the platelet suspension, the light is scattered by the platelets, thus reducing the relative amount of light transmitted. Upon addition of agonists (AA, ADP, thrombin, epinephrine) to the PRP, platelets aggregate to each other in a GP IIb/IIIa- dependent manner, resulting in a reduction of light scattering and thus increasing light transmission. So the more light transmitted, the greater the degree of aggregation. The level of aggregation is measured over five to ten minutes, to give an output curve showing aggregation response over time.

One of the major advantages of light transmission aggregometry is that it can be used to test multiple agonists thus providing valuable information on multiple signalling pathways. Limitations include the lack of comparability to physiological platelet aggregation as the separated platelets in PRP only form aggregates following the addition of soluble agonists under low shear conditions (stirring). In addition, light transmission aggregometry is time consuming, requires relatively large amounts of blood and expertise. It is only available in specialised laboratories, as sample processing and testing require experienced personnel.

2.2.2 Whole Blood aggregometry

This technique is similar to LTA. It was first described in 1980 by Cardinal et al and involves measurement of the change in electrical impedance or resistance between two electrodes following stimulation with an agonist in anticoagulated whole blood (108). An anticoagulated whole blood sample is diluted with saline in a 1:1 ratio, placed into a cuvette and stirred at 37°C between electrodes. The platelets adhere to the electrodes when they aggregate causing an increase in the impedance between the two electrodes. The impedance is read over time and the change in impedance is converted to % aggregation. The % aggregation is measured over five to ten minutes, to give an output curve showing aggregation response over time.

Less blood is required to carry out whole blood aggregometry and because there is less manipulation of blood, it is quicker and easier than LTA. In addition, given that no centrifugation of the blood sample is required, there is less likelihood for unwanted platelet activation and it avoids the loss of platelet subpopulations. The main limitation with this technique comes from the electrodes

as they tend to become covered in debris over time which can lead to greater intra-individual imprecision than would be seen in comparison to LTA. Indeed, the results from whole blood aggregometry appear to be less consistent than those achieved with standard LTA and the results are more difficult to interpet (109).

2.2.3 Shear-Induced Platelet Activation

The Platelet Function Analyser-100 (PFA-100) was first described in 1995 and was subsequently approved to evaluate acquired and congenital platelet dysfunction around 6 years later (110,111). The PFA-100® assay uses a relatively small sample of citrated whole blood and evaluates platelet function *in vitro*, under high-shear-rate conditions (5,000-6,000 s^{-1}). The instrument measures the fall in flow rate as platelets within citrated whole blood are aspirated through a capillary and begin to seal a microscopic aperture (147 μm) within a nitrocellulose membrane coated with collagen and epinephrine or collagen and ADP. Platelet function is measured according to the duration required for thrombotic occlusion of the aperture and is reported as the closure time (CT) (112). This reaction takes place within disposable cartridges that are inserted into the device. PFA-100 has certain advantages over many other platelet function tests. It is a rapid, whole blood assay that only requires a relatively small sample of blood and limited expertise. In addition it measures platelet function under conditions of shear stress which is an important modulator of platelet induced thrombosis. However the PFA-100 also has several disadvantages including poor correlation with LTA. Also, the closure time may be affected by haematocrit, platelet count and circulating vWF (111-113).

2.2.4 Thromboelastography

Thromboelastography (TEG) was developed by Hartert in 1951 and is a technique that measures both haemostasis and clot lysis in whole blood (114). Whole blood is incubated at 37°C in a heated rotating sample cup which contains a suspended pin connected to a detector system. The detector is either a torsion wire (TEG®) or an optical detector (ROTEM®). The cup and pin rotate relative to each other and as fibrin forms the transmitted oscillation from the cup to the pin (TEG®) or impedance of the pin oscillation (ROTEM®) is detected and a trace generated (115). The recent development of the Haemoscope® Thromboelastograph Platelet Mapping System has enabled TEG to be used to assess platelet function (116). The assay uses heparin as an anticoagulant to eliminate thrombin activity in the sample which overwhelms the effect of less potent agonists. Reptilase and factor XIIIa (activator F) are used to generate a cross-linked fibrin clot to isolate the fibrin contribution to the clot strength. The contribution of the ADP or TXA_2 receptors to the clot formation is then provided by the addition of agonists arachidonic acid and ADP (117). This modification of the traditional TEG technique has been shown to correlate well with LTA (117) however there are limited studies showing its beneficial applications in clinical practice.

2.2.5 Flow Cytometry

There is no doubt that one of the biggest advances in platelet function analysis has been the development of flow cytometry. Initially this technique was limited to the research settting however flow cytometric assays are now routinely used in the quantification of glycoprotein receptor density (i.e. diagnosis of deficiencies in platelet glycoproteins e.g. Glanzmann's thrombasthenia and Bernard Soulier

disease).Whole blood cytometry is performed on citrated blood samples using at least two monoclonal antibodies, each conjugated with a different fluorophore. Labelled platelets pass through a flow chamber with a focussed laser beam, which activates the fluorophore at the excitation wavelength. The emitted fluoresence and light scatter are detected and the intensity of the emitted light reflects the density of the antigen or cell characteristics being evaluated (118). A number of clinical applications of flow cytometry technique continue to emerge particularly with respect to the determination of platelet reactivity. However, flow cytometric platelet analysis is mostly limited to specialist centres of platelet biology and laboratory research. The main disadvantages of flow cytometry are the requirement for expensive cumbersome equipment, significant expertise both to perform and interpet the test and time-consuming sample preparation.

2.3 Novel Platelet Function Assay

Platelet function is highly complex involving multiple receptors and signalling pathways (119) but this is not reflected by most platelet function tests. LTA, the gold standard for platelet function testing, addresses the complexity to some extent because it enables the testing of multiple agonists over a range of concentrations; however the process is labour intensive and requires a considerable degree of expertise to perform. Platelet aging is an issue when using LTA to assay platelet function. The number of samples that can be analysed is limited to a maximum of eight channels. Most laboratories however use aggregometers that have only four channels, which means that only a batch of four samples can be tested simultaneously. As the duration of time from the initial blood draw to

testing increases, platelet function gradually declines in response to agonist induced stimulation (120). In addition, detailed platelet function testing requires that a range of soluble agonists are tested at multiple concentrations which requires a large blood sample and is extremely time consuming.

The Cardiovascular Biology Group at the Royal College of Surgeons in Ireland (RCSI) recently developed a new assay which addresses many of these issues. It is a modification of LTA using a 96-well plate which allows for accurate, rapid measurement of platelet aggregation in response to multiple platelet agonists at a range of concentrations, as evidenced by its application in a number of recently published studies (120-124). In my study, the average time from obtaining the blood sample to assay completion was 18 minutes; therefore platelet aging was not an issue.

2.3.1 Description overview

In brief, 180 μl of platelet rich plasma (PRP) is added to each well of a 96-well microtitre plate containing eight incremental concentrations of five different platelet agonists: thrombin receptor activating peptide (TRAP), arachidonic acid (AA), collagen (Coll), adenosine diphosphate (ADP), and epinephrine (EPI). The concentrations of the agonists used are 500, 375, 188, 83.8, 46.9, 23.4, 11.8, 5.86 μg/ml for AA; 190, 143, 71.3, 35.6, 17.8, 8.9, 4.45, 2.23 μg/ml for Coll; 20, 10, 5,2.5, 1.25, 0.625, 0.313, 0.156 μM for ADP and TRAP; and 20, 5, 1.25, 0.313, 0.078, 0.0195, 0.00488, 0.00122 μM for EPI.Control wells containing saline buffer, platelet-rich and platelet-poor plasma (PPP) are also prepared. Light transmission is measured using a 572nm filter at 0, 3, 6, 9, 12, 15 and 18 mins during which time the plate is constantly rotated at 1000 r.p.m. through a 0.1mm orbit between

measurements. The 96-well plate is then read using a Victor 3 Multilabel plate reader (Perkin Elmer,Wellesley, MA). Light absorbance values are normalised based on PRP and PPP control absorbance values, which represented 0 and 100% aggregation. Using this, the percentage aggregation response for each concentration of each agonist is calculated. These values are then plotted against the log values of the concentrations of agonist used with Graphprism software®.

2.3.2 'Novel' aspects of the assay

This platelet assay is novel for a number of reasons; the use of numerous agonists, numerous concentrations of agonists, length of time to perform assays and less blood required. As previously outlined, platelet aggregation is a highly complex sequence of events involving multiple receptors and signalling pathways (125). Most devices or assays use either a single agonist or a single concentration of agonist which seems relatively facile. Moreover, when vascular injury occurs *in vivo*, platelets come into contact with collagen, one of the essential components of the subendothelial matrix. When this occurs, platelets become activated and the subsequent release of thromboxane and ADP, and the binding of thrombin amplify platelet activation leading to aggregation. In addition, epinephrine is released from platelets contributing to thrombus growth (126). By using multiple agonists at different concentrations this novel assay aims to better reflect in part a similar environment to that when platelets aggregate *in vivo*.

Maximal platelet aggregation is routinely measured using high concentrations of agonist. This approach identifies gross platelet dysfunction and provides relatively high reproducibility and low variability in platelet aggregation responses. Multiple incremental concentrations of each agonist are included in this assay as recent

evidence suggests that using lower (submaximal) concentrations of agonist may be a better way of measuring platelet aggregation as this approach identifies subtle changes in platelet function which would otherwise be obscured by higher concentrations of agonist (127).

Standard LTA is limited by time constraints. Two to eight channel aggregometers have been developed to speed up assays, but even these multi-channel devices allow relatively small numbers of samples to be investigated simultaneously. This novel microtitre platelet aggregation (MPA) assay is rapid and uses comparatively less blood than LTA. Platelet function can be tested simultaneously in 2 individuals in 18 minutes following addition of PRP. To do the equivalent analysis using an 8 channel LTA could take up to 60 minutes for each individual.

2.3.3 Measures to reduce variability

A number of studies have shown that platelet aggregation to agonists is variable depending on both genetic and environmental factors (128,129) Factors that affect platelet response include smoking (130,131), whether an individual fasts prior to testing (132,133) caffeine (134), exercise (135), alcohol intake (136) and circadian rhythm (137,138). Peace *et al* recently looked at reducing intra-individual variation in platelet aggregation using this novel assay (124). Their results showed that there is wide variability in platelet aggregation within an individual. This intra-individual variability is most obvious in response to submaximal concentrations of agonists. They found that controlling for environmental factors and circadian rhythm significantly reduces intra-individual variability particularly in response to submaximal concentrations. These findings suggest that platelet function testing should only be performed in a fasting individual who has refrained from smoking,

caffeine consumption, alcohol, and intensive exercise. The application of these recommendations will provide more accurate results when testing platelet function using this method.

Another factor to consider when assessing platelet function in women is the timing of the menstrual cycle. Feuring *et al* demonstrated that platelet function is periodically altered during the ovarian cycle due to hormonal effects (139). One of the conclusions reached by Roell *et al* when looking at the effect of oral contraceptives (OC) and ovarian cycle on platelet function, was that progesterone may be the main mediator impacting on platelet reactivity. Using the PFA-100 system they assessed the mean closure time with Coll and EPI and found that it was significantly increased in the luteal phase compared to the follicular phase of the menstrual cycle in women both taking and not taking OCs (140). Therefore this evidence suggests that menstrual timing and/or progesterone levels should be evaluated in addition to hormonal medication history when measuring platelet reactivity in women.

2.4 Platelet Function Testing and Recurrent Miscarriage

2.4.1 Previous studies

The historical hypothesis is that women with RM are in a prothrombotic state. Despite the fact that platelets are central to thrombosis and that there is widespread empiric use of the antiplatelet agent aspirin in the RM population, there are a dearth of studies which assess platelet function testing in this clinical condition. A detailed literature search was performed which included online databases MEDLINE, EMBASE and OVID, Cochrane library reviews and handsearch of bibliographies of

all retrieved RM articles to date. Only two published studies which sought to evaluate RM with platelet function testing techniques were sourced.

Rai *et al* used the traditional TEG technique in their assessment of 494 women with unexplained RM over 18 months. Although the women were tested at random intervals during the menstrual cycle they were able to identify a proportion of women with RM with raised pre-pregnancy maximum clot amplitude (MA) as compared to control subjects. They concluded that a subgroup of the RM population is in a prothrombotic state outside pregnancy. They also concluded that results from their prospective observation of pregnancy outcome showed these women are at risk for future miscarriage in subsequent pregnancies (96). As the blood samples were not heparinised and other agonists were not used, the study was limited to evaluation of whole-blood haemostasis and not to evaluation of platelet reactivity which is the index of the functional capacity of platelets.

Beyan *et al* assessed 29 RM patients using LTA and found no difference in platelet aggregation response to three agonists (ADP, Coll and EPI) as compared to control subjects (141). However, the main limitation of this study was that only a single high concentration of each agonist was used in the test: ADP (5μM), Coll (0.2mg/ml) and EPI (10μM) which we know is inefficient to detect subtle changes in platelet function.

2.4.2 Aim of Proposed Study

Platelet reactivity refers to the ex vivo measurement of platelet responses to various agonists such as ADP, epinephrine, thrombin, arachidonic acid or collagen, thereby providing an index of platelet functional capacity. To date, the role of platelet reactivity has not been clarified in the RM population. The development of this

novel platelet function assay in our laboratory provided the opportunity to measure platelet reactivity in idiopathic RM. The aim of the proposed study was therefore, to critically evaluate platelet function in women affected by unexplained RM as compared to a tightly matched control group.

3 AUDIT OF PATIENT POPULATION

3.1 Background

There is a dedicated RM clinic in the Rotunda Hospital to which both private and public Rotunda patients and patients from peripheral hospitals are referred. At the clinic a comprehensive medical history is taken and routine RM investigations are performed. Referral for further appropriate investigations or treatment/counselling is arranged directly from the clinic. If pregnancy is subsequently achieved the patients then attend the clinic for weekly reassurance ultrasonography until twelve weeks gestation. The clinic is run by a senior Specialist Registrar in obstetrics and gynaecology, and is overseen by a Consultant Obstetrician & Gynaecologist.

In order to gain more insight into the proposed study population an audit of the dedicated RM clinic in the Rotunda Hospital was carried out.

Aim:

- To identify new referrals to the clinic and examine whether they met the correct criteria for definition of RM (i.e. three consecutive first trimester miscarriages).
- To review the investigation results of appropriate referrals
- To determine the rate of idiopathic cases of RM
- To evaluate the empiric use of aspirin in this cohort.

3.2 Methods:

The Rotunda Hospital outpatient computer database was used to identify all new referrals to this clinic from January to December 2008 inclusively. The individual patient charts were then reviewed. Patients were included in the review if they had experienced three documented consecutive first trimester miscarriages and had the appropriate investigations performed.

3.3 Results:

In total there were 54 new referrals to the RM outpatient clinic in 2008. Eleven patients did not meet the correct criteria for RM definition, most commonly lacking the 'consecutive' prerequisite. Also, four patients were pregnant at the time of referral and were therefore directly referred on to the antenatal clinic. Therefore there were 39 patients who met the correct criteria for definition, and all but one patient underwent appropriate RM investigations.

Table 3: Demographic details of RM clinic attendees.

Average age	34 years (range 22-44)
Rate of previous livebirths	55.3%
Median number of miscarriages	4 (range 3-7)
Underlying medical condition	21.1%
Family history of RM	13.2%
Smoking rate	31.6%

Underlying medical conditions included history of pulmonary embolism while taking the combined oral contraceptive pill, multiple sclerosis, severe asthma, morbid obesity, and PCOS. With respect to those that had previous livebirths, 71.4% had spontaneous vaginal deliveries.

Table 4. Causes of RM found in clinic attendees

Uterine anomalies	13.2% (n=5)
Raised ACA/LAC	5.3% (n=2)
Abnormal karyotype	7.9% (n=3)

Causes of RM were found in 10 patients (26.3%), and therefore 73.7% (n=28) remained 'unexplained'.

71.4% (20/28) of the idiopathic RM patients were commenced empirically on low dose aspririn therapy and in the subsequent year 40% (8/20) had achieved pregnancy (35% livebirth rate).

3.4 Discussion

Overall the number of patients referred who met the correct criteria for definition of RM, without significant medical history and who underwent appropriate investigation was relatively small. However, the results in Tables 3 and 4 are consistent with the findings of other studies examining the demographics and causes found in RM patient cohorts (43, 47, 52). In accordance with the ethos of the

Rotunda Hospital RM clinic the rate of empiric use of aspirin is high at 71.4% and the subsequent livebirth rate is consistent with the literature.

4 PLATELET REACTIVITY AND PREGNANCY LOSS

4.1 Introduction

The historical hypothesis behind the occurrence of recurrent miscarriage has been that affected women are already in a prothrombotic state before pregnancy begins. Evidence has been accumulating over the years that some cases of RM are the result of exaggerated haemostatic response as shown by investigations of placental thrombosis (90,91), thrombin-antithrombin complexes (92), circulating microparticles (93,94), and performance of thromboelastography in the unexplained RM population (95). Indeed there is also evidence of longterm prothrombotic risk (88, 89). It is often concluded that there is a subgroup of RM patients who are in a prothrombotic state and would likely benefit from antithrombotic treatment to improve outcome.

The major limitation of conventional hemostasis tests, such as measuring the levels of individual coagulation and anti-coagulation proteins, are that they ignore the fact that in vivo hemostasis is a dynamic process which involves the interaction of coagulation and fibrinolytic factors with cellular elements such as platelets and the blood vessel wall. One way of determining the thrombotic risk of this cohort of patients would be to assess *in vivo* platelet function which, has not been performed effectively to date. We have recently developed a novel platelet assay that uses multiple concentrations of different agonists and thus reflects the complexity of agonist-induced platelet aggregation in vivo (121-123). The aim of this study was to critically evaluate platelet function in an idiopathic RM cohort as compared to a matched control group.

4.2 Methods

4.2.1 Recruitment of patients

Ethical approval was obtained from the Rotunda Hospital Ethics Committee and the study complied with the Declaration of Helsinki. All patients were provided with written information at the time of recruitment (appendix 2.1) and written consent was obtained from all patients prior to phlebotomy (appendix 2.2).

4.2.1.1 Cases and Controls eligibility criteria

Cases were recruited by me from the Rotunda Hospital RM outpatient clinic. Eligibility criteria included patients who had experienced at least three consecutive first trimester miscarriages (with the same partner), and who had undergone routine RM investigation with no positive results. Routine investigations were performed in the RM clinic in accordance with ACOG and ESHRE guidelines. These investigations were full blood count (blood sugar level and thyroid function tests if additional risk factors were present), antiphospholipid antibodies (LAC and ACA), parental karyotypes and pelvic ultrasound and/or hysterosalpingogram and hysteroscopy (44, 76). The recruits had no significant medical disorders; in particular there were no conditions such as spherocytosis or thrombocytopenia which directly affect platelet function.

The control group was matched for age and ethnicity and had no known medical disorders, or personal or family history of venous thromboembolism. I recruited the control subjects both from the Rotunda Hospital day-operating list and family

planning clinic. The majority of women admitted for theatre were undergoing minor procedures such as Mirena coil insertion. The women attending the family planning clinic were contacted by me the preceding day and asked to fast. The control group also all had prior successful obstetric outcomes without a history of more than one miscarriage, stillbirth, intra-uterine growth restriction, pre-eclampsia or pre-term labour.

All subjects were provided with written instructions outlining preparatory measures (appendix 2.3), including the avoidance of medications such as aspirin or non-steroidal anti-inflammatories for a minimum period of 14 days prior to the platelet assay, the avoidance of alcohol, tobacco and vigorous exercise in the preceding 24 hours and all were fasting. None of the recruits were taking hormonal treatments such as oral contraception.

4.2.1.2 Details recorded

Details recorded included age (date of birth), ethnicity, parity (if previous livebirth), number of previous miscarriages, progesterone level and platelet count at time of platelet function test, and results of routine investigations. As previously specified these investigations included full blood count (blood sugar level and thyroid function tests if additional risk factors were present), antiphospholipid antibodies (LAC and ACA), parental karyotypes and pelvic ultrasound and/or hysterosalpingogram and hysteroscopy.

4.2.1.3 Timing of phlebotomy

The assay was performed at least 12 weeks since the last miscarriage. As the majority of the RM women were actively trying to conceive they contacted me on

day 1 of their menstrual cycle to arrange the platelet function test. This timing also ensured low follicular progesterone levels. Controlling the timing of the platelet assay also allowed reduction of assay variability by asking the patients to fast from midnight and to avoid caffeine, smoking and vigorous exercise the preceding 24 hours. Any anti-platelet drugs such as aspirin or non-steroidal anti-inflammatories (NSAIDs) were stopped two weeks prior to the test. These requirements were outlined for recruited patients in a pre-platelet assay information sheet (appendix 2.3). Regarding the control group, the majority of women were fasting for minor procedures. The women attending the family planning clinic were contacted the preceding day and asked to fast. As previously stated only those in the first half of the menstrual cycle were recruited.

Phlebotomy was performed after the subjects rested for 10mins. All samples were obtained before 10am to minimise variation due to circadian rhythm.

4.2.2 Experimental protocols and reagents

The complete description of the assay steps which I undertook including buffer constituents and preparation, agonist dilution and plate preparation are outlined in appendix 1 entitled 'Standard Operating Procedure (SOP) for the Microtitre Platelet Aggregation Assay'.

4.2.2.1 Phlebotomy technique and Blood Sample Preparation

I personally drew blood from all the recruited subjects. This involved collecting venous blood from the antecubital fossa through a 19-gauge butterfly needle. The first 5 ml was sent for full blood count and hormone profile. A further 27mls was collected into a syringe containing 3mls of 3.2 % sodium citrate at room

temperature, making 30mls total. All samples were obtained uncuffed to allow blood flow slowly and freely to avoid shear stresses on the platelets, as this can activate them and cause aggregation. I gently rocked the syringe back and forth for about 5-10 times to ensure adequate mixing of the citrate and the blood.

4.2.2.2 Platelet Rich Plasma Preparation

The first step involved centrifuging the blood sample. I transferred the blood in 5ml aliquots into 6 plastic test-tubes ensuring that each tube had equal measures of blood. This was essential to ensure the centrifuge was balanced and spun without agitation. I then spun the blood at 150g for 10 minutes. I aspirated the Platelet-Rich Plasma (PRP)) from the supernatant and placed it in a reagent reservoir. 11mls was the minimum required for full testing. Using a multi-channel pipette, I dispensed the PRP across wells in a 96-well plate (black isoplate ® with clear flat-bottomed wells, PerkinElmer) containing different concentrations of the agonists. Platelet-poor plasma (PPP) was prepared by centrifuging PRP for 1 minute in a microfuge. I then added 180 μL PPP to the wells of the control column using reverse pipetting. The completed plate was quickly placed in the Wallac plate-reader as aggregation begins immediately once PRP has been added to the first well.

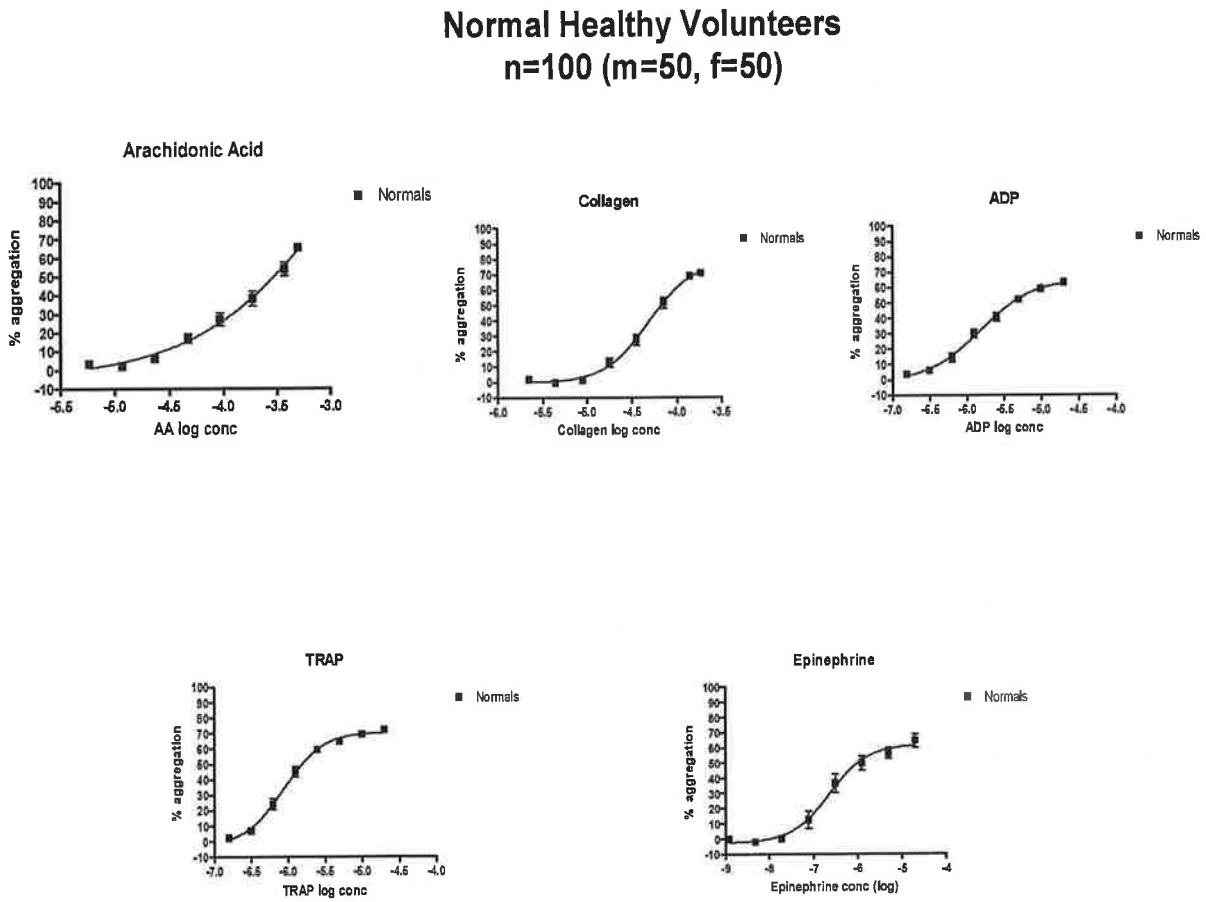
4.2.2.3 Reagents

Lysophilised arachidonic acid (AA), collagen (soluble calf skin) (Coll), adenosine-5 diphosphate (ADP) and epinephrine (EPI) were obtained from Bio/Data (Horsham, PA, USA) and were reconstituted with deionised water to give final stock concentrations of 5mg mL^{-1} AA, 0.19mg mL^{-1} Coll, 2 $x10^{-4}$ M ADP, and 1 x 10^{-4} M EPI. Epinephrine was further diluted to give a stock concentration of 200μM.

Thrombin receptor activating protein (TRAP) (200μM) was obtained from the Centre for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, RCSI, Dublin.

4.2.2.4 Platelet Aggregation

Light transmission was measured using a 572nm filter at 0, 3, 6, 9, 12, 15 and 18 mins during which the plate was constantly rotated at 1000 r.p.m. through a 0.1mm orbit between measurements. The 96-well plate was then read using a Victor 3 Multilabel plate reader (Perkin Elmer,Wellesley, MA). Light absorbance values were normalised based on PRP and PPP control absorbance values, which represented 0 and 100% aggregation. Using this, the percentage aggregation response for each concentration of each agonist was calculated using a conversion programme generated by Dr. Tedesco. These values are plotted against the log values of the agonist concentrations using Graphprism software®. The expected normal dose-response curves formulated for each agonist are shown below (in figures 3-7). These curves were generated by Peace et al during the development of the platelet function assay (121,124).



Figures 3, 4, 5, 6, 7. Normal dose response curves. The x-axis shows the concentrations of arachidonic acid, collagen, ADP, epinephrine and TRAP respectively, tested in platelet aggregation studies in 100 healthy volunteers. The y-axis shows platelet aggregation. Each data point represents mean aggregation (%) to each concentration.

To further analyse the aggregatory responses with respect to different agonist concentrations and time points I used graphprism® to calculate the EC_{50} results. The term **half maximal effective concentration** (EC_{50}) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. This is shown in figure 8. To do this I generated a dose-response curve per patient per agonist per time-point.

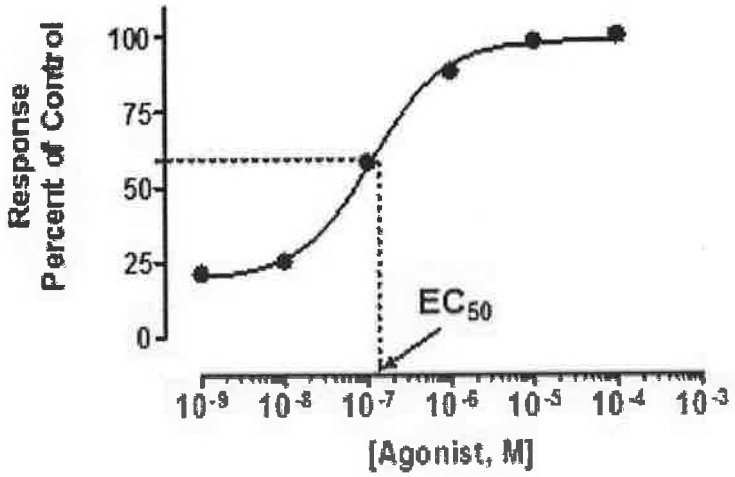


Figure 8. Explanation of EC_{50}

4.3 Statistical analysis

A two-way Anova was used to compare the maximum aggregation response to the different agonists for the RM group and Control group. The mean of the EC_{50} results were compared using student t-tests. Chi-squared tests were also performed to determine the difference between groups with respect to levels of platelet aggregation to each agonist at successive time points. A p value < 0.05 was used for statistical significance. Of note, analysis was also performed to adjust for multiple comparisons, using the Bonferroni method.

4.4 Results

4.4.1 Demographics cases / controls

Regarding the RM group they all met the definition of RM with at least 3 consecutive miscarriages. The group was limited to those who had experienced first trimester miscarriages and with a single partner. The entire RM group underwent routine RM investigations including full blood count (blood sugar level and thyroid function tests if additional risk factors were present), coagulation profile, antiphospholipid antibodies (LAC and ACA), parental karyotypes and pelvic ultrasound and/or hysterosalpingogram and hysteroscopy, all with normal results, and therefore were classified as 'unexplained RM'.

The control group was comprised of ethnicity and age-matched women with no known medical disorders, or personal or family history of venous thromboembolism. The control group all had prior successful obstetric outcomes without a history of more than one miscarriage, stillbirth, IUGR, PET or PTL. Again, as previously stated, the control group patients were not taking any hormonal oral contraceptives. The sample size of 30 cases and 30 controls was calculated to achieve a 5% level of significance and 80% power to detect at least a 20% difference between the two groups.Table 5 shows the demographic details of the RM and Control groups.

Table 5: Baseline characteristics of RM and control groups

	RM N=30	Controls N=30
Mean age (range)	35.0yrs (25-46)	36.6yrs (28-49) p=0.29
Ethnic origin	86.7% Caucasian (2 Asian, 2 African)	86.7% Caucasian (2 Asian, 2 African)
Number of Miscarriages	3 - 12	0 - 1
Previous Livebirth	54%	100%
Progesterone level (range)	3.95nmol/l (0.4-10.4)	4.81nmol/l (0.2-6.5) p=0.70

4.4.2 Comparison of two groups: different agonists at different concentrations

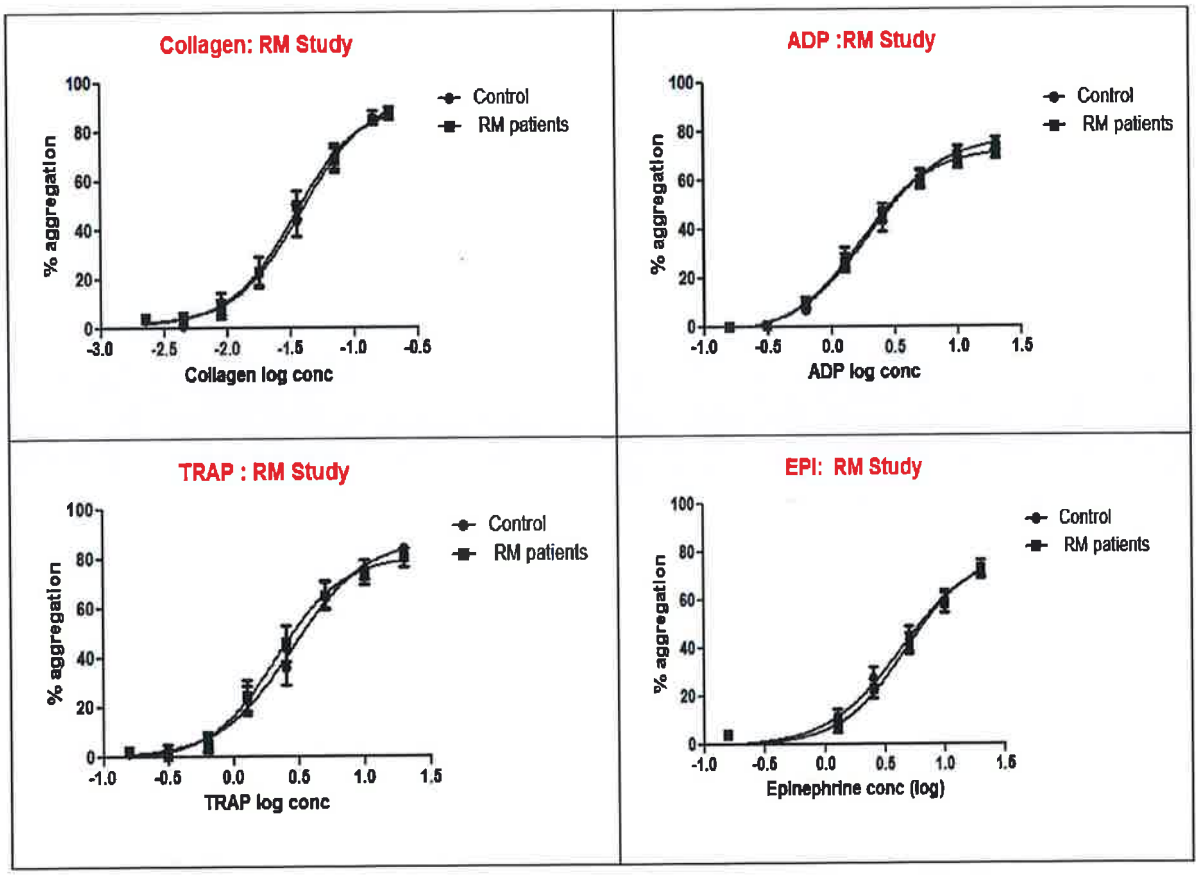
A dose-response curve was plotted for each agonist with respect to maximum aggregation achieved for each dose of agonist in order to compare the two groups. The graphs generated (figures 9 to 13) show no obvious difference between the RM patients and controls. This was confirmed with an Anova analysis. However they do highlight how tightly matched the two groups are and that the curve patterns are in keeping with the normal curves. This reinforces the accuracy of the platelet assay technique used in this study. However, there was an obvious difference evident with respect to AA as shown in figure 14.

A dose-response curve was performed for each individual patient in both groups for each agonist in order to calculate the EC_{50} for each subject for each agonist. Table 6 shows the mean EC_{50} results calculated from the maximum aggregation curves. The

lower mean EC_{50} result in the RM group for AA was statistically significant ($p< 0.0099$).

The AA response curves were then further analysed in terms of time points- T=3, T=9, T=15 and T=18 mins. Table 7 shows that the significant difference between the groups exists at the aggregatory response at T=18mins (test completion), ($p< 0.0045$).

Chi-square tests of association were also used for doses at which different percentages of aggregation achieved. A significant difference ($p < 0.0209$) was found between the groups in the dose at which 20% of the maximum aggregation was achieved (Table 8). For dose 0.18mg/ml AA 63% of patients versus 30% control subjects had reached 20% aggregation response.



Figures 9-13: Dose-response curves with agonists Coll, ADP, TRAP and Epi.

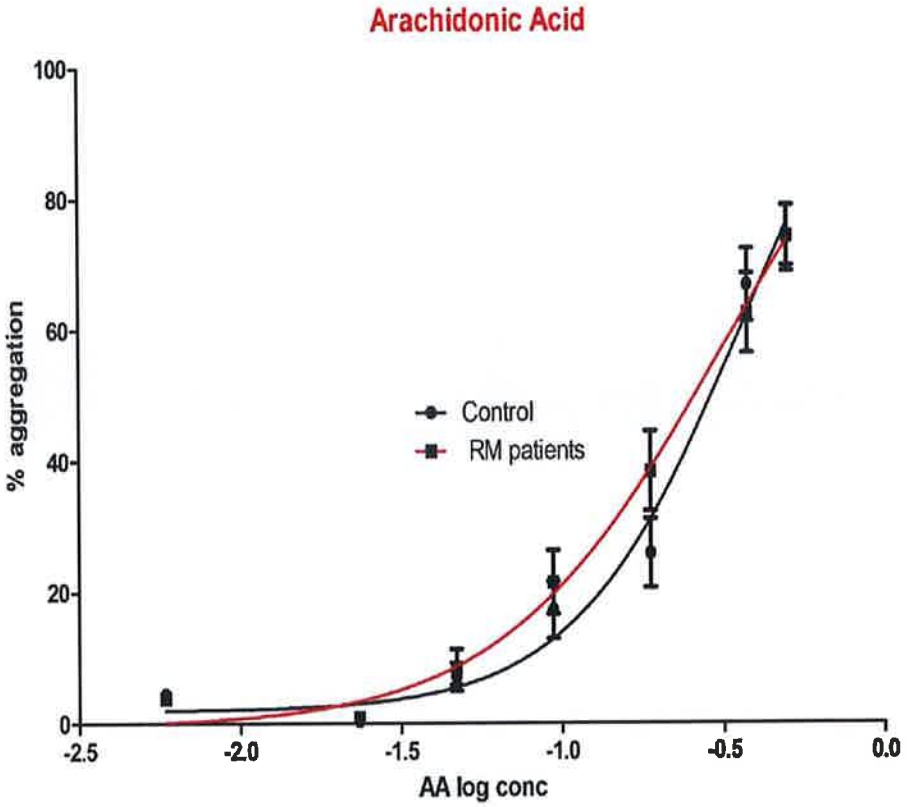


Figure 14: Dose-response curve showing maximum aggregation response to AA

Table 6: Comparison of the mean half-maximal effective concentration values for maximum aggregation for each group for each agonist.

Agonist	RM		CT		P value
	N	EC_{50}	N	EC_{50}	
AA	22	0.230	20	0.153	0.0099*
ADP	28	2.64	30	3.55	0.48
COL	28	0.005	25	0.004	0.90
EPI	26	2.56	28	3.35	0.62
TRAP	21	2.77	24	2.60	0.74

N indicates the number of subjects per agonist which allowed EC_{50} to be calculated from the dose-response curve generated.

Table 7: Comparison of the mean half-maximal effective concentration values for maximum aggregation for each group at test completion (T=18mins)

Time (mins)	RM		CT		P value
	N	EC_{50}	N	EC_{50}	
3	7	0.175	3	0.272	0.31
9	16	0.245	16	0.253	0.83
15	20	0.199	22	0.172	0.28
18	21	0.232	21	0.148	0.0045*

Table 8: Cross-tabulation for AA at time-point 18 giving frequencies of the dose at which 20% of maximum aggregation was achieved for both groups.

For example, at dose 0.09mg/ml 10 of the 27 RM subjects had reached 20% aggregation compared to only 5 of the 27 Control subjects. The remaining 3 subjects in each group did not achieve 20% maximum aggregation.

Dose mg/ml	RM	Control
0.01	0	1
0.05	4	2
0.1	6	3
0.2	7	2
0.4	5	17
0.5000	5	2
Total	27	27

4.4.3 Summary of results

Patients with a history of unexplained recurrent first trimester pregnancy loss demonstrate significantly increased platelet reactivity in the presence of AA. This has been shown with the significantly lower EC_{50} results generated for overall maximal response to AA indicating that the RM group need lower agonist dosage to achieve half maximal response. This is most significant at test completion

(T=18mins). The significant difference between the groups in their aggregatory response is also demonstrated with the increased frequency to achieve 20% response. Overall this indicates a greater aggregation response at submaximal doses of Arachidonic Acid. The dose-response curves and analysis showed no difference between the groups for the other agonists- ADP, EPI, Coll and TRAP. However the low variability of results plotted on these curves highlight that the groups have been tightly matched and follow the normalised curve patterns.

4.5 Discussion

The results of this study demonstrate that patients with a history of unexplained recurrent first trimester pregnancy loss have greater platelet aggregation to submaximal doses of Arachidonic Acid compared to an appropriate control group. The precise clinical correlation of these results is not clear but suggests a unique response specific to the cyclooxygenase pathway. These results agree with the accumulating evidence that some cases of RM are the result of exaggerated thrombotic response. Also the heightened response to AA in particular is interesting due to the therapeutic role of aspirin in RM management.

Platelet aggregation is a highly complex sequence of events involving multiple receptors and signalling pathways (125). Most devices or assays use either a single agonist or a single concentration of agonist which while useful, will not identify changes in response to submaximal concentrations of different agonists. Also maximal platelet aggregation is routinely measured using high concentrations of agonist. However recent evidence suggests that using lower (submaximal) concentrations of agonist may be a better way of measuring platelet aggregation as

this approach identifies subtle changes in platelet function which would otherwise be obscured by higher concentrations of agonist (127). By using multiple agonists at different concentrations this new assay more accurately reflects the environment in which platelets aggregate *in vivo*. To minimise variability enviromental (124) and hormonal (139,140) factors that may affect platelet function were taken into consideration. All the assays were performed before 10am and arranged according to the timing of the menstrual cycle to ensure low follicular progesterone levels. In addition to these efforts, the fact that subject recruitment, phlebotomy and assay performance was performed by a single operator further contributed to reduction in variability factors.

AA causes platelet aggregation through the thromboxane pathway. In vitro addition of AA to normal platelet rich plasma results in a burst of oxygen consumption, thromboxane formation and platelet aggregation (142). However, in the presence of aspirin or aspirin-containing compounds these reactions are absent (143). A number of units tend to extrapolate the management of APS to those with unexplained recurrent miscarriage using aspirin or a combination of aspirin and heparin while rightly or wrongly assuming an undiagnosed underlying thrombotic risk. The treatment of couples with idiopathic RM has traditionally been based on anecdotal evidence, personal bias and the results of small uncontrolled studies. Dolitzy *et al* aimed to address this issue recently and conducted a multicentred randomised prospective trial comparing aspririn and enoxaparin (low molecular weight heparin) in unexplained RM women. They found good outcomes in both groups with respect to livebirth rates and late pregnancy complications and recommended the use of either treatment modality in idiopathic RM citing the possibility of an unknown, undiagnosed prothrombotic condition in these women (144). The results of our study

lend further support to re-evaluating the benefit of low dose aspirin in a clearly defined cohort.

Further evaluation of this is the main recommendation for future research. We propose to assess the platelet aggregatory response in the RM group at different stages of achieved pregnancy. We also plan to use this novel assay to characterise platelet function in the three trimesters of pregnancy.

5 PLATELET FUNCTION AND NORMAL PREGNANCY

5.1 Introduction

Platelet reactivity refers to the ex vivo measurement of platelet responses to various agonists such as ADP, epinephrine, thrombin, arachidonic acid (AA) or collagen providing an index of platelet functional capacity. We have demonstrated that patients with a history of unexplained recurrent first trimester pregnancy loss demonstrate significantly increased platelet reactivity in the presence of AA (145). Future proposed research is the determination of platelet function in this cohort when pregnancy is achieved. However platelet function in normal pregnancies needs to be known to allow for comparison.

There is significant interest in the association between increased platelet reactivity and pregnancies complicated by hypertensive disorders such as pre- eclampsia (146-149). Emerging studies have also shown an association with gestational diabetes (150) and preterm labour (151). However, studies that have focussed on healthy uncomplicated pregnancies have shown conflicting results, such that platelet function in normal pregnancy remains poorly understood.

Various platelet function tests have been employed including use of platelet aggregometry, (149,152,153), shear-induced platelet activation (154,155), and flow cyometry (156-158) using various monoclonal antibodies. Only two of these cited studies sought to evaluate platelet function throughout the three trimesters of pregnancy (153,156).

Star *et al* used flow cytometry to evaluate platelet response to the agonists thrombin (5U/ml and 0.08U/ml) and U-46619 (a thomboxane A_2 analogue at doses 2μmol/l and 0.25 μmol/l) in 54 uncomplicated pregnancies divided into the three trimesters

(156). Monoclonal antibodies were directed against platelet membrane glcoproteins: 7E3 (fibrinogen receptor GPIIb/IIIa), S12 (α granule marker P-selectin) and 6D1 (von Willebrand factor receptor GPIb). They demonstrated that platelet reactivity was significantly altered in third trimester. They found that platelets from third trimester subjects bound significantly less 7E3 than platelets of controls or of first and second trimester subjects after stimulation with high dose thrombin ($p<0.05$ for all comparisons). Down-regulation of 6D1 on platelets after stimulation with high dose U-46619 was also significantly greater in third trimester subjects compared to the other groups ($p<0.05$).

In comparison, Norris *et al* who also assessed platelet function at different stages of normal pregnancy found that platelet response was significantly increased in the third trimester (153). They used a platelet counting technique to determine whole blood aggregation response to ADP (0.5μM), platelet aggregating factor (PAF) (0.05μM), collagen (0.5μg/ml), adrenaline (1.0μM), and AA (0.2mM) in twenty healthy pregnancies. The response to ADP, collagen, adrenaline and AA was found to be significantly increased in the third trimester with collagen and AA invoking the strongest platelet aggregation from as early as twenty weeks gestation.

Platelet function is highly complex involving multiple receptors and signalling pathways (119) but this is not reflected by most platelet function tests. The conflicting results from these studies may be as a result of the testing techniques employed or due to the use of either limited agonists or limited agonist concentrations. The new platelet function assay developed by the Cardiovascular Biology Group at the Royal College of Surgeons in Ireland (RCSI) is based on a modification of light transmission aggregometry (LTA) which is considered by most to be the gold standard of platelet function testing. This assay addresses the

complexity of platelet reactivity as it involves the use of a 96-well plate which allows for accurate, rapid measurement of platelet aggregation in response to multiple platelet agonists at multiple concentrations (120-124). This novel assay has provided the opportunity to characterise platelet function throughout the three trimesters of normal healthy pregnancy, which was the objective of this phase of the study.

5.2 Study Design

As with the previous study, ethical approval was obtained from the Rotunda Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Informed written consent was obtained from all patients prior to phlebotomy (appendices 2.4, 2.5). The phlebotomy technique was in adherence with the previously described method as was performance of the platelet assay, both of which I personally performed. The agonist TRAP was unavailable at the time of study performance and therefore omitted from the assay and analysis.

5.2.1 Patient population and design

Pregnant subjects were recruited according to their gestation. First trimester patients were recruited at time of booking visit or when attending for first trimester screening. Both second and third trimester patients were recruited from the low-risk antenatal clinic at the Rotunda Hospital. Only singleton pregnancies were included and all were undergoing routine antenatal care at the Rotunda Hospital Dublin. The

control subjects were comprised of age and ethnicity matched non-pregnant women who were not taking any medications including hormonal treatments such as oral contraception. Specific exclusion criteria for all groups included a history of thrombosis or vascular disorders, long term use of medication or chronic illness (such as diabetes mellitus, essential hypertension or renal disease), and any known platelet disorder including gestational or idiopathic thrombocytopenia. Smokers or those with reported previous illicit drug use were also excluded. All subjects were asked to abstain from medications such as aspirin or non-steroidal anti-inflammatories for a minimum period of 14 days prior to the platelet assay. The control patients and parous pregnant patients all had prior successful obstetric outcomes without a history of more than one miscarriage, stillbirth, intra-uterine growth restriction, pre-eclampsia or pre-term labour. Patient records were reviewed in the postnatal period to confirm that all pregnancies remained healthy without the aforementioned complications.

The sample size of 40 (10 controls and 10 cases per trimester) was calculated based on a 5% level of significance and 80% power to detect a 26% difference between the two groups (controls and single trimester cases).

5.3 Statistical analysis

Graph prism software was used to perform dose-response curves for each agonist and resulting maximum aggregation for each dose. This was performed for each agonist and each group. The graphs generated allowed comparison between each trimester and the control group and also between the trimesters. EC_{50} values and

maximum aggregation responses for each study group for each agonist were also calculated and analysed.

The non-parametric Kruskal-Wallis test was used to study differences between trimesters, for each of the agonists. It is an ANOVA-like test for differences between the study groups including differences which may not represent trends. Median values of the comparison criteria need to be assessed in combination with the Kruskal-Wallis test.

5.4 Results

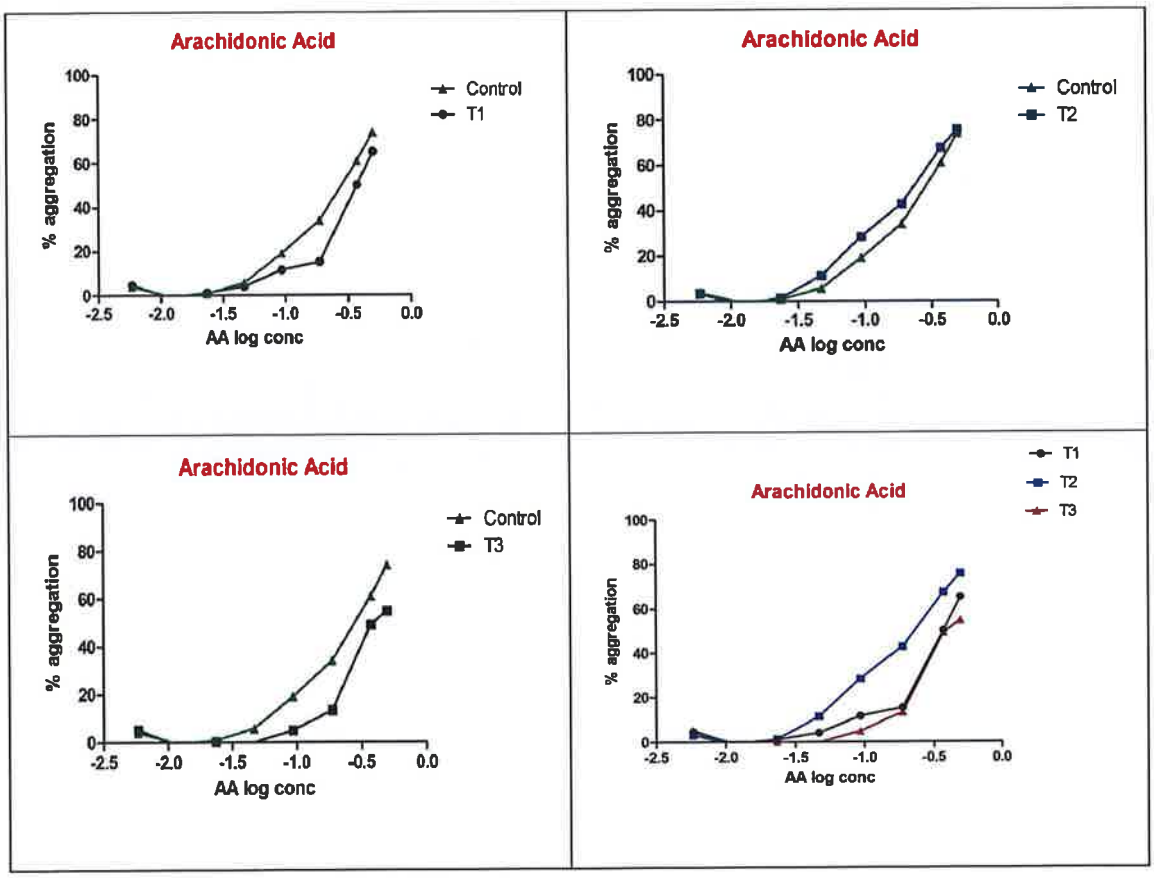
In total there were ten women recruited in each trimester and ten healthy nonpregnant controls. Each pregnancy was evaluated postnatally with chart review to ensure that the pregnancy remained without complication. One patient was omitted from the first trimester group due to intra-uterine death of the fetus related to Trisomy 21. One patient was omitted from the second trimester group due to the development of severe pregnancy induced hypertension and subsequent placental abruption. One patient was also omitted from the third trimester group due to development of late onset pre-eclampsia. This left nine patients in each group for analysis.

Table 9. Baseline characteristics of each group in pregnancy study

Mean	Trimester 1 N=9	Trimester 2 N=9	Trimester 3 N=9	Control N=10
Age (yrs) p=0.25	34.44 (29-37)	30.67 (19-43)	32.55 (28-43)	35.22 (28-43)
Gestation (weeks)	12.11 (11-13)	21.88 (20-26)	36.11 (31-40)	
Platelet Count (10^{12}/l) p=0.88	252.78	265.33	253	264.89

The following figures show the dose-response curves generated for each agonist for each group. The graphs plotted compare each trimester to the control group as well as to each other. EC_{50} values and maximum aggregation achieved were then calculated to allow statistical comparison. The mean values and statistical analysis p values are outlined in tables 10 and 11 respectively.

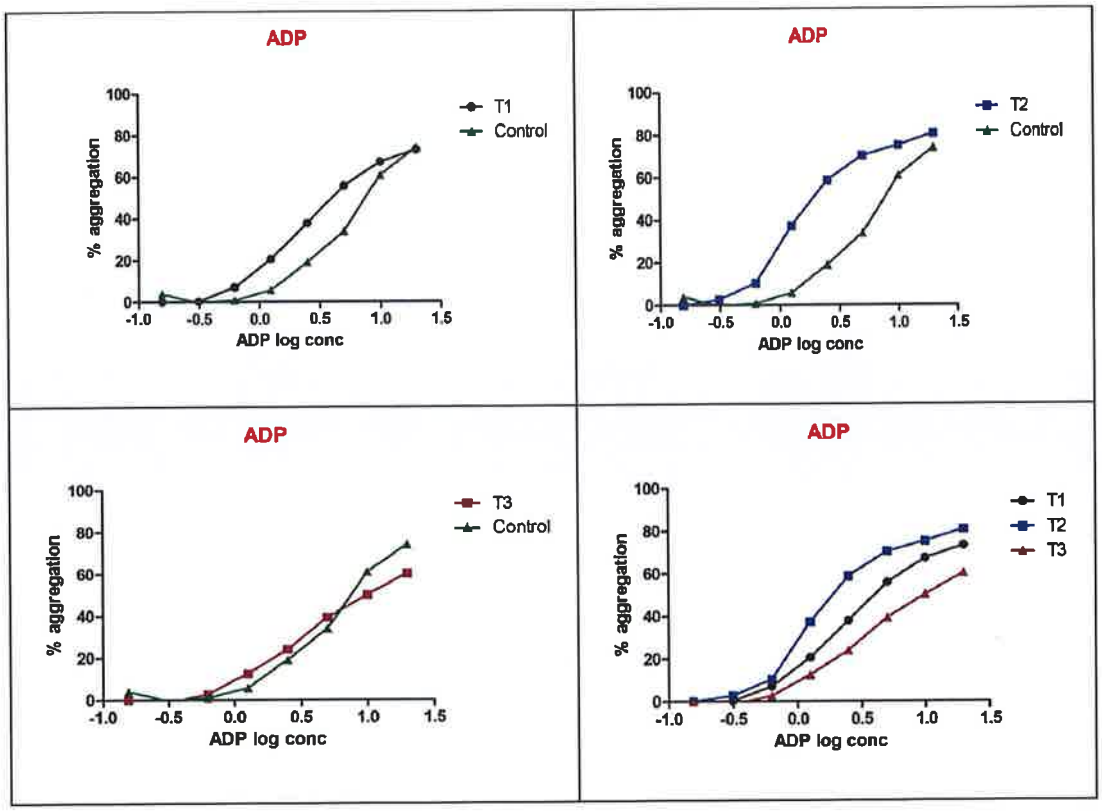
5.4.1 AA-induced platelet aggregation



Figures 15-18: Arachidonic Acid dose response curves

These graphs show that the control non-pregnant group had greater aggregation response overall to the AA doses when compared to each trimester. When comparing the three trimesters the aggregation response in the second trimester was greater than the first trimester which in turn was greater than the response in the third trimester.

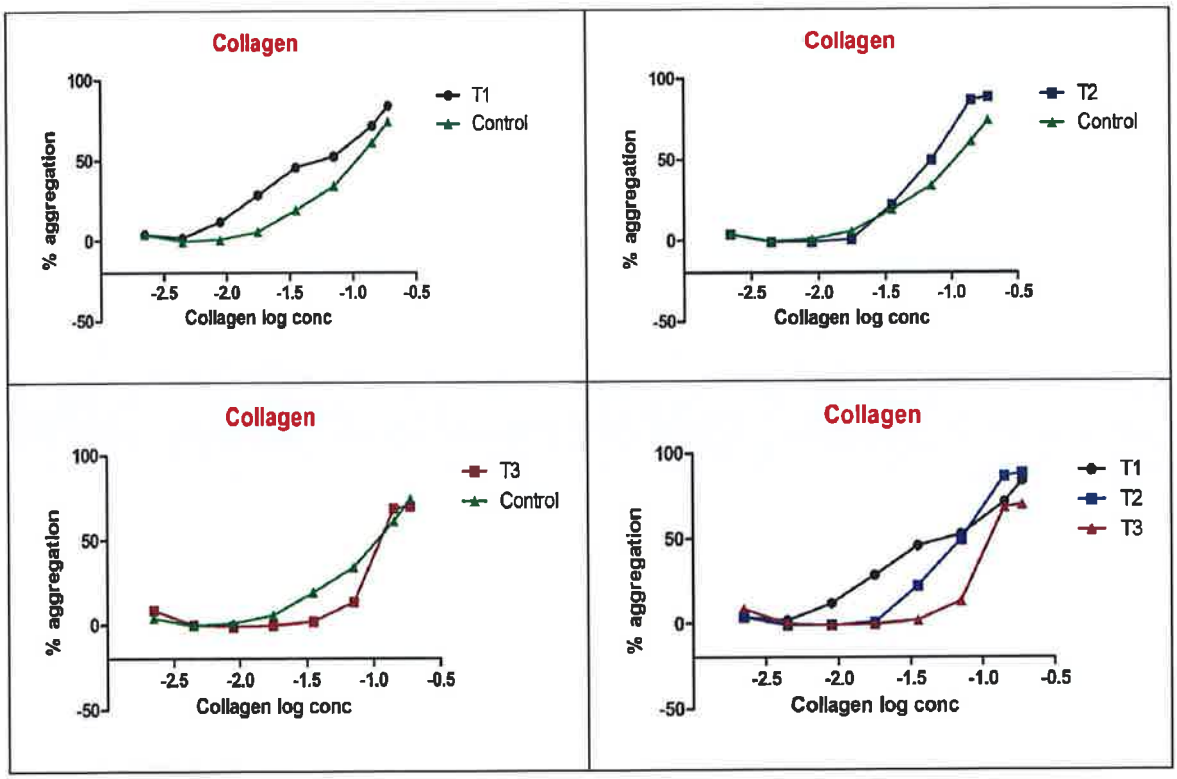
5.4.2 ADP-induced platelet aggregation



Figures 19-23: Adenosine Diphosphate dose response curves

These graphs show that both Trimester 1 and 2 had increased aggregation response to ADP doses when compared to the control group. The response of the control group and Trimester 3 were similar. When comparing the three trimesters the aggregation response in the second trimester was greater than the first trimester which in turn was greater than the response in the third trimester.

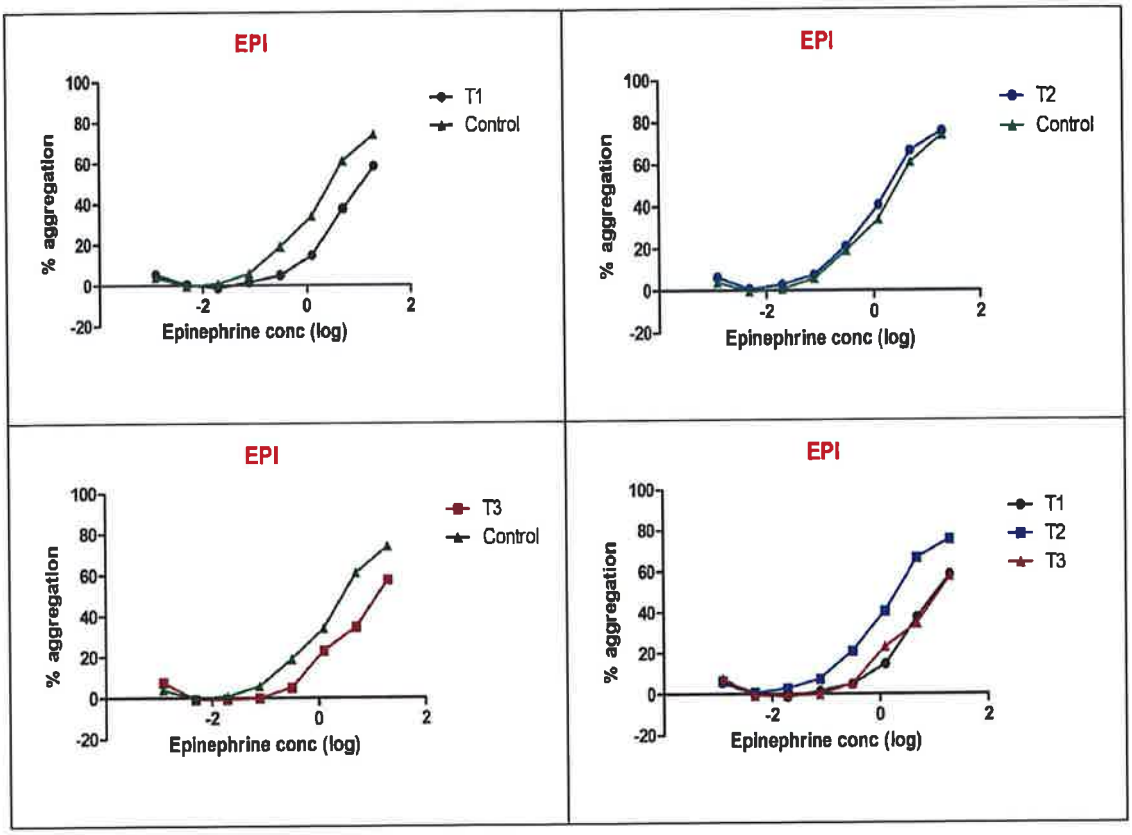
5.4.3 Collagen- induced platelet aggregation



Figures 24-28: Collagen dose response curves

These graphs show that again Trimester 1 and 2 had increased aggregation response collagen when compared to the control group. Trimester 3 was actually less than the control group. When comparing the three trimesters the aggregation response in the first trimester was greater than the second trimester which in turn was greater than the response in the third trimester .

5.4.4 Epinephrine- induced platelet aggregation



Figures 29-33: Epinephrine dose response curves

These graphs show that the control group had increased aggregation response to Epinephrine as compared to both Trimester 1 and 3 however it was very similar to Trimester 2. When comparing the three trimesters the aggregation response in the second trimester was greater than the similar response in the first and third trimester.

5.4.5 Statistical analysis of differences between trimesters

The basic interpretations of the graphs show that the second trimester of pregnancy demonstrated the greatest platelet reactivity with a marked reduction in Trimester 3. The pregnant patients demonstrated greater aggregation response to ADP and collagen than the non-pregnant controls; however this was the opposite for AA and Epi. Median Values of Individual Dose-Response EC_{50} values and maximum aggregation achieved were calculated (Table 10).

With respect to analysis of EC_{50} values there was statistical significance for ADP and EPI. The differences relate to Trimester 2 being significantly less than Trimester 1 and 3. With respect to maximum aggregation values the response to collagen was significantly decreased in the third trimester with a 24% drop in maximal aggregation (p-value=0.0054). There was a similarly large drop of 18% in maximal aggregation response to ADP in the third trimester however this only reached borderline significance (p-value=0.0686).

Table 10: Median Values of Individual Dose-Response EC_{50} values and maximum aggregation achieved

	Trimester	Agonist			
		AA	Coll	ADP	EPI
EC_{50}	1	0.23	0.03	3.21	6.59
	2	0.12	0.06	1.34	1.00
	3	0.22	0.08	4.93	7.95
Max aggregation	1	82.80	89.20	79.70	62.80
	2	82.50	91.20	82.40	81.20
	3	64.30	67.30	64.20	56.60

Table 11: Statistical Significance of Difference in Trimester results.

(P-values from Kruskal-Wallis Test)

	Agonist			
	AA	Coll	ADP	EPI
EC_{50}	0.1996	0.1478	**0.0119**	**0.0235**
Max aggregation	0.3171	**0.0054**	**0.0686**	0.2579

5.5 Discussion

The most obvious and interesting observation was the marked reduction in platelet reactivity in the third trimester group as compared to the rest of pregnancy and non-pregnant subjects which was in agreement with the findings by Star *et al* (156). Our study also revealed that the first trimester showed less platelet reactivity than expected. Normal platelet counts and the absence of aspirin or NSAID usage was confirmed in these pregnancy groups. It is believed that several cytokines or growth factors are increased in normal pregnancy, which may contribute to altered platelet function (159). These results are interesting when considering the findings of a study performed by Bar *et al.* who explored the effects of cytokines on platelets according to pregnancy timing (160). They assessed the effects of cytokines IL-1β and tumour necrosis factor α (TNF-α) on ADP-induced platelet aggregation measured by LTA and platelet P-selectin expression by flow cytometry in non-pregnant patients and at different stages of normal pregnancy. IL-1β and TNF-α had no effect on platelet aggregation and P-selectin expression in early pregnancy. In comparison, both cytokines had a stimulatory effect in non-pregnant effect women ($p<0.05$) and an inhibitory effect in the third trimester of pregnancy ($p<0.05$).

For each agonist used in our study, the maximum aggregation response occurred in the second trimester. This finding was also demonstrated by Hayashi *et al* while evaluating platelet function in different stages of pregnancies complicated by pre-eclampsia compared to normal pregnancies (161). Using LTA to measure platelet aggregation induced by 5 μM ADP and 5 μg/ml Collagen they demonstrated that the aggregatory response increased significantly in gestational weeks 20-30 and decreased in the gestational week 35 in normal pregnant women. They proposed that

'*perhaps the platelets that exhibit hyperfunction in aggregation earlier in pregnancy may have been gradually consumed towards the term of pregnancy*'.

The pregnant state must achieve a fine balance between haemorrhage and excessive thrombosis. The results of our study may simply reflect the adaptive nature of platelets in normal pregnancy. Less reactivity may occur in the first trimester to allow successful implantation at a time when the dramatic changes in the fetomaternal interface may expose maternal platelets to significant pro-thrombotic factors. The third trimester 'under-reactivity' may be an adaptive measure in accordance with Virchows triad with reduction in prothrombotic risk in response to increased vascular wall injury and circulatory stasis.

The cross-section design of this study is inferior to a longitudinal design, in which the same subjects are tested repeatedly and each serves as her own control. Another limitation is the relatively small patient number as the element of chance must be taken into consideration with our results. However, by using this novel assay and clearly defining gestational timing we have highlighted the evolving nature of platelet reactivity in pregnancy. A further large study is planned to characterize platelet function longitudinally in normal pregnancy which will then allow more accurate comparison to pregnancies affected not only by RM but other complications such as PET and IUGR.

6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The platelet is a highly complex cell involving multiple receptors and signaling pathways. Platelets have a central role in cardiovascular thrombosis. As a result, therapies targeting the key pathways of platelet activation have established an important role in cardiology. Aspirin which targets the arachidonic pathway has been the foundation of antiplatelet therapy for over fifty years. There is no debate that aspirin use attenuates the risks of myocardial infarction, stroke, and vascular related deaths in patients with cardiovascular disease. Morbidity and mortality has been further reduced with the addition of ADP antagonist clopidogrel in dual antiplatelet therapy (DAPT) (163). Recent research and development has been driven by the emergence of the phenomenon 'aspirin resistance' (164). This has involved further evaluation of the role of platelet function tests in monitoring antiplatelet therapy and predicting clinical outcomes.

In contrast, little is known about platelet function in pregnancy and the potential of antiplatelet therapy in obstetric practice. The lack of understanding regarding the role of platelets in complicated pregnancies is evident in recurrent miscarriage (RM). Platelet function has not been fully evaluated in this condition and the widespread empiric use of aspirin for treatment of women affected with RM is a controversial issue.

RM is a distressing problem that is increasing in prevalence. Beyond the pregnancy risks there are also implications for the health of these women both during and beyond their reproductive years. Despite investigation a significant proportion of cases of RM remain unexplained. Unknown cause adds further to the distress associated with this condition. The historical hypothesis behind the occurrence of

recurrent miscarriage has been that affected women are already in a prothrombotic state before pregnancy begins. Despite this theory and the controversy surrounding aspirin usage in this population there are a paucity of studies which evaluate platelet function in this cohort.

Platelet reactivity refers to the ex vivo measurement of platelet responses to various agonists such as arachidonic acid (AA), ADP, collagen, thrombin and epinephrine providing an index of platelet functional capacity. The recent development of a novel platelet assay developed in RCSI which optimally reflects the environment in which platelets react *in vivo,* provided an opportunity to critically evaluate platelet function in idiopathic RM. Identification of a tightly controlled cohort was paramount in undertaking this study. Strict inclusion criteria mandated that all subjects met the correct RM definition, had normal prior investigations and a single partner. On the other hand patients were excluded if there was any history of medical or clotting disorders and they were tightly controlled with respect to their menstrual cycle and other platelet function variability factors.

Patients with a history of unexplained recurrent first trimester pregnancy loss demonstrated significantly increased platelet reactivity in the presence of AA. This is shown with the significantly lower EC_{50} results generated for overall maximal response to AA indicating that the RM group need lower agonist dosage to achieve half maximal response. This is most significant at test completion (T=18mins). The significant difference between the groups in their aggregatory response is also demonstrated with the increased frequency to achieve 20% response. Overall this indicates a greater aggregation response at submaximal doses of AA.

The heightened response to AA in particular is interesting due to controversy surrounding the therapeutic role of aspirin in RM management. Numerous clinical

studies have attempted to address the role of aspirin in RM however they have been limited by patient numbers, randomization and a clear definition of RM. The results of our study lend further support to aspirin usage however it should be limited to a clearly defined cohort. Future proposed research is the determination of platelet function in this cohort when pregnancy is achieved however platelet function in normal pregnancies needs to be known to allow comparison.

Following a detailed literature review of platelet function in pregnancy it became clear that, despite the presence of a number of studies who evaluated platelet reactivity with different modalities, platelet function in pregnancy has not been efficiently evaluated. The conflicting results from previous studies may be as a result of the testing techniques employed, the use of either limited agonists or limited agonist concentrations and the lack of clear definition with respect to pregnancy timing. For this reason the novel platelet assay provided an opportunity to more clearly define platelet function in the three trimesters of normal healthy pregnancy. The study yielded interesting results including the peak of aggregation occurring in the second trimester rather than increasing towards term as previously believed. The results highlighted the evolving nature of platelet reactivity and the significant influence of gestational timing.

With respect to the aggregatory response to AA, the non-pregnant control group was more reactive than the pregnant groups. The evaluation of this phenomenon will be very interesting to evaluate in RM pregnancies considering the findings in the non-pregnant RM patients. Indeed, the comparison of our normal pregnancy findings to other complications of pregnancy may help guide obstetric management in the future. The limited cross-sectional design of this study is acknowledged and a longitudinal study with greater numbers would be optimal. A further large study is

planned to characterize platelet function longitudinally in normal pregnancy which will then allow more accurate comparison to pregnancies affected not only by RM but other complications such as PET and IUGR.

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APPENDIX 1:

Standard Operating Procedure (SOP) for the Microtitre Platelet Aggregation Assay

(Please read carefully before starting)

1) **Set-up of plate reader: (these instructions apply when using a Victor 3TM Multilabel plate reader (Perkin Elmer, Wellesley, MA, USA) with Wallac software. However these settings can be chosen in most plate readers with sufficient specifications.**

- First switch on the Wallac (large green switch at back).
- Then switch on computer (always do this after switching on the Wallac or else the computer can fail to detect the Wallac machine).
- Choose the pre-set protocol which should have standard settings that become the default settings. These settings are as follows
    - Temperature – 37°C
    - Wavelength – Absorbance @ 572nm
    - Shake Pattern – OrbitaL
    - Shaking diameter – 1mm
    - Protocol duration – 18 minutes divided into 5 standard timepoints (0, 3, 9, 15 and 18) minutes

- Double click the Wallac Manager Program as found on the desktop. This will take a bit of time to initiate itself and you will hear the Wallac make its initiation noises.
- Click on the "Temperature" tab.
- Set the heating plate to "ON"
- Set the temperature to 37°C
- Click on the "Apply" button
- After a minute, confirm this has worked by looking at the temperature graph. It will display the current Wallac temperature (blue line) and the temperature it is aiming for (horizontal dotted line). The blue line grows slowly and will be seen to be rising towards the dotted horizontal line as the heating plate warms up.
- Perform any calibrations (e.g. pH meter) during the warm-up period.

2) Buffer preparation

Preparing the 50mls of JNL:

- JNL is an artificial solution of salts, dextrose and pH buffers, used to match the physiological environment for platelets.
- Remove JNL ingredients (JNL A, B, D and E and the ACD) from fridge and check them for cloudiness, impurities and expiry date (they each last 4 weeks if stored in fridge). Keep them refrigerated at all times.
- Obtain a clean dry 200ml beaker, add 5mls JNL-A using a sterile pipette.

- Using a fresh sterile pipette each time, add 5mls JNL-B and 5mls JNL-D.
- Using a 1ml sterile pipette, add only 0.5mls JNL-E (i.e. 1/10th of the others).
- Add distilled water to achieve a volume of 40 - 45mls approx.
- Allow the solution to get to room temperature as this will affect the pH measurements in the next step.
- The solution made up so far will have a pH of about 8.8 (approximately). Using

  the pH meter to monitor your progress, carefully add ACD solution until the pH just reaches 7.35. Use a small 3ml plastic "transfer pipette". Note 4mls should get you very close; however add the ACD slowly; drop by drop. Stir the solution whilst doing this to ensure homogeneity.
- Add distilled water to make up to a final volume of 50mls. This can be done in a

  measuring cylinder to be more accurate.
- Unstable pH may indicate bacterial contamination of JNL ingredients
- Use it that day only: once made up, it only lasts for a few hours.

Constituents of Buffer Ingredients

	Component	FW	Amount	FC	Dilution
JNL A	60mM Dextrose	180.2	5.4g/500ml	6mM	10X
JNL B	1.3M NaCl	58.44	37.99g/500ml	130mM	10X
	90mM Na Bicarb	84.01	3.78g/500ml	9mM	10X
	100mM Na Citrate (Tribasic,dehydrate)	294.1	14.7g/500ml	10mM	10X
	100mM Tris base	121.14	6.06g/500ml	10mM	10X
	30mM KCl	74.56	1.12g/500ml	3mM	10X
JNL D	8.1mM KH_2PO_4 (monobasic anhydrate)	136.1	0.55g/500ml	0.81mM	10X
JNL E	90mM $MgCl_26H_2O$	203.3	1.83g/100mls	0.9mM	100X

ACD contains 38mM citric acid anhydrous (FW=192.12g/M, 0.73g/100ml), 75mM sodium citrate tribasic dihydrate (FW=294.1, 2.2g/100ml), 124mM dextrose (also known as D-glucose FW=180.16, 2.23g/100ml).

3) Preparing the syringe for collecting blood:

- Obtain a 50ml syringe.
- Remove the 3.2% sodium citrate solution from the fridge.

- Draw up the correct dose of 3.2% sodium citrate into the syringe, such that the volume of sodium citrate is one tenth of the final volume of blood.

  Therefore:

  - For a female patient, we need 30mls blood, so draw up 3mls Sodium Citrate.
  - For a male patient, we need 40mls blood, so draw up 4mls Sodium Citrate.

- Put the sodium citrate solution back in the fridge.
- Remove any air bubbles from the syringe so that the correct amount of blood will be drawn when the blood is collected.
- It is recommended that the syringe containing the sodium citrate is gradually warmed up to 37°C to avoid excessive platelet activation.

4) Making up fresh stock of the agonists:

This only needs to be done when you run out of agonist (Arachidonic Acid [AA], Collagen [Coll], Adenosine Di-Phosphate [ADP], Epinephrine [Epi] and Thrombin-Related Activating Peptide (TRAP) Always check expiry dates for each agonist which should be clearly indicated from the manufacturer's bottle or from the date written on the eppendorf container by the person who prepared it.

- Remove the vial containing lyophilised powdered agonist from the fridge. The exception is TRAP, which is in concentrated liquid form and is kept frozen. This agonist is stable despite thawing and re-freezing.
- Make up new ampoules using distilled water:

For AA, Coll and ADP:

- Open the glass ampoule (the metal capping first and then the grey soft rubber bung), taking care to avoid losing any of the fine powder within.
- Measure 0.5mls distilled water accurately.
- Add the water to the ampoule and replace the rubber bung.
- Shake the ampoule so that all the powder is fully dissolved (including any stuck to the rubber bung), leaving only a clear colourless fluid (Collagen is slightly cloudy). Label it with the date it was re-constituted.
    - For Coll and ADP: store in the fridge.
    - For AA: divide the re-constituted AA into 54ìL aliquots in Eppendorf 0.5ml "safe-lock" tubes, so that they can be frozen and individually used when required. One vial of freshly made AA which makes 9 eppendorfs. Store these in the freezer in an upright position. N.B. Biodata © recommend that arachidonic acid cannot be refrozen and so only use a single sample for each test.

For Epinephrine:

- Follow the steps above for ADP to make up an ampoule of fullstrength epinephrine, which is 1mM.
- Dilute this down to the assay-strength of 200ìM in a plastic 1.5ml "safe-lock" tube. This can be done by transferring 200ìL into the safe-lock tube and adding 800ìL of distilled water (i.e. a 1:4 dilution).

- Label and date. Store in the fridge.

For TRAP

- Take an ampoule of frozen full-strength TRAP from the freezer (10mM in 20ìL).
- Add 380 ìL distilled water (making a total of 400ìL at 500ìM strength). Transfer this to a 1.5ml plastic "safe-lock" tube. Add 600ìl distilled water to make a total volume of 1000ìL, at strength 200ìM, which is our final desired strength.
- It's easiest to keep the ADP, Col and epinephrine together in the small cardboard box in the fridge so that they don't get mixed up or lost amongst the un-prepared ampoules.
- Keep TRAP and AA frozen when not in use.

5) Preparing the 96-well plates:

- The plates used to test platelet samples have 96 Perspex-bottomed plastic well (12 columns of 8). Of these, the test uses 48 wells (6 columns, each with 8 rows) per patient, each with varying amounts of reagents in a 20ìL volume. Each new plate needs to have the reagents accurately measured and inserted in their appropriate wells.
- When adding liquid to a well at any point, always touch the pipette tip to the bottom edge of the well and inject slowly.
- Avoid any air bubbles whenever possible.
- Avoid contact with the sides of the wells.

- Avoid spraying liquid above the wells, as droplets can contaminate nearby wells.
- As you work, try to hold the plate at an angle so any fluid will sit at the bottom edge of a well, allowing you to see the pipette tip easily.
- Add JNL to appropriate wells. This is described for columns 1-6 (columns 7-12 will correspond respectively).
- Pour the JNL into a clean dry plastic dispensing trough
- Dispense JNL using "reverse-pipetting": over-fill the pipettes by fully depressing the pipette plunger to the 2nd stop prior to immersing the tips in the JNL. This draws up more than the required amount into each pipette.
- Then dispense the JNL into the wells by pressing the plunger but only to the 1st stop, without over-pressing it. Keep your thumb down at the 1st stop as you withdraw from the wells. Then get more JNL for the next set of wells.
- Using the multi-channel pipette, first add 10iL of JNL to upper 7 wells of column 4 (i.e. rows A to G – leave the H-well empty).
- Add 20iL JNL to the upper 7 wells (i.e. A to G: H-well stays empty) of columns 3, 4 (these will then have 30 iL), 5 and 6.
- Add 20iL JNL to the upper 6 wells (i.e. A to F: leave the G and H wells empty) of columns 1 and 2.
- Add 20iL JNL to the well H6. Add 10iL to wells G1 and G2
- Return the remaining JNL to its beaker and cover and store away in the fridge.
- You can follow your own method if you prefer, but the final volumes of JNL in the plate layout should be:

	1	*2*	*3*	*4*	*5*	*6*
A	20	20	20	**30**	20	20
B	20	20	20	**30**	20	20
C	20	20	20	**30**	20	20
D	20	20	20	**30**	20	20
E	20	20	20	**30**	20	20
F	20	20	20	**30**	20	20
G	**10**	**10**	20	**30**	20	20
H	0	0	0	0	0	**20**

AA:

- Reverse pipette 20µL of (thawed) AA into well H1.
- Reverse pipette 30µL of AA into well G1.
- G1 already had 10µL of JNL, so it will now have 40µL of fluid.
- Mix these thoroughly by drawing up and dispensing with a 20 µL pippette. This is done about 10 times to ensure adequate mixing of the AA and the JNL while avoiding air bubbles.
- Remove 20µL from G1 and transfer it to F1. Again, draw the fluid in and out 10 times to ensure mixing.
- Remove 20µL from F1 and transfer it to E1, then mix as above.
- Repeat this 20µL transfer along the rest of column 1, mixing well each time and avoiding air bubbles.
- Discard the final 20µL drawn from well A1.
- Each well in column 1 should now have 20µL of AA at various dilutions.
- Visually, they should all be similar in terms of the quantity of fluid present

and the appearance; the fluid should coat the well's plastic base, giving it
an oily appearance.

Coll:

- Reverse pipette 20µL of Coll into well H2.
- Reverse pipette 30µL of Coll into well G2.
- G2 already had 10µL of JNL, so it will now have 40µL of fluid.
- Mix these thoroughly by drawing up and dispensing with a 20 µL pippette. This is 10 times to ensure adequate mixing of the Coll and the JNL while avoiding air bubbles.
- Remove 20µL from G2 and transfer it to F2. Again, draw the fluid in and out 10 times to ensure mixing.
- Remove 20µL from F2 and transfer it to E2, then mix as above.
- Repeat this 20µL transfer along the rest of column 2, mixing well each time and avoiding air bubbles.
- Discard the final 20µL drawn from well A2.
- Each well in column 2 should now have 20µL of Coll at various dilutions. Visually, they should all be similar in terms of the quantity of fluid present and the appearance.

ADP/TRAP:

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

- Reverse pipette 20µL of ADP/TRAP into well H3.

- Reverse pipette 20μL of ADP/TRAP into well G3.
- G3 already had 20μL of JNL, so it will now have 40μL of fluid. Mix these thoroughly by drawing up and dispensing with a 20 μL pippette.
- Remove 20μL from G3 and transfer it to F3. Again, draw the fluid in and out 10 times to ensure mixing.
- Repeat this 20μL transfer along the rest of column 3, mixing well each time and avoiding air bubbles.
- Discard the final 20μL drawn from well A3.
- Each well in column 3 should now have 20μL of ADP/TRAP at various dilutions.

EPI:

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

- Reverse pipette 20μL of Epi into well H4.
- Reverse pipette 10μL of Epi into well G4.
- G4 already had 30μL of JNL, so it will now have 40μL of fluid. Mix these thoroughly by drawing up and dispensing with a 10 μL pipette. This is done about 15 times to ensure adequate mixing.
- Remove 10μL from G4 and transfer it to F4. Again, draw the fluid in and out 15 times to ensure mixing.

- Repeat this 10μL transfer along the rest of column 4, mixing well each time and avoiding air bubbles.
- Discard the final 10μL drawn from well A4.
- Well G4 to A4 will now each contain 10μL of liquid. This needs to be reduced down to 20μL. Withdraw and discard a further 10μL from these wells in column 4 so they all end up with a 20μL volume (No need to touch well H4 since it will already have the exact 20μL volume)
- All the wells in column 4 should now have 20μL of Epi at various dilutions.

Column 6 will have just the JNL to act as a control. These will all already have 20iL of fluid. Thus all the wells (in columns 1 to 6) should now all have 20iL of fluid. They should all have a very similar appearance in terms of volume and as a clear colourless ball of fluid at the base of the well (except for the oily-looking AA of column 1).

The addition of (raw, unmixed) agonists is summarised below:

	1 -	*2 -*	*3-*	*4-*	*5-*	*6*
A						
B						
C						
D						
E						
F						
G	**30**	**30**	20	**10**	20	0
H	20	20	20	20	20	0

To do two patients on a single plate (i.e. to do a "double plate"), repeat the above JNL and agonist preparations for columns 7 to 12.

6) Collecting a blood sample

- prepare the necessary equipment:
  - a warmed syringe with the correct amount of sodium citrate, with no air bubbles
  - tourniquet
  - 19G butterfly needle
  - 5ml syringe
  - alcohol swab
  - cotton ball
  - sticking plaster
- attach the 5ml syringe to the butterfly
- place the tourniquet on the upper arm and clean the sample site with alcohol
- insert the needle until a flashback of blood is seen
- remove the tourniquet
- slowly draw 5mls of blood and discard
- remove this syringe and attach the 50ml syringe

- Slowly draw the blood sample to the exact amount required (total 30mls for women, 40mls for men).
- Blood should flow slowly and freely to avoid sheer stresses on the platelets, as this can activate aggregation
- Remove the needle; apply cotton wool and then a plaster.
- Gentle rock the syringe back and forth about 5 times to ensure adequate mixing of the citrate and the blood.
- Record the date and time that the blood is taken.

7) Centrifugating the blood sample

- Transfer the blood in 5ml aliquots into plastic test-tubes (6 tubes used for women, 8 tubes for men). Do this by tilting the test-tubes and allowing the blood to slowly run from the top of the test-tube down along the side of the tube into its base.
- Each tube should have the same amount of blood in them. This is essential to ensure the centrifuge is balanced and spins without agitation. Use plastic pipettes to correct any discrepancies in volume.
- Screw on the caps of the tubes and transfer them into the centrifuge.
- Place the test-tubes in a balanced fashion inside the centrifuge.
- Close the lid and set it to spin at 150g for 10 minutes
- Press the start button firmly.
- Check that the centrifuge starts by looking at the display
- Once finished, slowly and carefully remove the test-tubes and sit them vertically into a holding rack.

8) Collecting Platelet-Rich Plasma (PRP)

- Using a plastic pipette, remove the PRP from each test-tube, avoiding any red blood cells and the thin layer of white blood cells.
- Hold the test-tube vertically and try to keep the pipette tip just below the surface of the PRP to avoid collecting other blood cells.
- If you get too close to the other blood cells, you can see the different type of fluid being drawn into the pipette. Stop and quickly squeeze out a few drops so that the contaminating cells are removed.
- Add the PRP to a clean 50ml plastic test-tube by running the PRP along the side of the tube.

9) Obtaining the measurements

- Measure the amount of PRP obtained. 8.64mls is the minimum required for full testing.
- Transfer 1ml of PRP into an eppendorf and spin at high speed to remove any platelets. This will give you 1ml of Platelet-Poor Plasma (PPP).
- Add 180 iL PPP to well H6, G6, F6 and E6 using reverse pipetting.
- Run the Wallac's set-up wizard to set it to the 3-9-15-18 program. Choose columns 1 to 6 to be "measured" if only one patient is being tested and set the rest to "empty".
- Enter in the sample details, such as the identity of the sample, PRP volume, Platelet count etc. (Platelet count is not adjusted) Gently pour the PRP into a clean dry trough

- Using reverse pipetting, add 180 ìL PRP to the remaining wells, one column at a time. Do this by holding the pipettes almost horizontally across the tops of the well and allow the PRP to run from the top of each well, down the wall of each well, into the base where it will mix with the agonists.
- Do this quickly as aggregation begins immediately once PRP has been added to the first well.
- Quickly place the plate in the Wallac
- Click on the start button
- Once completed, save the data to the appropriate file.
- All data will be displayed as raw values in light absorption units. These values should then be converted into % aggregation using the following equation:

% Aggregation = 100 x (PRP – Well) / (PRP – PPP)

Log values of agonists to calculate dose-response curves

AA	Coll	ADP	Epi	TRAP
-2.232	-2.652	-0.806	-2.913	-0.806
-1.931	-2.351	-0.505	-2.311	-0.505
-1.630	-2.050	-0.204	-1.709	-0.204
-1.329	-1.749	0.097	-1.107	0.097
-1.028	-1.448	0.398	-0.505	0.398
-0.727	-1.147	0.699	0.097	0.699
-0.426	-0.846	1.000	0.699	1.000
-0.301	-0.721	1.301	1.301	1.301

APPENDIX 2

2.1

Patient Information Leaflet and Consent Form

Protocol Title: **What is the role of hyper-reactive platelets in the aetiology of recurrent miscarriage?**

Principal Investigators:

- Dr. Karen Flood: SPR Obs/Gynae Rotunda Hospital – RCSI unit.
- Professor Fergal Malone: Consultant Obs/Gynae, Rotunda Hospital - RCSI unit.
- Dr. Michael Geary: Master, Rotunda Hospital.
- Dr Edgar Mocanau, Consultant Gynae , Rotunda Hospital - HARI unit.
- Professor Dermot Kenny, RCSI, St.Stephens Green.
- Dr Aaron Peace , RCSI, St.Stephens Green.

Telephone No: 01- 8786070.

We would like to ask you to take part in a research study, which will be carried out at the
Recurrent Miscarriage Clinic in the Rotunda Hospital.

This research is being done, in an attempt to understand the association between platelets (clotting cells) and recurrent miscarriages. The study may show doctors how to better manage patients with this problem.

As part of routine investigation of Recurrent Miscarriage blood samples are taken at the first visit to the clinic (12 weeks after miscarriage). The only extra step that is different from routine care will involve taking a small amount (30ml) of additional blood from your forearm, from the same needle. Blood samples will be obtained in

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

We will respect completely your right to confidentiality. The samples will be processed without being labeled with your name or medical history. Regarding medical details obtained they will be discarded after the study has been completed and published. Your medical records will not leave the Rotunda Hospital.

Before you make a decision to participate or not, please read carefully the information provided and take the time to ask questions. If you wish to discuss any aspect of the study with any of the study researchers or with family and friends, or your GP please take the time to do so. Please ensure that you clearly understand the risks and benefits of participating in the study.
You may change your mind at any time and decide to withdraw before the start of the study or even after the study has commenced. You do not have to justify your decision to any person involved in the study. Your decision to participate or not in the research will have no affect on your care in the hospital.
If you have any further questions about the study, you may contact the main study investigator at the number below:

Name **Dr. Karen Flood**

Address: *Dept. of Obstetrics and Gynaecology, RCSI Unit, Rotunda Hospital, Parnell Square, Dublin 1*

Tel: *01- 8786070* *bleep: 635*

2.2

CONSENT FORM

Please tick the appropriate answer.

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

  Yes No

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

- I understand that other research scientists may view any experimental data arising from my participation in this study with delegated authority from the Rotunda hospital or RCSI and that my identity and medical details will remain anonymous. Yes No

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

  Yes No

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

  Yes No

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

  Yes No

_____

Patient Signature and dated

_____

Name in block capitals

To be completed by the Principal Investigator or his/her nominee.

I the undersigned have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, the experimental nature of the treatment, as well as the possible benefits and have invited him/here to ask questions on any aspect of the study that concerned them.

_____

Signature, qualifications and date

_____

Name in block capitals

In accordance with Good Clinical Practice if there is a dependent relationship between the Physician and the Subject then another physician should obtain consent. Likewise the person obtaining consent should be fully conversant with the study and be suitably trained and qualified.

3 copies to be made: 1 for patient, 1 for PI and 1 for research records.

2.3

Pre-platelet function test information sheet

Eligible for recruitment:

3 consecutive miscarriages -meeting the definition of Recurrent Miscarriage (RM).

No proven cause for RM: normal blood results, normal uterine cavity, normal parental cytogenetics..

No known platelet disorder e.g spherocytosis, thrombocytopenia

Once recruited:

Need to be off aspirin and folic acid for two weeks.

To avoid ibuprofen/neurofen in the preceding two weeks (Paracetamol acceptable).

Ring Day 1 of menstrual cycle to arrange blood test appointment.

Day of testing:

Morning blood test

Fasting from midnight the day before (water allowed)

To avoid alcohol, coffee and vigourous exercise for preceding 24hrs.

2.4

Patient Information Leaflet and Consent Form

Protocol Title: **What is the role of platelets in pregnancy?**

Principal Investigators:

- Dr. Karen Flood: SPR Obs/Gynae Rotunda Hospital – RCSI unit.
- Dr Aaron Peace, RCSI, St.Stephens Green.
- Professor Fergal Malone:, Rotunda Hospital - RCSI unit.
- Dr. Michael Geary: Consultant Obs/Gynae, Rotunda Hospital.
- Professor Dermot Kenny, RCSI, St.Stephens Green.

Telephone No: 01- 8786070.

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

This research is being done, in an attempt to understand normal platelet function in pregnancy. We wish to compare platelet activity in normal healthy pregnancies to abnormal pregnancies such as those complicated with recurrent miscarriage, pre-eclampsia and growth restricted fetuses. The study may show doctors how to better manage patients with these problems.

Participation includes a blood test during each of the three trimesters of pregnancy. Blood samples will be obtained in the outpatient department at the Rotunda Hospital and the analysis will be carried out and at the Rotunda Hospital.
The only risk associated with participating in this study is that of taking a blood sample, which may cause bruising at the point the needle goes into your arm. Some patients may feel light- headed or dizzy. As previously stated obtaining a blood sample is needed as part of the routine investigation so there will only be one needle prick required. A qualified healthcare professional will obtain the blood.

We will respect completely your right to confidentiality. The samples will be processed without being labeled with your name or medical history. Regarding medical details obtained they will be discarded after the study has been completed and published. Your medical records will not leave the Rotunda Hospital.

Before you make a decision to participate or not, please read carefully the information provided and take the time to ask questions. If you wish to discuss any aspect of the study with any of the study researchers or with family and friends, or your GP please take the time to do so. Please ensure that you clearly understand the risks and benefits of participating in the study.
You may change your mind at any time and decide to withdraw before the start of the study or even after the study has commenced. You do not have to justify your decision to any person involved in the study. Your decision to participate or not in the research will have no affect on your care in the hospital.

2.5

CONSENT FORM

Please tick the appropriate answer.

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

  Yes No

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

- I understand that other research scientists may view any experimental data arising from my participation in this study with delegated authority from the Rotunda hospital or RCSI and that my identity and medical details will remain anonymous. Yes No

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

  Yes No

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

  Yes No

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

  Yes No

_____

Patient Signature and dated

_____

Name in block capitals

To be completed by the Principal Investigator or his/her nominee.

I the undersigned have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, the experimental nature of the treatment, as well as the possible benefits and have invited him/here to ask questions on any aspect of the study that concerned them.

_____

Signature, qualifications and date

_____