

# **Platelet function and response to low-dose aspirin in pregnancy**

Thesis submitted in accordance with the requirements of the University  
of Liverpool for the degree of Doctor in Philosophy

*by*

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*July 2017*

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## **ABSTRACT**

### **Platelet function and response to low-dose aspirin in pregnancy**

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Pre-eclampsia is a serious multisystem disorder unique to pregnant women and associated with significant maternal and perinatal morbidity and mortality worldwide. Despite the investment of decades of basic science and clinical research, the pathophysiology of pre-eclampsia has been incompletely illustrated.

In low doses, the cyclooxygenase inhibitor and antiplatelet, aspirin, can redress the thromboxane/prostacyclin imbalance observed in pregnancies affected by pre-eclampsia. Low-dose aspirin is currently recommended for high risk pregnancies in many countries, despite affording only modest overall risk reduction. It has been demonstrated that aspirin-treated individuals experience variable antiplatelet and clinical effects, with ‘non-responders’ having a preponderance for adverse clinical outcomes.

The aim of this research was to investigate whether aspirin non-responsiveness exists in pregnant women at high risk of pre-eclampsia and assess whether platelet response to aspirin relates to markers of placental function and/or adverse clinical outcomes. An additional aim was to conduct an unbiased genome-wide assessment of genetic factors which may influence an individual’s response to aspirin. This was made possible by first establishing reference ranges for cyclooxygenase-selective platelet function in pregnancy and developing nuclear magnetic resonance and liquid chromatography mass spectrometry protocols to detect aspirin metabolites and determine adherence. Women at high risk of pre-eclampsia, according to National Institute of Health and Care Excellence criteria, were assessed longitudinally for adherence and platelet function.

With exact adherence assessments and cyclooxygenase-selective platelet function testing, aspirin non-responsiveness could not be identified. Additionally, there were no significant associations between platelet response to aspirin, markers of placental function, genetic factors and adverse clinical outcomes. However, a significant proportion of women exhibited variable response to low-dose aspirin, changing their response status throughout their pregnancies. This variable response strongly suggests suboptimal aspirin adherence and/or suboptimal dosing in this population. With the recent findings of increased reduction in the risk of pre-eclampsia with higher doses of aspirin, there is now a valuable opportunity to deepen our understanding of the pharmacokinetics and pharmacodynamics of aspirin in pregnancy. Advances in technology available for genomics and access to biobanked maternal DNA from high-quality, well-phenotyped cohorts provide a strong foundation from which to examine pre-eclampsia disease genomics.

## **ACKNOWLEDGEMENTS**

It would not have been possible to complete my thesis without the love and unwavering support from my husband Damian, particularly as much of the analysis and writing was completed during our first pregnancy. I am also thankful to my parents and brother, Joe for their steadfast belief in me over the years.

I am forever grateful for the mentorship and insights from my supervisors Ana and Zarko. They have encouraged me to grow as both a clinician and academic and continue to whole-heartedly support me in developing plans for my future. In meeting them I found positive clinical and academic role models who constantly challenge me to be better.

I was privileged to receive a Research Training Fellowship from Wellbeing of Women to support this work and my academic career development. I am very thankful I was afforded the opportunity for this experience, which has enriched my life and ultimately changed the course of my career. I also appreciate the award I received from the University of Liverpool Technology Directorate voucher scheme, which enabled collaborations with experts in nuclear magnetic resonance and mass spectrometry within the University.

I wish to thank Dr Marie Phelan and Professor Lu-Yun Lian, Dr Sujan Penchala and Professor Saye Khoo, and Dr Eunice Zhang and Andrea Jorgensen for their assistance with specialised analyses in nuclear magnetic resonance, mass spectrometry and for supporting me in learning to analyse genomic data, respectively.



## ABBREVIATIONS

11-DHTXB <sub>2</sub>	11-Dehydrothromboxane B <sub>2</sub>
AA	Arachidonic acid
ADAM-12	A-disintegrin and metalloprotease-12
ADP	Adenosine diphosphate
ADRA2A	Adrenoreceptor alpha 2A
AERD	Aspirin exacerbated respiratory disease
ARU	Aspirin reaction units
ASA	Acetylsalicylic acid
ASPREE	Aspirin for evidence-based PRE-eclampsia prevention
ATP	Adenosine triphosphate
AU	Aggregation units
BMI	Body mass index
BMPR1A	Bone morphogenetic protein type 1a
BP	Blood pressure
CAD	Coronary artery disease
CE	Carboxylesterase enzymes
CGA	Candidate gene approach
CGS	Candidate gene study
COX	cyclooxygenase
CRL	Crown-rump length
CVD	Cerebrovascular disease
EDD	Expected date of delivery
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FA	Formic acid
FDA	US Food and Drug Administration
FPR	False positive rate
GCP-L	Good clinical practice laboratory
GP	Glycoprotein
GWAS	Genome-wide association study
HELLP	Haemolysis Elevated Liver Enzymes and Low Platelets syndrome
H-ESI	Heated electrospray ionisation source
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HQC	High quality control
IMPROVED	Improved pregnancy outcomes via early detection study
JMJD1C	Jumonji domain containing 1C
LC: MS	Liquid chromatography mass spectrometry
LD	Linkage disequilibrium
LDA	Low-dose aspirin
LQC	Low quality control
LTA	Light transmission aggregometry
MAP	Mean arterial pressure
MDR1	Multidrug resistance 1
MoM	Multiples of the median

MPV	Mean platelet volume
MQC	Medium quality control
MRV11	Murine retrovirus integration site 1 homolog
NGS	Next generation sequencing
NICE	National Institute for Health and Care Excellence
NMR	Nuclear magnetic resonance
NPA	Negative percent agreement
NSAID	Non-steroidal anti-inflammatory drug
OPA	Overall percent agreement
PAPP-A	Pregnancy-associated plasma protein A
PEAR1	Platelet endothelial aggregation receptor 1
PIGF	Placental growth factor
PPA	Positive percent agreement
PPI	Proton pump inhibitor
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised controlled trial
rpm	Revolutions per minute
SA	Salicylic acid
sFlt-1	FMS-like soluble tyrosine kinase-1
SHH	Sonic hedgehog
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
SUA	Salicyluric acid
SVIL	Supervillin
TCI	cryoprobe triple resonance cryoprobe
TEG	Thromboelastography
TX	Thromboxane
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
TXB <sub>2</sub>	Thromboxane B <sub>2</sub>
VEGF	Vascular endothelial growth factor
VWF	Von Willenbrand Factor
WBA	Whole blood aggregometry
WGS	Whole genome sequencing
WHO	World Health Organisation

# **CHAPTER 1: Introduction**

### **1.1 Pre-eclampsia, clinical context**

Pre-eclampsia is a serious endothelial disorder of pregnancy with multisystem manifestations. It is characterised by hypertension occurring after 20 week's gestation associated with significant proteinuria (30mg/DL or 0.3 g or greater in 24 hours) (Davey and MacGillivray, 1988, ACOG, 2013). Pre-eclampsia usually presents in the late second or the third trimester of pregnancy and has a vast spectrum of severity. Severe manifestations tend to be associated with earlier gestational age at onset and are dependent on the organ systems involved (NHBPEP, 2000, NICE, 2010a). Severe early onset disease can present from 20 week's gestation, prior to fetal viability, and accordingly is associated with severe maternal and perinatal morbidity and mortality. Worldwide, pre-eclampsia affects 2-8% of women at low-risk and 15% of women at high-risk according to risk-factor- based screening methods (Bujold et al., 2010). Severe disease, where the major maternal and perinatal disease burdens are concentrated, is known to affect 0.5% of pregnancies in the developed world, but this incidence is doubled in developing countries (Tuffnell et al., 2005, Villar et al., 2006). Pre-eclampsia is consistently in the top five causes of maternal morbidity and mortality worldwide and causes significant perinatal morbidity and mortality (Roberge et al., 2013, MMBRACE, 2014).

### **Pathophysiology of pre-eclampsia**

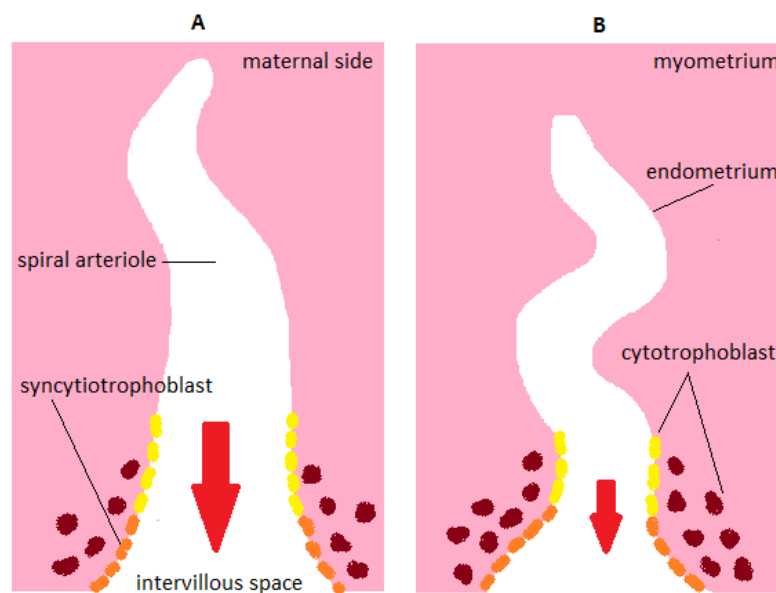
Despite sustained efforts during the last four decades and development of a large body of basic science and clinical research, the pathogenesis of pre-eclampsia remains only partially elucidated. However, it is understood that poor placentation is key to the manifestation of clinical disease at later gestations of pregnancy. The heterogeneity of placental characteristics and clinical disease observed supports the theory of a multifactorial model of causation for inadequate placental invasion with interlinked genetic, immunological and environmental determinants. Pre-eclampsia has been

described as immunologically-mediated, with lack of recognition of the fetus and placenta by the maternal immune system. There is some evidence to suggest that the human leukocyte antigen system may be important, as women with pre-eclampsia were found to have reduced HLA-G and HLA-E (Colbern et al., 1994). Several candidate genes have also been described that appear to increase susceptibility to developing pre-eclampsia (Walker, 2000, Moffett-King, 2002, Mutze et al., 2008, Nilsson et al., 2004).

During normal placental development the cytotrophoblast invades into the maternal myometrium and the spiral arterioles progressively lose their endothelial and muscular layers. This process means that with a well-invaded cytotrophoblast, maternal spiral arterioles lose their tortuosity and widen in calibre (Figure 1). Additionally, due to the loss of surrounding vessel musculature these vessels are minimally responsive to vasoconstrictive stimuli. The overall result is maturation of low-resistance vessels to facilitate maternal-fetal vascular supply. With normal placentation this is sustained with advancing gestational age and fetal demands (Figure 1).

In pregnancies that are destined to be affected by pre-eclampsia, the vascular remodelling process is incomplete. During the first wave of placental invasion (first trimester), there is inadequate invasion of the cytotrophoblast (Figure 1). This is combined with insufficient re-modelling of the uterine spiral arterioles during subsequent placentation (second trimester), resulting in diminished placental vascular supply (Figure 1). The resulting under-perfusion of the placental vascular bed has been implicated as the trigger for endothelial injury within the vascular bed (Redman and Sargent, 2005). Subsequent reperfusion injury results in placental ischaemia and villous immaturity and promotes loss of placental material into the maternal circulation, which further perpetuates the cycle of maternal endothelial cell dysfunction (Missfelder-Lobos et al., 2002). These placental events trigger a systemic cascade including; generalised vasoconstriction, formation of

microthrombi and an overall reduction in circulating blood volume. These systemic consequences have been termed the 'maternal vascular response' and are also thought to be related to derangements in thrombotic and coagulation pathways. The effect on platelets is marked, with increased platelet activation and increased production of thromboxane A<sub>2</sub> (TXA<sub>2</sub>), a platelet-derived vasoconstrictor and platelet aggregator. This effect is compounded by reduced endothelial cell production of the protective vasodilator and platelet inhibitor, prostacyclin (Baker et al., 1996, Mills et al., 1999, Walsh, 1985). The maternal vascular response results in a widespread inflammatory response and vascular hyperpermeability, responsible for the plethora of downstream clinical manifestations. Hyperpermeability causing capillary leakage can precipitate cerebral oedema and eventual eclampsia, similarly capillary leakage can cause pulmonary oedema which is also exacerbated by diminished renal function secondary to under-perfusion. Hepatic dysregulation and oedema causes deranged liver function and liver capsule irritation along with consumption and sequestration of platelets which may culminate in Haemolysis Elevated Liver Enzymes and Low Platelets (HELLP) syndrome.



**Figure 1:** Normal (A) and abnormal (B) placentation.

The pathogenesis of pre-eclampsia is intimately linked to that of intrauterine fetal growth restriction (IUGR) and a high crossover of these conditions is observed. Clinical manifestations may be isolated maternal or fetal, however a combination of both factors with unequal weighting is more frequently encountered clinically. The spiral arterioles are the primary supply to the fetal circulation, and the maladaptation seen as a direct consequence of inadequate invasion can mean the available vascular supply becomes prematurely outstripped by the demands of the developing fetus (Sankaran and Kyle, 2009). The fetus will generally compensate initially, prioritising metabolic demands, reducing growth velocity and redistributing its circulation accordingly to cope with the in-utero environment (Sankaran and Kyle, 2009). However, there are intrinsic limits to each pregnancy affected by such inadequate placental invasion and maladaptation. Eventually a fetal compensatory threshold is reached. The poorly invaded placenta is often affected by cumulative formation of microthrombi and is vulnerable to acute vascular accidents, these can manifest as placental abruption, with or without fetal demise in-utero. Similarly, fetal death in-utero may occur silently as the available vascular supply becomes terminally outstripped.

In recent years a new theory has emerged, stating that the placental dysfunction seen in pre-eclampsia is a secondary manifestation due to impaired cardiovascular function in response to the circulatory demands of pregnancy (Thilaganathan, 2017). It is proposed that placental syndromes, including pre-eclampsia and IUGR, may be reflections of intrinsic placental pathology or extrinsic effects exerted on the placenta (Thilaganathan, 2017). Whilst it is not disputed that both severe early onset pre-eclampsia and IUGR are driven by intrinsic placental processes, late-onset disease is more likely related to extrinsic factors. This may offer a plausible explanation for the spectrum of disease manifestations, and differential performance of screening strategies and preventative treatment. Early and late onset pre-eclampsia may in fact be two distinct entities. Weight

is added to the argument as many risk factors for pre-eclampsia including, hypertension (evidence of cardiovascular disease), pre-existing diabetes, obesity and advancing maternal age are also risk factors for cardiovascular disease (Magnussen et al., 2007). In some apparently healthy postpartum women ventricular remodelling can be observed, with associated subclinical diastolic dysfunction (Melchiorre et al., 2016, Savu et al., 2012). These effects are exaggerated in women who developed pre-eclampsia.

This perspective on causation is of relevance to screening and preventative approaches. However, particularly important are the implications for future pregnancy counselling and long-term cardiovascular health. Women affected by placental syndromes are known to be at increased risk of chronic hypertension, myocardial infarction and stroke (Fraser et al., 2012). Population-based studies indicate that increased cardiovascular morbidity in women previously diagnosed with pre-eclampsia is likely related to risk factors that pre-date pregnancy (Romundstad et al., 2010).

### **Screening for pre-eclampsia**

The last twenty years have seen a significant drive to move screening for pre-eclampsia into the first trimester of pregnancy to allow earlier stratification of care, though this may be at the cost of slightly less effective prediction when compared to second trimester screening, dependent on the specific method (Costa et al., 2008). Methods assessed include risk-factor based prediction, use of ultrasound techniques and a vast array of biomarkers have been proposed. Using a combination approach appears to offer the more reliable risk assessment (Sharp and Alfirevic, 2014, Costa et al., 2008, Kenny et al., 2014).

Assignment of risk status based on specific risk factors is the longest established method for pre-eclampsia risk prediction, incorporated into national evidence-based guidelines and in widespread clinical use (NICE, 2010a). Such methods are based on a risk-score, for



example in the National Institute of Clinical Excellence (NICE) guideline risk factors are stratified into; a) 'high risk factors'; pre-existing hypertension or diabetes, previous hypertension in pregnancy, chronic renal disease and autoimmune diseases and b) 'moderate risk factors'; nulliparity, BMI greater than 35, maternal age greater than 40, inter-pregnancy interval greater than 10 years and multiple pregnancy (NICE, 2010a) (Table 1). Low-dose aspirin (LDA) is recommended for any woman with a single high-risk factor or two or more moderate risk factors (Table 1). Detection rates of up to 34% of pre-eclampsia cases (for a false positive rate, FPR of 5%) have been reported when using detailed assessment of maternal characteristics in isolation (Myers et al., 2013). However, the risk factors included have low individual odds ratios (Steegers et al., 2010, North et al., 2011). Additionally risk-factor based models perform more poorly in nulliparous women, in whom the majority of pre-eclampsia occurs, where they predict a third of cases (North et al., 2011).

	<b>Moderate risk factors</b> (≥2 require LDA in pregnancy)	<b>High risk factors</b> (≥1 require LDA in pregnancy)
<b>Personal and family characteristics</b>	Nulliparity BMI ≥ 35 Maternal age > 40 years Family history of pre-eclampsia in a first degree relative	Not applicable
<b>Pre-existing medical conditions</b>	Not applicable	Chronic renal disease Chronic hypertension Diabetes mellitus Autoimmune disease (systemic lupus erythematosus)
<b>Obstetric factors</b>	Inter-pregnancy interval of ≥ 10 years Multiple pregnancy	Personal history of hypertension in pregnancy

**Table 1:** Risk factors triggering eligibility for low dose aspirin to prevent pre-eclampsia (NICE, 2010a).

The pathophysiology of pre-eclampsia, centred on inadequate cytotrophoblast invasion and retention of the spiral arteries' vasoconstrictive abilities, underpins the rationale for Doppler ultrasound assessment of the uterine arteries to identify pre-eclampsia

susceptibility. The maternal uterine arteries lie proximal to the spiral arteries and can be readily identified with colour-flow Doppler ultrasound. Increased resistance in the uterine arteries can provide an indication of increased placental resistance and poor placental invasion in women at risk of developing pre-eclampsia later in pregnancy. These signs can be reliably detected during the first trimester (Poon et al., 2009). A meta-analysis suggested that this method can predict 47.8% (for 7.9% FPR) of early onset pre-eclampsia cases and 26.4% (for 6.6% FPR) of pre-eclampsia overall (Velauthar et al., 2014). This can be further improved by combining uterine artery Doppler examination with maternal factors, where 81% (for 10% FPR) of clinically important cases (necessitating delivery prior to 34 weeks) can be predicted in the first trimester (Poon et al., 2009).

Additional first trimester ultrasound techniques have also been described, including two-dimensional measurement of placental surface area and assessment of placental bed vascularity (Suri et al., 2013, Hafner et al., 2013). These techniques indicate comparable prediction to uterine artery Doppler. However, due to the high level of skill required to perform these examinations, they have limited practical utility.

For high-risk populations, second trimester uterine artery Doppler assessment, compared to first trimester assessment, can provide more accurate risk prediction. A systematic review of diagnostic test accuracy of uterine artery Doppler to predict pre-eclampsia revealed that an increased uterine artery Doppler pulsatility index with notching of the waveforms is the best predictor of pre-eclampsia, with a positive likelihood ratio of 21.0 for high risk women (Cnossen et al., 2008).

In recent years much effort has been focussed on the identification of biomarkers with predictive capacity for pre-eclampsia (Wu et al., 2015). In support of the placentally-mediated pathophysiology of pre-eclampsia, most biomarkers with good predictive capacity relate to placental growth, vascular development, or are released by the

placenta. At present, no single biomarker has been identified with sufficient predictive capacity to be used in isolation for pre-eclampsia screening and there is a need for large-scale high quality investigation of biomarkers to predict pre-eclampsia (Wu et al., 2015).

Pregnancy-associated plasma protein A (PAPP-A) is an enzyme synthesised by the syncytiotrophoblast. Due to its interactions with the insulin-like growth factors, PAPP-A is important for development and function of the placenta. Levels below 0.4 multiples of the median (MoM) are associated with increased risk of pre-eclampsia, though it is not useful as a predictor in isolation due to poor positive predictive capacity (D'Antonio et al., 2013). Similarly, other biomarkers that have been used for aneuploidy screening, inhibin-A, corticotropin releasing hormone, and activin have been assessed and their positive likelihood ratios are unreliable for clinical use in pre-eclampsia screening.

Placental growth factor (PlGF) is a glycosylated dimeric glycoprotein, a member of the vascular endothelial growth factor family. In pregnancy PlGF is synthesised by the villous and extravillous trophoblast and is important for placental angiogenesis and vascular adaptation. With healthy placentation, levels peak at 26-30 week's gestation then fall gradually during the third trimester. In the presence of placental dysfunction, PlGF falls more rapidly and at earlier gestations. PlGF is now recommended by NICE to rule out pre-eclampsia in women presenting with hypertension in the second and third trimesters and has utility in guiding outpatient follow-up and timing of delivery (NICE, 2016). However, low PlGF does not have sufficient predictive capacity to be clinically useful in the first trimester. It is more promising when used in combination models.

Other markers involved in angiogenesis including fms-like soluble tyrosine kinase-1 (sFlt-1), VEGF, the angiopoietins, A-disintegrin and metalloprotease-12 (ADAM-12) and endoglin have limited capacity to predict pre-eclampsia (Villa et al., 2013, Akolekar et al.,

2010, Schneuer et al., 2014, Goetzinger et al., 2013). However, endoglin has been incorporated successfully in combination models (Foidart et al., 2010).

More clinically useful predictive capacity has been achieved by combining risk factor assessment with uterine artery Doppler examination and biomarkers with variable detection rates dependent on the specific combination selected. First trimester predictive capacity of 75-96.3% of early onset pre-eclampsia cases (for a 10% FPR) has been described when biomarkers (including PLGF, PAPP-A, inhibin-A) are combined with uterine artery Doppler examination plus or minus risk factor assessments or MAP (Audibert et al., 2010, Scuzzocchio et al., 2013, Foidart et al., 2010, Akolekar et al., 2013). A multicentre prospective cohort study assessed combinations of biomarkers and clinical factors for prediction of pre-eclampsia in nulliparous women sampled at 14 to 16 weeks (Kenny et al., 2014). Predictive accuracy was moderate for pre-eclampsia overall (AUC 0.68, 95% CI 0.63-0.74) with a model incorporating PIGF, mean arterial pressure (MAP), BMI, assessment of fruit intake and mean uterine artery resistance index. Prediction was improved for pre-eclampsia diagnosed prior to 34 weeks (0.78 95% CI 0.58-0.99), using a model combining angiogenin/PIGF ratio, MAP, any pregnancy loss prior to 10 weeks and mean uterine artery resistance index. However, the confidence intervals were wide due to only 28 cases of early onset disease of 5623 women in the cohort (Kenny et al., 2014).

The IMproved PRegnancy Outcomes Via Early Detection (IMPROVED) study is a phase IIa clinical study aiming to develop an early pregnancy predictive test for pre-eclampsia using a panel of maternal proteomic and metabolomic markers (Navaratnam et al., 2013). Recruitment of 5000 low-risk nulliparous women across Europe is currently ongoing and due to complete during 2017.

## 1.2 Platelets

### Platelet adhesion, activation and aggregation

Platelets, anucleate cells unique to mammals, are derived from the fragmentation of megakaryocyte precursors present in bone marrow. Through the processes of adhesion, activation, aggregation and platelet interactions with components of the coagulation cascade, they contribute to stable blood clot formation. Exhausted platelets are sequestered from the circulation and are removed by phagocytosis that occurs in the liver and spleen. In the pregnant state this sequestration also occurs within the placenta. In healthy adults 10% of circulating platelets are replaced daily (McNicol and Israels, 2003). In healthy non-pregnant individuals the average platelet lifespan is 8-9 days (McNicol and Israels, 2003).

Events such as blood vessel injury or atherosclerotic plaque rupture result in exposure of collagen and Von-Willenbrand Factor (VWF) to circulating platelets. The process of stable clot formation starts with adhesion of circulating platelets to the exposed subendothelial collagen and Von Willenbrand Factor, via the glycoprotein (GP) 1a/IIa and GP 1b/V/IX receptors. Platelet adhesion triggers platelet activation, causing morphological changes in the platelet and the release of intracellular calcium. This increased intracellular calcium encourages platelet to fibrinogen binding via conformational changes to the GP IIa/IIIb receptors . Adenosine diphosphate (ADP) is released from intracellular granules within activated platelets, and this promotes the activation of adjacent platelets (Roth and Majerus, 1975) . The enhanced action of phospholipase A<sub>2</sub> increases production of arachidonic acid. The conversion of arachidonic acid to TXA<sub>2</sub> is catalysed by the platelet enzymes cyclooxygenase (COX-1) and thromboxane synthase. TXA<sub>2</sub> increases fibrinogen receptor expression within the activated platelet's membrane to promote binding. TXA<sub>2</sub> released from activated platelets binds to the membranes of surrounding platelets causing activation, and combines with ADP, fibrinogen and factor V, also released to

further promote the cycle of platelet activation in the proximity (Roth and Calverley, 1994). TXA<sub>2</sub> also acts as a vasoconstrictor, reducing local blood flow to promote stabilisation of the establishing blood clot (McNicol and Israels, 2003).

### **Platelet response in normal pregnancy and pre-eclampsia**

Pregnancy represents a transient physiological state of increased endothelial stress (Juan et al., 2011). Heightened platelet function is physiologically important to support coagulation of maternal blood around the time of delivery. During normal pregnancy there is evidence of increased platelet turnover, with exhausted platelets continually sequestered and removed from circulation by the placenta, liver and spleen, and increased release of new platelets from bone marrow (Fay et al., 1983). Accordingly, increased numbers of immature platelets, with higher Mean Platelet Volumes (MPV), are detectable in maternal blood (Ahmed et al., 1993). Platelet immaturity predisposes them to aggregate at lower levels of stimuli, perpetuating the cycle of activation, aggregation, exhaustion and placental consumption. The net effect is a slight reduction in the total number of circulating platelets with advancing gestation (Fay et al., 1983). In pregnancies affected with hypertensive disorders the observed physiological reduction in circulating platelet count and increase in MPV may be enhanced (Stubbs et al., 1986, Hutt et al., 1994), with markers of increased platelet activation present in maternal plasma, changes which may be detected as early as the first-trimester (Banzola et al., 2007, Bosio et al., 2001). Trimester-specific reference ranges for platelet count and MPV, in comparison to those for non-pregnant individuals are shown in Table 2.

These observations in hypertensive pregnancies appear to support a mechanism including excessive intravascular platelet activation associated with increased placental removal of circulating platelets. Preservation of platelet count in stable pre-eclampsia may therefore reflect diminishing placental function.

In pregnancies affected by pre-eclampsia, placental TX production is enhanced (Walsh, 1985). Concurrently production of its opponent prostacyclin, a vasodilator and inhibitor of platelet aggregation, may be reduced, leading to an important imbalance (Walsh, 1985). Increasing thromboxane dominance promotes utero-placental and systemic vasoconstriction (Walsh, 1985). TX's effects on platelets perpetuate platelet activation and stimulate further TXA<sub>2</sub> production, which eventually leads to the clinical manifestations of pre-eclampsia (Walsh, 1985).

Observation of this imbalance in opposing prostaglandins in pre-eclampsia led to the hypothesis that antiplatelet agents such as aspirin may have a role in preventing the condition. Low dose aspirin (LDA) favours inhibition of TX production with minimal effect on prostacyclin, which can serve to redress the ratio of these two prostaglandins (Schiff et al., 1989, Sibai et al., 1989). LDA has been shown to reduce plasma, serum and placental TX (Schiff et al., 1989, Sibai et al., 1989, Spitz et al., 1988, Thorp et al., 1988).

	Non-pregnant adult	First trimester	Second trimester	Third trimester
Platelet count ( $\times 10^9/L$ )	165-415	174-391	155-409	146-429
MPV ( $\mu m^3$ )	6.4-11.0	7.7-10.3	7.8-10.2	8.2-10.4

**Table 2:** Platelet count and MPV in healthy non-pregnant adults and pregnant women throughout the trimesters (Abbassi-Ghanavati et al., 2009).

### 1.3 Aspirin

#### History

Aspirin is one of the oldest medications still in modern use (Figure 2, Table 3). Aspirin is also one of the most widely investigated medications and despite its longevity, is evaluated in up to 1000 clinical trials per annum (Table 3). The first records of aspirin-related compounds, 'salix', derived from willow tree bark, were documented on papyrus scrolls used by Egyptian physicians in 1534 BC and Hippocrates recommended a solution made with willow tree leaves for analgesia in labouring women (Figure 2) (Ebers, 1534).

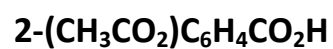
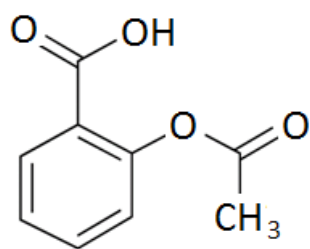
The first translation of this knowledge into modern practice began in Oxford in 1758 when Reverend Edward Stone consumed, and later in 1763 successfully trialed willow tree bark for relief of headaches, myalgia and fever in his parishioners suffering the effects of rheumatic fever (Stone, 1763). Throughout the 1800s significant efforts were made by European researchers to isolate the active compound, salacin (salicylic acid, SA, Figure 4) from willow and then to make this tolerable for oral administration (Figure 3). In the 1890's chemists working at Bayer were successful in discovering that the addition of an acetyl group to salicylic acid removed the bitter taste and reduced gastro-oesophageal irritation associated with the drug. The result was acetylsalicylic acid (ASA, Figure 3), which was patented by Bayer and two years later named aspirin and introduced for sale (Gerhardt, 1853, Zundorf, 1997). Sixty-eight years later the antiplatelet effects of aspirin were first described (Weiss and Aledort, 1967). In 1971 two research groups, Vane/Smith, and Willis were able to independently demonstrate the antiplatelet action of aspirin, and clinical research investigating aspirin's antiplatelet effects to reduce the risk of cardiovascular events gained momentum (Smith and Willis, 1971, Vane, 1971) .

Though aspirin was initially used as an analgesic and antipyretic, its antiplatelet effects mean it has become one of the most frequently prescribed medications worldwide, taken by more than 50 million people for prevention of cardiovascular disease alone, with approximately 40,000 tons administered annually. Aspirin's broad clinical effectiveness, cost-effectiveness and safety profile have led to its inclusion in the World Health Organisation (WHO) Essential Medicines list for basic healthcare systems (WHO) .

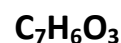
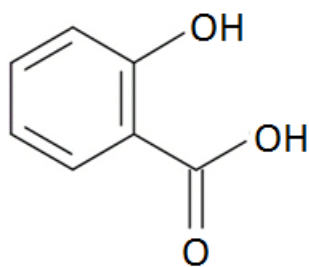




**Figure 2:** Timeline of sentinel events in the history of aspirin



**Figure 3:** Chemical and linear formulae of acetylsalicylic acid.



**Figure 4:** Chemical and linear formulae of salicylic acid.

Mechanism of action	Therapeutic/ Preventative	Condition
Antiplatelet	Therapeutic  Preventative	Acute coronary syndrome, transient ischaemic attack, stroke Atrial fibrillation, ischaemic heart disease, cerebrovascular disease, peripheral arterial disease, antiphospholipid disease, pre-eclampsia and in-utero fetal growth restriction
Anti-inflammatory/Analgesic	Therapeutic	Rheumatoid arthritis, osteoarthritis, giant cell arteritis, Kawasaki disease
Antipyretic	Therapeutic	Acute viral or bacterial febrile illness
Novel mechanisms	Preventative	Colorectal cancer, prostate cancer, minor evidence for endometrial and breast cancer

**Table 3:** Overview of modern clinical uses of aspirin.

## **Pharmacokinetics**

ASA is a weak acid that is completely absorbed, mostly undissociated, in the upper gastrointestinal tract following oral administration of immediate release formulations (FDA, 2016). Low gastric pH favours absorption of ASA by passive diffusion, with the remainder of the drug absorbed more slowly in the higher pH environment of the small intestine. Enteric formulations have delayed absorption, with some evidence of erratic and incomplete absorption (Grosser et al., 2013, Haastrup et al., 2015, Bhatt et al., 2017).

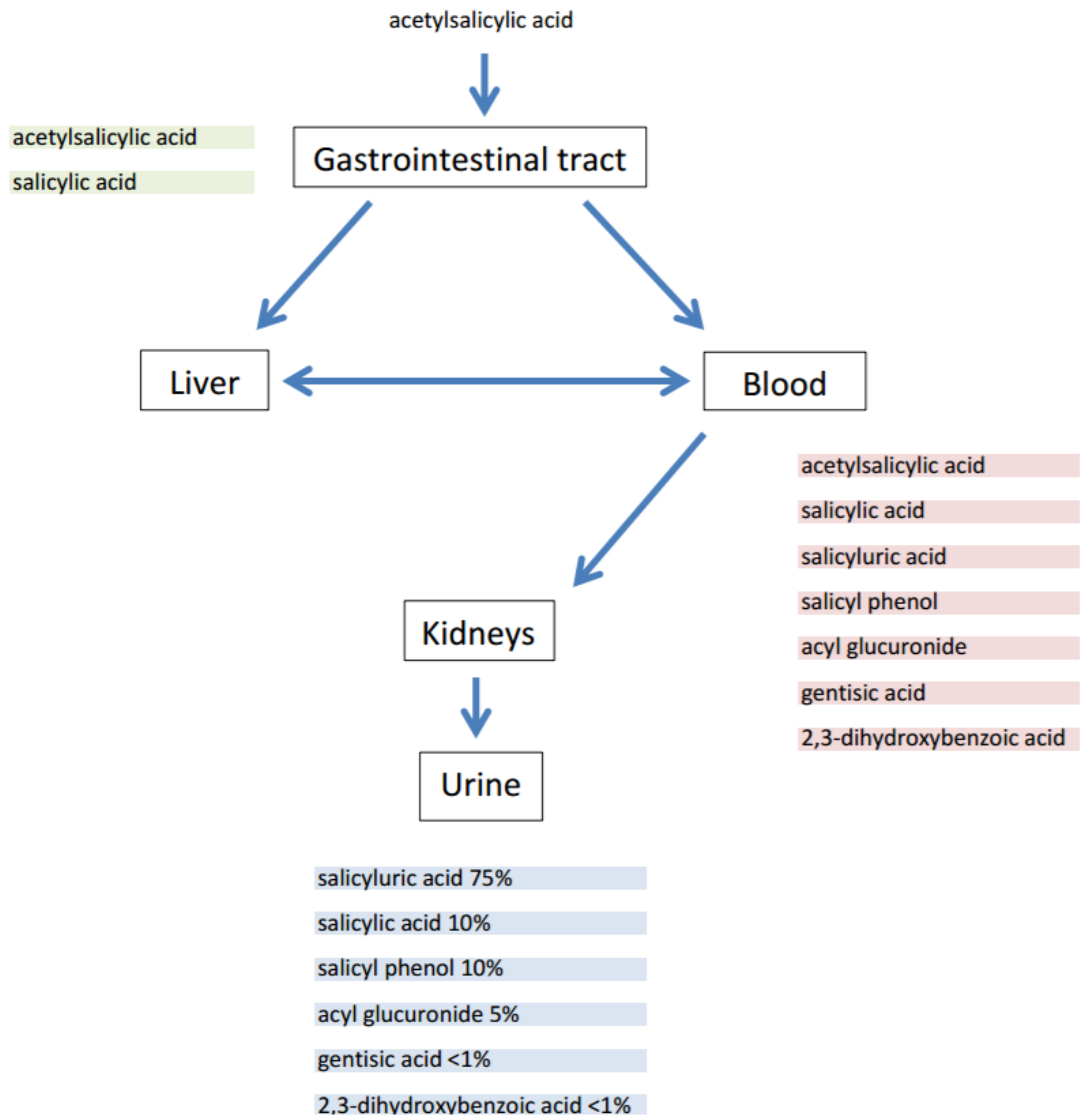
ASA undergoes rapid hydrolysis by carboxylesterases (CEs) initially in the mucosa of the stomach, and also by plasma, erythrocyte and hepatic CEs. CEs deacetylate and inactivate ASA, to produce the metabolite, SA (FDA, 2016) (Figure 5). The hepatic CE isoform 2 accounts for the major proportion of first-pass deacetylation of ASA. This CE is also present within the gastrointestinal tract, therefore may have a role in pre-systemic inactivation of ASA (Tang et al., 2006, Yang et al., 2009). Subsequently, with oral dosing, systemic bioavailability of ASA is between 40 and 50%, with a short half-life of 15-20 minutes (Ali et al., 1980). With immediate release ASA formulations, peak plasma levels are achieved within 40 minutes of ingesting a single oral dose (Abaci et al., 2006, Bochner et al., 1988). Different enteric-coated formulations may vary in time to achieve peak plasma ASA concentrations as much as 4-8 hours (Ali et al., 1980, Bochner et al., 1988, de la Cruz et al., 2002).

The rapid conversion of ASA to SA is of significance for biochemical assessment of LDA adherence as ASA is generally not measureable more than 2 hours post administration. The SA produced is more stable with a half-life of 6 hours after ingestion of low-doses of ASA. SA is widely distributed across many compartments and tissues, including placenta and fetal tissues, but is largely concentrated in plasma, in-keeping with its low volume of distribution (FDA, 2016). SA protein binding is concentration-dependent, at low

concentrations (<100mcg/ml), 90-95% of SA is protein bound (FDA, 2016). SA undergoes hepatic metabolism by the microsomal enzyme system; at low doses, 90% of SA is conjugated via the saturable glycin pathway, with metabolites undergoing urinary excretion.

The salicylates undergo renal excretion, the primary urinary metabolite is salicyluric acid (SUA, 75%). Additionally, some SA is excreted unchanged (10%), a phenolic glucuronide (10%), an acyl glucuronide (5%) and other minor metabolites are also excreted in urine (FDA, 2016) (Figure 4). Elimination of low-dose salicylates is constant in relation to their plasma concentration, following first order pharmacokinetics. With high salicylate doses, elimination occurs via parallel first and zero order pharmacokinetics (Levy, 1965). Elimination of free salicylates increases with a shift of the urinary pH into the alkaline range (pH >5.5), which has been exploited for therapeutic use to clear salicylates in aspirin poisoning (FDA, 2016).

Very few studies have assessed pregnancy-specific pharmacokinetics of aspirin. However, the physiological changes in pregnancy, particularly increases in intestinal transit times, plasma transport proteins, volume of distribution and glomerular filtration rate mean that differences in pharmacokinetics are plausible. A study assessing single dose pharmacokinetics of 75mg LDA, in the third trimester identified decreased uptake rate and increased volume of distribution but no effect on the rate of conversion of ASA to SA or elimination (Rymark et al., 1994).



**Figure 5:** Overview of the generation of aspirin’s metabolites.

### Pharmacodynamics

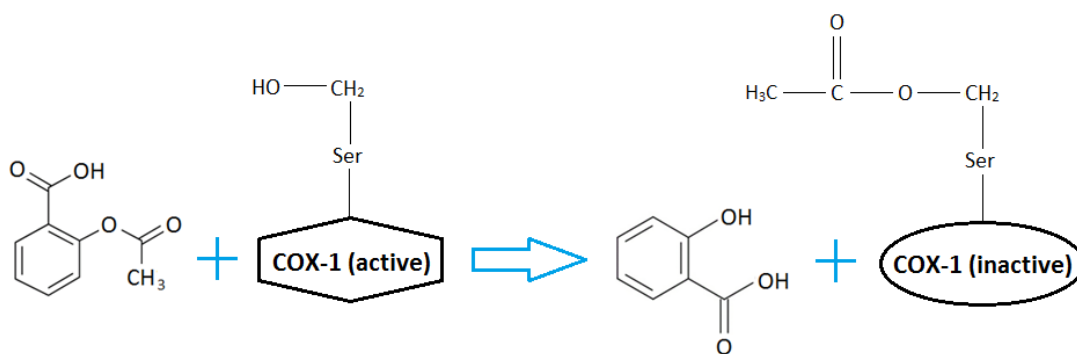
ASA’s principal pharmacological target is the COX-1 enzyme, a membrane-bound glycoprotein expressed on the endoplasmic reticulum of many cells, including platelets. COX-1 (the constitutive isoform) is responsible for catalysing the production of cyclic prostanoids, including the vasoconstrictor and pro-platelet aggregator,  $\text{TXA}_2$  and vasodilator, prostacyclin, via oxidation of arachidonic acid. Via this function COX-1 mediates normal platelet function, protection of the gastric mucosa and regulation of

renal perfusion (Morita et al., 1995) (Smith, 1992). COX-2 is the inducible isoform associated with inflammation, though it does have some discrete constitutive functionality.

ASA is 170 times more potent for COX-1 than for the COX-2 isoform, despite significant shared amino acid sequencing (Vane et al., 1998). COX-2 inhibition is dose dependent and occurs with increasing ASA doses (Vane et al., 1998). ASA selectively acetylates the serine residue (Ser529 of COX-1, Ser516 of COX-2), altering enzyme structure and preventing binding of arachidonic acid to the Tyr385 catalytic site (Roth and Majerus, 1975). The resultant inhibition of prostaglandin H-synthase production leads to irreversible inhibition of COX-1 activity with inhibition of COX-1-dependent TXA<sub>2</sub> generation (Figure 4) (Roth and Majerus, 1975, Roth and Calverley, 1994).

COX-1 inhibition by aspirin is rapid and occurs pre-hepatically within the portal system, with inhibition of platelet activity evident within one hour of ingestion for non-enteric coated formulations (Roth and Majerus, 1975, Patrono et al., 1998). This is often prior to ASA being detectable in peripheral blood (Pedersen and Fitzgerald, 1984). COX-1 inhibition is saturable at low doses (0.45mg/kg, 60-150mg), with once daily dosing shown to reduce serum Thromboxane B<sub>2</sub> (TXB<sub>2</sub>) the stable metabolite of TXA<sub>2</sub>, by a minimum of 95% within 5 days of commencing aspirin (Roth and Majerus, 1975, Roth and Calverley, 1994, Eikelboom et al., 2012). Platelets lack the capacity to regenerate their COX enzymes following inhibition by aspirin. Subsequently, saturation of platelet COX-1 inhibition accumulates rapidly and remains inhibited for the lifespan of the platelet (7-10 days in healthy adults) (Patrignani et al., 1982). Additionally, systemically circulating ASA acts on platelet precursors, megakaryocytes, pro and pre platelets in bone marrow, meaning during ASA treatment a proportion of platelets enter the peripheral circulation already COX-1 suppressed.

Overall platelet COX-activity is restored by 10% per day due to platelet replenishment from bone marrow precursors (Burch et al., 1978). It has been demonstrated that if a minimum of 20% of platelets possess normal COX activity, overall platelet activation and function can also register within the normal range (Bradlow and Chetty, 1982, Patrono et al., 1985). In healthy individuals this may occur in as little as 48 hours following cessation of low-dose aspirin, but may be more rapid in states of enhanced platelet turnover, including after coronary artery bypass surgery, in diabetes and pregnancy (Rocca et al., 2012, Paikin et al., 2015). It is also important to note,  $\text{TXA}_2$  is produced in smaller quantities via COX-independent pathways and from non-platelet sources, including monocytes and macrophages. These pathways have considerable inter-connections (Roth and Calverley, 1994).



**Figure 6:** ASA inhibition of COX-1

### Dose selection

The move towards preferential use of LDA for prevention of cardiovascular and cerebrovascular disease has been driven by the aim of using the lowest effective dose to inhibit platelet COX-1, spare endothelial COX, and also to reduce the risk of dose-related adverse effects. Targeting the inhibition of platelet COX-1, with suppression of  $\text{TXA}_2$  formation whilst reducing inhibition of prostacyclin, is thought to protect the vessel wall against thrombosis. Additionally, in pregnancy, this strategy would serve to redress the

imbalance in the TXA<sub>2</sub>: prostacyclin ratio shown in pregnancies that subsequently develop pre-eclampsia (Walsh, 1985, Schiff et al., 1989, Thorp et al., 1988).

During the 1970s and 1980s work in rabbit models demonstrated that high doses (100-200mg/kg) of aspirin inhibit both TXA<sub>2</sub> and prostacyclin, as evidenced by the measurement of stable prostaglandin metabolites (TXB<sub>2</sub> and 6-keto-prostaglandin PGF<sub>1α</sub>) or by proxy measurements of arachidonic acid-stimulated platelet aggregation and ADP-stimulated platelet aggregation (Ellis et al., 1980, Louie and Gurewich, 1983, Kelton et al., 1978). In contrast, low doses of aspirin (1-10mg/kg) were shown to favour inhibition of platelet TXA<sub>2</sub>, whilst sparing prostacyclin in vessel walls. Assessment of the effect of high dose SA, showed no effect on either prostaglandin, supporting the accepted understanding that ASA is responsible for aspirin's antiplatelet, antithrombotic effects. These findings were found to have parallels in humans, where low doses of 150mg of aspirin produced complete inhibition of COX-dependent platelet aggregation and 75mg consistently produced major inhibition of these pathways (Ellis et al., 1980, Louie and Gurewich, 1983).

The potential for sex-related differential inhibition of TXA<sub>2</sub> and prostacyclin and effects on platelet aggregation were also investigated, both in a rabbit model, and in humans (Buchanan et al., 1983). There were no sex-related differences in the degree or duration of TXA<sub>2</sub> and prostacyclin inhibition and no measurable differences in collagen-induced platelet aggregation. Studies in pregnant women demonstrated that aspirin doses as low as 20mg benefitted the TXA<sub>2</sub>: prostacyclin ratio (Martin et al., 1996). Strong inhibition of platelet TXA<sub>2</sub> with no significant effect on prostacyclin was evident with doses of 50mg and 60mg (Viinikka et al., 1993, Loudon et al., 1992). No effect on bleeding time was evident when 20mg aspirin doses were taken, whereas dose-related increases in bleeding



time were noted in pregnant women with doses between 40mg and 80mg (Martin et al., 1996).

Rabbit models also showed that reduction in thrombosis was only associated with use of low doses of aspirin, with some evidence for transient pro-thrombogenic effects, within 150 minutes of a vascular injury stimulus, specifically when high doses were administered (Kelton et al., 1978). However, the authors acknowledge that the high doses required to produce pro-thrombogenic effects, when translated to equivalent human doses, are much higher than those in clinical use. Subsequently, preventative and therapeutic doses in clinical use would not be expected to potentiate thrombosis in humans (Kelton et al., 1978).

Prior to 1985 high doses of aspirin (500-1500mg) in divided doses were frequently used in clinical practice. However, it has subsequently been demonstrated that in humans maximal suppression of platelet aggregation can be achieved with LDA, in the range of 40-160mg once daily (Bochner and Lloyd, 1995). Importantly, this also translates into equivalent clinical effectiveness between LDA and high doses of aspirin (ATT, 2002, Baigent et al., 2009). A meta-analysis which included 145 RCTs of antiplatelet therapy examined three daily dose ranges, <160mg (7 RCTs), 160-325mg (12 RCTs), 500-1500mg (30 RCTs) and found no statistically significant difference in the risk reductions for vascular death, myocardial infarction or stroke. However, in the low dose range there was insufficient evidence to assess the effect of daily doses below 75mg (APT, 1994). A further meta-analysis in 2002 examined a broad range of low to high dose regimens, most centred on a 75mg-325mg dose bracket, administered once daily. Equivalent risk reduction was observed for low and high dose regimens (ATT, 2002).

When considering dose selection it is also important to consider that the primary adverse effects of aspirin are dose-related. Escalation of doses to 300mg doubles the risk of

gastrointestinal bleeding associated with LDA (75mg daily). The risk is increases five-fold with the use of high doses, in the 1.8-2.4g range (APT, 1994).

### **Efficacy for prevention of cardiovascular and cerebrovascular disease**

Antiplatelet agents have become pivotal in both primary and secondary prevention of coronary artery disease and cerebrovascular disease worldwide (Table 3). Antiplatelet uses of aspirin in the UK are summarised in Table 4. In 2002 a collaborative meta-analysis was conducted to assess the effectiveness of antiplatelet agents, including aspirin, for patients at high-risk of occlusive vascular events (ATT, 2002). The meta-analysis included 287 randomised trials with 135,000 patients involved in comparisons of antiplatelet agents to placebo and 77,000 patients involved in comparisons of different antiplatelet agents against each other. The results showed aspirin reduced the risk of; non-fatal myocardial infarction by a third, non-fatal stroke by a quarter, any serious event by approximately a quarter and vascular death by one sixth (ATT, 2002). When used for secondary prevention following myocardial infarction or stroke the absolute risk reduction was 36 per 1000 with aspirin treatment for two years in both cases. A risk reduction of 22 per 1000 was also proved for other high-risk patients, including those with peripheral arterial disease ( $p=0.004$ ) and atrial fibrillation ( $p=0.01$ ) (ATT, 2002). These original findings were supported by a meta-analysis of individual patient data they completed in 2009 which included data from 16 secondary prevention RCTs with 17,000 participants a high risk of occlusive vascular events. This analysis demonstrated long-term LDA use was associated with risk reductions of one fifth in coronary events and total stroke, with no significant increase in the risk of haemorrhagic stroke (Baigent et al., 2009).

An individual patient data meta-analysis in 2009 assessed the risks and benefits of LDA for primary prevention of occlusive vascular disease. Six primary prevention RCTs with 95,000 participants were included, the analysis demonstrated a 12% reduction in serious vascular events (myocardial infarction, stroke and vascular death) driven by a one fifth reduction in non-fatal myocardial infarction, but with non-significant effects on stroke and vascular death. However, use of LDA for primary prevention was also associated with a

significantly (43%) increased risk of major gastrointestinal/extracranial bleeding (Baigent et al., 2009). Subsequently, aspirin is not routinely advocated for use in primary prevention of myocardial infarction or stroke and current advice is that bleeding tendency should be assessed on an individual basis (USPSTF, 2017).

### **Efficacy for prevention of cancer**

Over the last three decades some evidence has emerged to suggest a role for aspirin in reducing the risk of certain cancers, and prolonging life in particular in colon and prostate cancer. A recent systematic review and meta-analysis identified four randomised controlled trials (RCTs), a meta-analysis and 42 observational studies (Elwood et al., 2016). The review demonstrated a 24% reduction in risk of colon cancer and 11% reduction in prostate cancer attributable to aspirin. There were no statistically significant risk reductions for other cancers identifiable within the studies available for this review. There was significant heterogeneity of study design and cancers assessed. Some studies with conflicting results were also excluded from the meta-analysis, meaning these findings should be viewed with caution. The authors concluded that aspirin may be useful as an adjunctive treatment for cancer, though large scale RCTs are needed to evaluate aspirin in individual cancer types.

Despite the controversies of LDA for primary prevention of cardiovascular disease, the emergence of data to suggest reduction in risk of colorectal cancer prompted a re-evaluation of risks and benefits of aspirin prescription in populations with no prior cardiovascular history. The US Preventative Services Task force has issued a recommendation statement for individuals with a greater than 10% 10-year risk of cardiovascular disease (USPSTF, 2017). They recommend LDA for primary prevention of colorectal cancer for individuals aged 50-59 years and advise an individualised decision be made on LDA for those aged 60-69 years (Werner et al., 2015).



Clinical Area	Clinical Situation	Dose	Guideline
Cardiovascular Disease	Acute Myocardial Infarction	300mg	NICE 2010 (NICE, 2010b)
	Acute unstable angina	300mg	
	Secondary prevention of Myocardial Infarction	75mg	
	Atrial Fibrillation, primary prevention of Myocardial Infarction	75mg	NICE 2007 (NICE, 2007)
Cerebrovascular Disease	Acute ischaemic stroke	300mg	NICE 2008 (NICE, 2008b)
	Transient Ischaemic Attack	300mg	
	Secondary prevention of stroke/Transient Ischaemic Attack	75mg	NICE 2008 (NICE, 2008a)
Peripheral Arterial Disease	Secondary prevention	75mg	NICE 2008 (NICE, 2008a)
High-risk Pregnancy	Antiphospholipid Syndrome	75mg from conception, combined with low-molecular-weight heparin	RCOG 2011 (RCOG, 2011)
	High-risk for pre-eclampsia	75mg from 12 weeks	NICE 2010 (NICE, 2010a)
	High-risk for Small for Gestational Age infant	Consider 75mg <16 weeks if at high-risk for pre-eclampsia	RCOG 2014 (RCOG, 2013)

**Table 4:** Clinical indications for antiplatelet therapy with aspirin in adults in the UK.

### **Efficacy for prevention of pre-eclampsia**

In 2007 A Cochrane systematic review and an individual patient data meta-analysis examined the effectiveness of aspirin in reducing the risk of pre-eclampsia for pregnant women deemed to be at high risk (Duley et al., 2007, Askie et al., 2007).

The Cochrane review compared any antiplatelet agent, including aspirin, with either placebo or no antiplatelet to reduce the risk of hypertension in pregnancy and its complications (Duley et al., 2007). A pragmatic comparison was made irrespective of dose, duration of treatment and combination with any other treatment, including anticoagulants. 59 RCTs were reviewed including 37,560 women. 43 of 59 studies evaluated aspirin to reduce the risk of pre-eclampsia and 38 of these studies assessed low versus high-dose aspirin for this indication. The systematic review demonstrated significant reduction in the risk of pre-eclampsia, delivery prior to 37 week's gestation and fetal and neonatal deaths by 17%, 8% and 14%, respectively. A subgroup analysis by risk for pre-eclampsia (high-risk women versus moderate risk women) was conducted. The high-risk subgroup included women with pre-existing hypertension, renal disease, diabetes and autoimmune disease and those with a previous history of severe pre-eclampsia. The moderate risk subgroup included teenagers, nulliparous women, those with multiple pregnancies, those with increased BMI, family history of pre-eclampsia and positive uterine artery Doppler or roll-over test screens. This subgroup analysis concluded that there was a significant risk reduction in pre-eclampsia with aspirin in both high and moderate risk groups of 25% and 14%, with numbers needed to treat of 19 and 119, respectively. Interestingly, no particular risk factors associated with significantly higher attributable risk were identified, despite the clear increase in absolute risk difference for composite high and moderate risk groups.

The individual patient data meta-analysis evaluating antiplatelet agents for prevention of pre-eclampsia identified a 10% reduction in the risk of pre-eclampsia and a 10% reduction in preterm birth with aspirin LDA. However, it did not demonstrate a clear difference in the effectiveness of aspirin for any particular subgroup due to small numbers represented in the groups (Askie et al., 2007). Interestingly, there was also no difference in risk reduction between women who commenced LDA treatment prior to and after 20 week's gestation.

The Cochrane review included a subgroup analysis comparing the effects of different doses of aspirin (Duley et al., 2007). Doses of 75mg and below were compared to doses above 75mg, and shown to be associated with 12% and 36% reductions in the risk of pre-eclampsia, respectively (Duley et al., 2007). The escalation in risk reduction noted between these dose brackets introduced the idea that higher doses, still in the low-dose range, may be more effective choices for prevention. Additionally, a third subgroup of 296 women from five studies assessed higher dose LDA, greater than 75mg plus dipyridamole which was associated with a 70% risk reduction in the risk of pre-eclampsia compared to placebo (Duley et al., 2007). During the development of a new NICE guideline a subgroup analysis was performed by aspirin dose (60mg daily, 75mg daily, 100mg and 150mg) (NICE, 2010a). This analysis incorporated studies from the Cochrane systematic review. This analysis demonstrated statistically significant reduction in risk of pre-eclampsia of 8% and 35% with 60mg and 75mg, respectively. However, reduction in risk was not statistically significant for the 100mg and 150mg groups (NICE, 2010a). This may, at least in the case of the 150mg group, be attributable to the small number of studies (n=3) available for inclusion.

Recently, the combined multimarker screening and randomised patient treatment with Aspirin for evidence-based PRE-eclampsia prevention (ASPREE) study, has evaluated the



efficacy of 150mg LDA once daily (O'Gorman et al., 2016, Rolnik et al., 2017). ASPRE was a double-blind placebo controlled RCT evaluating LDA in women at high risk of pre-eclampsia between 11 and 36 weeks (O'Gorman et al., 2016, Rolnik et al., 2017). Participants underwent detailed screening at 11-13 weeks using maternal factors, serum PAPP-A, PlGF, MAP and uterine artery Doppler assessment and underwent longitudinal biomarker and fetal growth surveillance (O'Gorman et al., 2016, O'Gorman et al., 2017). The primary outcome, preterm preeclampsia occurred in 1.6% of the aspirin group and 4.3% of the placebo group (odds ratio, OR, in the aspirin group 0.38; 95% CI 0.20-0.74;  $p=0.004$ ) (Rolnik et al., 2017). There were no significant differences in the profile of adverse events observed between the groups (Rolnik et al., 2017). Though the ASPRE cohort is an arguably well-characterised cohort, these findings strongly support some of the earlier signals in the literature of enhanced risk reduction with escalation of aspirin dose.

Other strategies for prevention of pre-eclampsia include careful optimisation of pre-existing medical problems known to confer particularly high risks. This may include attaining hypertensive control, optimising renal function and normalising BMI (NICE, 2010a). Vitamin supplementation is not advocated where the woman is following a balanced diet and does not have particular vitamin and/or mineral deficiencies. However, calcium supplementation may be beneficial for some women and this decision should be taken on an individual basis as further assessment of calcium via large studies is required (Villar et al., 2006).

Appraisal of these sentinel analyses and addition of a recently conducted meta-analysis provide the current statistics appropriate when counselling high-risk women, that LDA confers a 10% risk reduction for pre-eclampsia and a 20% risk reduction for fetal growth restriction (Askie et al., 2007, Xu et al., 2015). In the UK, NICE reviewed the evidence for

aspirin in prevention of pre-eclampsia, conducted a cost-effectiveness analysis and published new guidance, 'Hypertension in Pregnancy' in 2010. NICE recommends prescription of 75mg aspirin to pregnant women at high-risk of pre-eclampsia (NICE clinical guideline 107, August 2010). Numerous risk factors for pre-eclampsia have been identified, based on obstetric and medical and family history (Duckitt and Harrington, 2005). According to the NICE guidance, to qualify as high-risk, women should have at least a single high-risk factor (Table 1) or at least two moderate risk factors (Table 1).

LDA is now widely recommended in developed countries from 12 week's gestation for primary and secondary prevention pre-eclampsia for pregnant women deemed to be at high-risk. However, uncertainties remain regarding the optimal dose of aspirin and most effective gestation to initiate this preventative treatment. Additionally, it remains unclear which women derive most benefit from preventative treatment with aspirin.

#### **Efficacy for prevention of adverse pregnancy outcomes in pregnant women with antiphospholipid syndrome**

Low-dose aspirin is also recommended for prevention of adverse events for pregnant women with antiphospholipid syndrome (Tables 3 and 4). Antiphospholipid syndrome is defined as antiphospholipid antibodies plus adverse pregnancy outcomes or thrombotic events (RCOG, 2011). Two percent of women have evidence of circulating lupus anticoagulant, anticardiolipin or anti-B2 glycoprotein-I antibodies, compared to 15% investigated for recurrent miscarriage (Rai et al., 1995) . Antiphospholipid syndrome adversely affects trophoblastic differentiation and later function, causes a local inflammatory response and maternal vascular response resulting in micro and macrothrombi of the placental vasculature (Out et al., 1991). These effects result in the clinical manifestations of pregnancy loss, fetal growth restriction, pre-eclampsia and preterm birth. A meta-analysis of randomised-controlled trials for treatments

demonstrated a 54% reduction in miscarriage rate, and subsequent improved live birth rate with aspirin combined with low-molecular weight heparin thromboprophylaxis (Empson et al., 2005).

### **Safety and adverse effects**

Aspirin-mediated inhibition of prostaglandin synthesis, responsible for its desirable effects as an antiplatelet can also lead to significant adverse effects. These relate to bleeding and interference with normal prostaglandin functions causing adverse gastro-oesophageal and renal effects.

Aspirin's most frequently reported adverse effect is gastric irritation, reported by up to 40% of individuals, gastric ulcers occur in up to 2.6% of individuals (Awtry and Loscalzo, 2000). An overview of randomised trials assessed a broad dose range (30mg-1300mg) of once daily aspirin doses has demonstrated that the observed gastro-oesophageal effects escalate with increasing dose (Roderick et al., 1993). Enteric coated formulations do not appear to reduce gastro-oesophageal effects, but have been associated with diminished platelet effects and may reduce bioavailability (Grosser et al., 2013). In 2013 a study of 400 healthy volunteers compared immediate release and enteric coated 325mg dose formulations for arachidonic acid stimulated platelet aggregation, serum TXB<sub>2</sub> and urinary 11-dehydrothromboxane B<sub>2</sub>. The study found delayed and reduced drug absorption in 49% of participants who took enteric coated aspirin, compared to 0% in the immediate release group (Grosser et al., 2013). All participants who initially exhibited reduced response to aspirin responded appropriately following repeated doses, extension of their post-dose interval or when their samples were spiked ex-vivo with aspirin.

A meta-analysis of 16 randomised controlled trials including 55,462 trial participants showed a statistically significant increase in the risk of haemorrhagic stroke with aspirin use which occurred on a background of a decrease in strokes overall (He et al., 1998).

The low doses of aspirin used in prevention of cardiovascular, cerebrovascular disease and used in pregnancy weakly inhibit renal prostaglandins and subsequently present minimal risk to renal function or blood pressure homeostasis. However, daily doses

exceeding 1500mg have been shown to cause significant inhibition of renal prostaglandin synthesis resulting in decreased sodium excretion particularly for patients with heart failure (Mene et al., 1995).

Whilst aspirin intolerance can affect up to 20% of individuals, true hypersensitivity to aspirin is estimated to affect 0.6-2.4% (Lambrakis et al., 2011). Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can precipitate bronchospasm and urticarial reactions in subgroups of patients. Up to 20% of patients with asthma may experience these effects, with preponderance for women and individuals aged between 30 and 40 years. Bronchospasm, conjunctival irritation and flushing emerge with variable timing ranging from minutes to hours following aspirin ingestion (Lambrakis et al., 2011).

Seven percent of asthmatics, and up to double the number of severe asthmatics, develop aspirin exacerbated respiratory disease (AERD) (Kowalski et al., 2013, Rajan et al., 2015). AERD is characterised by asthma and chronic rhinosinusitis with development of nasal polyps and exacerbations of both conditions triggered by aspirin use (Kowalski et al., 2013). AERD is thought to be due to abnormal arachidonic acid metabolism, with the accumulation of pro-inflammatory leukotrienes (Hamad et al., 2004). Management includes symptomatic treatment, use of leukotriene antagonists and potential for aspirin desensitisation therapy (Hamad et al., 2004).

Urticarial response to aspirin is less well characterised, but may occur with or without angioedema, with an increased risk for patients with known chronic idiopathic urticaria (Grattan, 2003). The mechanisms for these effects have not been conclusively determined but may relate to derangements in leukotriene synthesis. This theory is supported by evidence that leukotriene-receptor antagonists inhibit aspirin-induced urticaria and angioedema (Lee, 1992). Patients who have experienced such a previous adverse reaction

to aspirin, or have a pre-existing history of a type I allergy or delayed T-cell mediated hypersensitivity are advised to avoid NSAID exposure.

In pregnant women ASA and its active metabolite, SA, are capable of transplacental passage and the effectiveness of aspirin in preventing platelet clumping and placental microthrombi relies on this access to the placental vasculature. However, there has been no substantiated evidence of teratogenicity and low dose aspirin has a good safety profile for mother and fetus.

A systematic review did not identify any increase in the risk of adverse effects on pregnancy outcome with LDA (Duley et al., 2007). Many large cohort and case control studies examining use of LDA in the first trimester report no association of aspirin with fetal structural or developmental abnormalities (Slone et al., 1976, Klebanoff and Berendes, 1988, Werler et al., 1989, Norgard et al., 2005). A meta-analysis of 22 studies supported no overall increase in congenital malformations associated with LDA use, but indicated an increase in gastroschisis (OR 2.37; 95% CI, 1.44 – 3.88) in neonates born to mothers who had used higher dose aspirin (325mg daily) during the third trimester (Kozer et al., 2002). This finding could not be replicated in a subsequent population-based study and the potential increase in risk of gastroschisis with escalating doses remains uncertain (Norgard et al., 2005).

In 2007 an individual patient data meta-analysis found no association between LDA treatment and risk of maternal bleeding complications haemorrhage, placental abruption and post-partum haemorrhage (Askie et al., 2007). These findings were supported by those of a systematic review published in 2013 included an analysis of harms of aspirin (ACOG, 2013). 21 RCTs and two observational studies were included in the analysis of harms, no increase in maternal bleeding complications were identified. No association has

been found between the use of LDA in pregnancy and epidural anaesthesia-related complications (Sibai et al., 1995).

Two systematic reviews have found no association between antenatal use of LDA and neonatal intraventricular haemorrhage or other neonatal bleeding (Duley et al., 2007, ACOG, 2013). Additionally, there is no evidence of premature closure of the ductus venosus in neonates born to mothers prescribed LDA throughout the third trimester (Di Sessa et al., 1994, Schiessl et al., 2005, Wyatt-Ashmead, 2011).

Since the introduction of national evidence-based clinical guidelines for the use of aspirin to reduce the risk of pre-eclampsia in 2010, there has been more widespread and sustained clinical use of aspirin in pregnancy (NICE, 2010a). During this time the excellent safety record has been further strengthened. However, a limitation consistently acknowledged by the authors of influential systematic reviews and meta-analyses is that many trials lack long-term follow-up data. A recent cohort study found maternal LDA use was not significantly associated with mortality, cerebral lesions, cerebral palsy, or global cognitive impairment in children by age 5 years who were born prior to 33 week's gestation (Marret et al., 2010). The same study demonstrated reductions, at the limit of significance, total behavioural activities (adjusted OR 0.44 95% CI: 0.19-1.02) and hyperactivity (adjusted OR 0.43 [95% CI: 0.17-1.05) (Marret et al., 2010). However, due to lack of reporting in many trials, the possibility of rare harms attributable to LDA in pregnancy cannot be entirely excluded.

Whilst aspirin is endorsed for safe use in pregnant women, when taken by children it is associated with the risk of triggering Reyes Syndrome, a rare acute non-inflammatory encephalopathy and hepatotoxicity that affects children and young people recovering from viral illness. The absolute risk of triggering Reye's syndrome with aspirin is unknown and no safe age has been outlined for children, though the condition is rare after age 18

years (Sullivan et al., 2000). Subsequently, the FDA advises aspirin is not administered as an antipyretic less than 19 years of age, and the British Healthcare Regulations Agency advises to avoid aspirin less than 16 years, except when advised by a doctor (Macdonald, 2002, NRSF, 2005).

### **Drug-drug interactions**

Drug-drug interactions of aspirin can be segregated into those related to its pharmacodynamics, and interactions due to other synergistic mechanisms (Russo et al., 2016). As aspirin does not rely on specific drug transporters or cytochrome P450 enzymes for its metabolism there is a much lower risk of pharmacokinetic-related interactions than for many other drugs and none impacting safety and/or efficacy have been described. There was previously concern that concomitant use of proton pump inhibitors (PPIs) with aspirin may increase the risk of cardiovascular events due to higher stomach pH inhibiting aspirin absorption (Giraud et al., 1997). As aspirin is not absorbed solely via the stomach in humans this did not translate into increased clinical risk (Garcia Rodriguez et al., 2014). However, as with other drugs it is advisable to avoid concurrent ingestion of antacids, as they have the potential to delay absorption of aspirin (BNF, 2017).

In addition to acetylating the serine 529 residue in the catalytic site of COX-1 ASA is known to acetylate arginine 120 at the mouth of the COX-1 site which is a docking site for other NSAIDs containing a carboxylic acid moiety (Mancini et al., 1995). This can produce competitive inhibition of aspirin by some NSAIDs including, dipyron and ibuprofen when ingested concurrently (Dannenberg et al., 2016, Schuijt et al., 2009, Yokoyama et al., 2013). Though these interactions may reduce aspirin's inhibitory effects, the clinical significance of this information is incompletely understood, with some conflicting evidence (Russo et al., 2016). It is important to consider that this pharmacodynamic interaction impacting aspirin's antiplatelet effects may increase the cardiovascular risks



associated with use of certain NSAIDs (Bhala et al., 2013). However, NSAIDs are used on a large scale worldwide both acutely and chronically for a variety of conditions. The FDA and the European Medicines Agency (EMA) published guidelines on the safe use of aspirin and ibuprofen (EMA, 2015, FDA, 2006). Patients are advised to take ibuprofen at least 8 hours prior to aspirin or to wait 30 minutes after aspirin ingestion, however both regulatory agencies state that occasional ibuprofen use during aspirin treatment is unlikely to be detrimental.

Concurrent use of other NSAIDs or corticosteroids with aspirin, each with an individual risk of gastric irritation, may produce an additive effect. Similarly, risk of bleeding complications should be considered and patient's carefully monitored, when other antiplatelet agents or anticoagulants, particularly coumarins, and aspirin are co-prescribed (BNF, 2017). Additionally, when selective serotonin reuptake inhibitors (SSRIs) are co-administered with aspirin there is potential for synergistic antiplatelet effects. SSRIs decreased inter-granule serotonin, decreasing platelet aggregation. SSRIs alone and in combination with NSAIDs, including aspirin are known to increase bleeding sequelae (Cheng et al., 2015, Anglin et al., 2014, Labos et al., 2011).

### **Aspirin non-responsiveness**

During the last 20 years it has become apparent that individuals do not experience equivalent benefits from LDA. A significant proportion of individuals exhibit suboptimal response to aspirin referred to interchangeably, as 'aspirin non-responsiveness', 'aspirin resistance' and 'aspirin treatment failure' Non-responsiveness to aspirin is associated with a burden of increased morbidity and mortality (Snoep et al., 2007, Krasopoulos et al., 2008). A systematic review and meta-analysis including 20 studies of aspirin resistance demonstrated a higher incidence of further cardiac events and cardiac death, with odds

ratios of 3.85 (95% CI 3.08–4.80 ( $p < 0.001$ )) and 5.99 (95% CI 2.28–15.72 ( $p < 0.003$ )), respectively (Krasopoulos et al., 2008).

Aspirin non-responsiveness has been defined biochemically, clinically and a minority of authors describe definitions with combining both biochemical and clinical factors. Biochemical aspirin non-responsiveness is defined as insufficient suppression of COX-mediated platelet activation in aspirin treated individuals, where platelets retain the ability to produce TXA<sub>2</sub>. This can result from incomplete COX blockade or TXA<sub>2</sub> production via alternative pathways. Clinical definitions of aspirin resistance focus on recurrent adverse events despite aspirin treatment. However, there is currently no universally accepted definition which unifies laboratory and clinical findings.

The reported prevalence of aspirin non-responsiveness ranges from 5% to 65%, varying with the assay used and the populations studied (Michelson, 2004, Weber et al., 2002, Snoep et al., 2007, Marshall et al., 1997, Eikelboom et al., 2002). This variability in prevalence stems from the diversity of platelet assays using a range of agonists. In fact, true biochemical aspirin resistance, with sustained platelet TXA<sub>2</sub> production despite confirmed aspirin adherence, is rare and it is vital that COX-specific platelet function assays are selected to assess response to aspirin.

When considering aspirin non-responsiveness it is essential to discriminate suboptimal adherence from true aspirin non-responsiveness to begin to explore other causal mechanisms. Causal mechanisms proposed include pharmacokinetic, pharmacodynamic and genetic factors (Gum et al., 2001, Maree and Fitzgerald, 2007). States of enhanced platelet turnover, including during immediate postoperative periods and in pregnancy have been implicated. Recently, the concept of aspirin non-responsiveness has been extended to high-risk obstetric populations, where sustained platelet activity despite LDA

has been linked to subsequent pre-eclampsia and fetal growth restriction (Sullivan and Elder, 1993, Rey and Rivard, 2011, Wojtowicz et al., 2011, Caron et al., 2009).

Importantly, at present, significant controversies remain across all speciality areas regarding the optimal means of identification of aspirin non-responsiveness and management strategy in affected individuals (Fitzgerald and Pirmohamed, 2011).

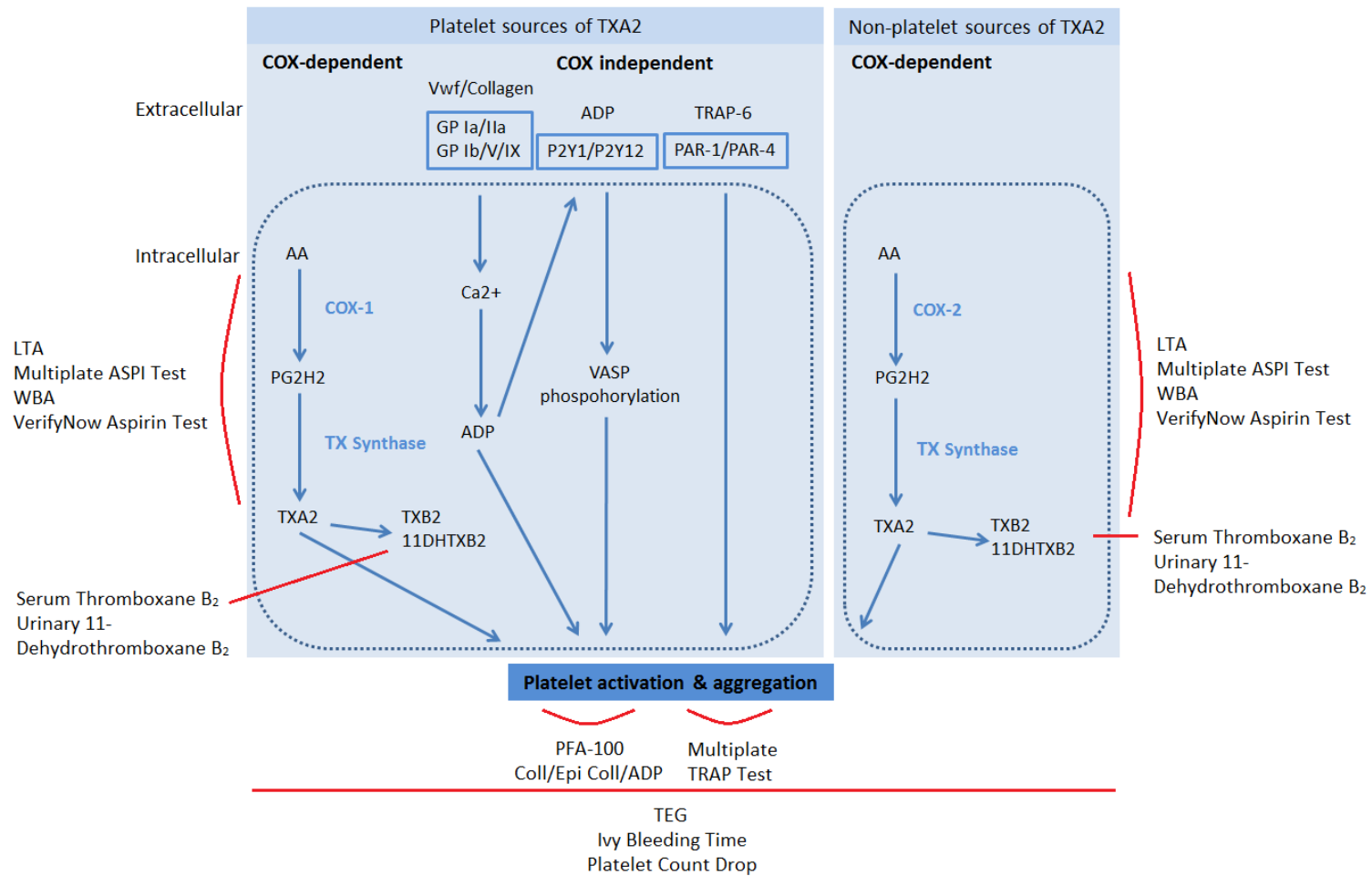
### **Platelet function assays and assessment of platelet response to aspirin**

A range of platelet function assays are now commercially available, but suffer from limited reproducibility and poor agreement between assays. With appropriate dosing and reliable adherence to aspirin therapy, low-dose aspirin provides complete inhibition of the COX-1 pathway, aspirin's primary target, in over 99% of individuals (Grosser et al., 2013, Becker et al., 2006). Consequently, assays that target the COX pathways demonstrate lower inter-individual variability.

COX-specific assays include Light Transmission Aggregometry (LTA) with arachidonic acid induction (Figure 7). This method is based on detection of light that passes through platelet rich plasma containing aggregated platelets, it is a laboratory-based technique requiring significant operator skill. VerifyNow™ utilises the theory of LTA but allows assessment using whole blood samples within a closed point-of-care system and requires minimal training. Additionally, the stable serum and urinary metabolites of TXA<sub>2</sub>, TXB<sub>2</sub> and 11-dehydrothromboxane B<sub>2</sub> (11-DHTXB<sub>2</sub>) respectively, can be quantified using enzyme-linked immunosorbent assay (ELISA) techniques (Figure 6).

Agonists including adenosine diphosphate (ADP), collagen and epinephrine act via alternative pathways and may produce high-levels of platelet activation despite complete inhibition of the platelet COX-1 pathway. Subsequently, platelet activation and function assays that rely on ADP, collagen or epinephrine for platelet activation are considered to assess primarily non COX-specific pathways (Figure 7). Non-COX specific assays include

the PFA-100™ System, a point-of-care system using citrated whole blood. This assay measures time taken for platelet aggregation to occlude a micro-aperture within collagen/epinephrine (Coll/Epi) or collagen/ADP (Coll/ADP) coated cartridges. Thromboelastography (TEG) measures speed and strength of clot formation as an indirect assessment of the contribution of platelet aggregation to stable clot formation (Figure 7). Bleeding time measurements are a traditional method of assessing platelet activation and function and still in widespread clinical use in haematology (Figure 6). They provide a pragmatic in-vivo assessment of time taken for blood to clot in a superficial linear wound made in the patient's forearm. Platelet counts approximate the impact of platelet activation, aggregation, sequestration and destruction on total circulating platelets (Figure 7). All non COX-specific assays demonstrate a high-degree of inter-individual variability, both prior to, and following aspirin dosing. However, due to the multiple pathways involved in platelet activation, non-COX specific assays may better reflect the global platelet response in-vivo in addition to providing information about in-vivo milieu.



**Figure 7:** Platelet and non-platelet thromboxane generation and COX-dependent and independent platelet function assays.

### Reference ranges for platelet function in pregnancy

To our knowledge, no studies have previously developed pregnancy-specific reference ranges for available platelet function tests. Due to the circulatory changes in pregnancy and the altered platelet parameters, it is vital to refer to pregnancy-specific reference ranges when assessing platelet response to aspirin in pregnancy. Between October 2012 and April 2014 we completed a cross-sectional study assessing a range of commercially available platelet function assays (Table 6) in healthy aspirin naïve pregnant women at low risk of pre-eclampsia between 11+<sup>0</sup> and 20+<sup>6</sup> week's gestation. Data from 87 women with confirmed normal term pregnancy outcomes were used to establish 95% reference ranges for each assay to enable work on platelet response to aspirin in pregnant populations (Table 6).

	Urinary 11-DHTXB <sub>2</sub> (pg/mg)	Platelet solutions (m/f)	Multiplate ASPI test (AUC)	Multiplate ADP test (AUC)	Multiplate ADP 3.3 test (AUC)	PFA-100 Coll/Epi (seconds)
<b>Mean</b>	6655	1249	101	64	48	106
<b>95% reference interval</b>	2927-16084	760-2108	59-185	29-158	20-111	69-164
<b>95% CI (lower limit)</b>	2517-3414	690-838	53-65	26-34	17-24	64-75
<b>95% CI (upper limit)</b>	13393-19385	1886-2359	163-210	130-195	94-131	151-183

**Table 6:** Reference ranges for platelet function in pregnancy.

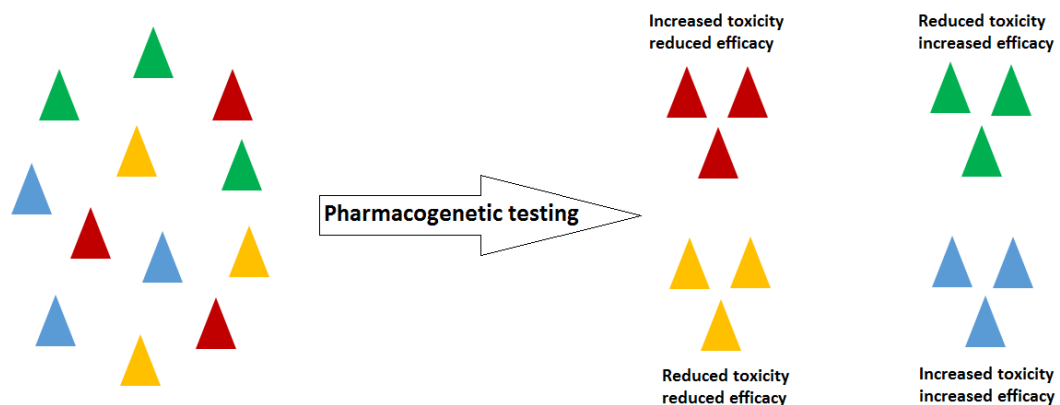
#### **1.4 Pharmacogenomics**

Pharmacogenetics is the study of variability in drug response due to hereditary factors and typically concerns genes known to govern metabolism and drug targets (Nebert, 1999). Pharmacogenomics, a more recently favoured term, implies genome-wide systematic consideration of inherited and acquired intra and inter-individual variability which may include assessment of gene expression and products in relation to individual drug response. However, in practice both terms are often used interchangeably (Pirmohamed, 2001). Most genetic variants of relevance determine drug metabolism or disposition, occur in genes encoding specific drug targets (signal transduction modulators or receptors) or relate to the pathogenesis of disease (Ring and Kroetz, 2002).

The human genome is by nature variable, with identifiable differences occurring approximately every 500-1000 bases (Roses, 2000). The single nucleotide polymorphisms (SNPs) may be synonymous, with preservation of the original polypeptide chain, or non-synonymous, resulting in an altered polypeptide. Additionally, SNPs in non-coding sequences can affect important regulatory processes, splicing or gene expression. In the field of pharmacogenomics, most efforts have focussed on identification of SNPs and their potential for individual profiling. This can lead to the ability to stratify patient groups, or more ambitiously, this information can be used within a systems biology approach, to personalise therapy. The most recent term introduced to describe this concept is precision medicine.

Within clinical pharmacology, pharmacogenomics is highlighted as a priority area for future research. The over-arching clinical aim is to apply pharmacogenomics knowledge to deliver the right drug to the right patient and at the right dose, improving the safety and efficacy of therapeutic agents (Figure 8). There are also important applications for industry, with more accurate selection of target populations and potential to streamline the lengthy drug development process and enhance progress of bringing new drugs to

market. The value of pharmacogenomic-guided prescribing is already recognised by regulatory authorities. To date, the FDA have included information on genomic biomarkers within the labels of 144 drugs, including frequently prescribed anticoagulants and antiplatelet agents. The majority of these biomarkers have applications for genotype-guided dosing, drug exposure and efficacy and risk of specific adverse drug reactions. In addition, several markers provide insight into mechanisms of drug action, polymorphic drug targets and the disposition of genes. The 100,000 genome project is now underway in the UK, aiming to embed genomic medicine into the NHS framework (GE, 2017).



**Figure 8:** Stratification of target patient groups with stratified medicine approach.

### Study designs

There are several approaches to identify important genetic factors, including assessment of candidate genes, genome-wide assessments and, more recently whole genome sequencing approaches.

Candidate gene approaches (CGA) are hypothesis driven and focus on identification of variants in plausible genes and examining genetic associations with the phenotype of interest (Wilkening et al., 2009). In the context of pharmacogenetics candidate genes are often those implicated by the drug's pharmacokinetic or pharmacodynamic properties or relate to disease phenotype. Phenotypes include clinical symptoms and signs, drug efficacy or adverse drug reactions. Candidate gene studies (CGS) most often follow a case-



control design, a range of SNPs can be assessed within these formats from a few to several thousands, within both coding and non-coding gene regions. Arrays can be customised to fit the candidates selected by the researcher, it may also be possible to conduct genome-wide CGS providing SNPs selected are represented in standard arrays (Peters et al., 2010).

The success of the CGA depends on appropriate selection of candidate genes, underpinned by accurate a priori understanding of the drugs mechanism of action and targets. However, there is widespread variability in the human genome and it is accepted that our current understanding is relatively limited in this regard. Importantly, this particular approach will not cover variants in sequences outside genes. CGS can be cost-effective as a first analysis, allowing use of resources for targeted assessment of variants and is said to increase the a priori odds of producing relevant findings (Wilkening et al., 2009). For investigation of rare variants that would perhaps not meet stringent significance levels applied in GWAS, this approach may still be beneficial. Assessment of genetic associations using these methods is useful for post-GWAS analysis, including re-sequencing of the candidate regions highlighted in GWAS.

GWAS is an unbiased, data-driven approach that is hypothesis generating. GWAS uses fixed arrays to allow genome-wide assessment for association between variants and phenotype of interest (Peters et al., 2010, Motsinger-Reif et al., 2013). The Wellcome Trust Case Control Consortium (WTCC) has published recommendations for researchers embarking on GWAS (WTCCC, 2007).

In contrast to CGS, GWAS allows investigations to move beyond a priori knowledge to identify potentially novel regions of interest, new genes of interest or associations outside genes. This has the potential to improve understanding of phenotypes. GWAS allows researchers to effectively exclude contributions of unidentified genes to their chosen drug response phenotype, meaning that even negative GWAS yield important information.

Direct investigation of variants that impact clinical response means pharmacogenomic findings through GWAS may be more amenable to clinical translation.

Though costs are continuously reducing, GWAS remains expensive and data requires expert computation and analysis. Safeguarding against systematic errors using strict quality control (QC) validation and replication of findings are vital to avoid attributing value to spurious associations (WTCCC, 2007). GWAS examines hundreds of thousands to over a million SNPs simultaneously. Large sample sizes are required to combat statistical corrections for multiple testing, which force strict significance thresholds. Additionally, current arrays are designed to detect variants with minor allele frequency (MAF) of 0.05 or greater, suited to the common disease/common variant hypothesis, but insufficient to assess for multiple rare variants that may interact to influence phenotype, the alternative hypothesis (Altshuler et al., 2008). For these reasons GWAS are primed to detect common variants responsible for moderate to large effect sizes. Even with advancing array technology, the power required to detect less common variants (MAF <0.005), may mean prohibitively large sample sizes would be required. There remains a real risk of discounting true associations that do not meet the strict significance thresholds and combining GWAS data in meta-analysis is advised. It is important to note that SNPs associated with phenotypes of interest may not necessarily be causative, but may be in linkage disequilibrium (LD) and inherited with causal variants. Post-GWAS analysis including, interpretation of prior knowledge of candidate genes, re-sequencing and functional assessments can add value to variants identified by GWAS.

Next generation sequencing (NGS) or whole genome sequencing (WGS) describes high-throughput modern sequencing techniques and can be used for in depth assessment of limited numbers of genes, whole exome examination or whole genome examination, mapped to the human reference genome (Goodwin et al., 2016). WGS requires specific

infrastructure and expertise and needs to run with large batches of samples to be cost-effective. As a result, WGS is more suited to supra-regional or national coordination.

Specific to pharmacogenomics, WGS has huge potential to examine for the effects of variants with MAF <0.005 on phenotype, including the interactions between panels of variants, known to be problematic with GWAS. WGS allows assessment of variants in non-coding and non-gene regions, and can subsequently offer insight into regulatory sequences and untranslated regions. WGS is also well-placed to elucidate the variome, evolutionary variants accumulated within populations over relatively short time periods, which may be particularly relevant when considering population exposure to commonly used drugs (Pavlopoulos et al., 2013).

#### **Successes in cardiovascular medicine**

Warfarin, widely used for anticoagulation, is a racemic mix of R and S enantiomers. Warfarin antagonises vitamin K epoxide reductase complex subunit 1 (VKORC1). Warfarin's potent S-enantiomer is metabolised and inactivated by CYP2C9. Warfarin has a narrow therapeutic window, with wide variation in individual daily dose requirements, and patients spend approximately a third of their time outside the therapeutic range (Caraco et al., 2008). Response must be closely monitored via the international normalised ratio (INR) to avoid dose-related adverse effects.

The non-coding *VKORC1* SNP rs9923231 (-1639G>A; G3673A) is associated with lower warfarin dose requirements in several ethnic groups and is responsible for 20-25% of dose variation in Caucasians, and Asians (Johnson et al., 2011). *CYP2C9* allelic variants \*2 (rs1799853) and \*3 (rs1057910) encode enzymes with reduced activity, associated with prolonged warfarin half-life and reduced therapeutic dose requirements (Johnson et al., 2011). A European RCT recently evaluated the utility of genotype-guided warfarin dosing, compared to standard care. Use of an algorithm incorporating clinical and genetic factors

resulted in increased time spent within the therapeutic range, both with intention-to-treat (ITT) and per-protocol analysis (7%  $p=0.001$ , 6.6%  $p=0.001$ , respectively) an effect which was more beneficial with increasing number of variants present in the individual (Pirmohamed et al., 2013). Additionally, genotype-guided dosing has been shown to be a cost-effective strategy for the UK health system (Verhoef et al., 2016). A pilot study is now being undertaken with NWC AHSN, to assess the feasibility of clinical implementation of point-of-care genotyping and genotype-guided warfarin dosing using a combined clinical and genetic internet-based algorithm.

Clopidogrel is an important antiplatelet agent associated with highly variable clinical responses. Clopidogrel's active metabolite binds to platelet membrane purinergic P2Y<sub>12</sub> receptors to irreversibly inhibit ADP-dependent platelet aggregation. CYP2C19 is involved in Clopidogrel metabolism and formation of the active metabolite. *CYP2C19* loss of function alleles \*2 (rs4244285) and \*3 (rs4986893) are represented in African, Asian and Caucasian populations and result in decreased levels of clopidogrel's active metabolite and increased high-on-treatment platelet reactivity (HTPR) (Holmes et al., 2011). Meta-analyses have reliably demonstrated an association with adverse events (Wurtz et al., 2013, Mega et al., 2010, Hulot et al., 2010). Importantly the deleterious effects of \*2 and \*3 heterozygotes on clopidogrel response can be partially compensated by dose escalation, with evidence of a gene dosage effect with these variants as the same is not true for homozygotes (Mega et al., 2011). At present, the clinical utility of *CYP2C19* genotyping remains controversial and routine genotyping is not currently supported. However, it is recommended that alternative antiplatelets are prescribed for patients undergoing percutaneous coronary interventions (PCI) with known *CYP2C19* loss of function variants (Johnson et al., 2017). Experience with genotype-guided dosing in warfarin may, in some regards, translate to clopidogrel via point-of-care genotyping and algorithmic incorporation of clinical and genetic factors dictating response

Despite promising evidence, cardiovascular pharmacogenomics-based testing has encountered adoption and implementation challenges. A significant amount of work remains if these proven associations are to be successfully progressed into the clinical environment.

### **Potential for pharmacogenomics in obstetrics**

Whilst 65% of pregnant women take a drug other than vitamins or iron salt in pregnancy, there are safety and/or efficacy concerns regarding the use of more than 90% of drugs in pregnancy (Andrade et al., 2004, Mitchell et al., 2011, Adam et al., 2011). Pharmacokinetic and pharmacodynamic knowledge specific to pregnancy is lacking for most drugs, with doses selected on the strength of data derived from healthy males and only 10% of registered trials in pregnancy collecting pharmacokinetic/pharmacokinetic data (Endicott and Haas, 2012). For these reasons this is an important area in which to outline genomic determinants of drug response. Cell-free fetal DNA has entered the mainstream and the fetal genome can now also be sequenced non-invasively (Kitzman et al., 2012). The value of both maternal and fetal genotyping in disease susceptibility, particularly pre-eclampsia has already been highlighted (GOPEC, 2005). Technological advances have introduced the possibility of in-utero assessment of pregnancy-specific disease susceptibility and impact of drugs on the fetus. There are certain advantages to conducting pharmacogenomics studies in pregnant populations. Experience and expertise already accumulated in other research disciplines can be translated into this population. Pregnant women are generally engaged and motivated with the expectation of a high degree of healthcare contact, all of which may serve to ease participation in clinical research. Additionally, there is the advantage of a shorter timeframe to realise clinical end-points.

Pharmacogenomics in pregnancy is a developing field. The pharmacogenomics of many commonly prescribed drugs, including aspirin, have not been assessed in these populations. However, key examples of the value of pharmacogenomic information for prescribing in pregnancy are now emerging. Codeine, frequently prescribed for postpartum and postoperative pain relief, is metabolised by CYP2D6 to morphine and UGTB7 facilitates its excretion. CYP2D6 is induced in pregnancy and variant alleles are associated with different metabolic activity (Wadelius et al., 1997). Women possessing variants denoting them as ultra-rapid codeine metabolisers, especially when combined with a *UGTB7* variant causing diminished excretion, are at risk of personal toxicity and toxicity for their breast-fed infants (Madadi et al., 2009). As a direct result the FDA and EMA have advised against the use of codeine for breastfeeding women (FDA, 2007, EMA, 2013). However, other drugs commonly used in pregnancy, are also metabolised by highly polymorphic cytochrome P450 enzymes, including beta blockers and SSRIs (CYP2D6), nifedipine (CYP3A family) and indomethacin (CYP2C9, CYP2C19) (Haas, 2014). Genomic variants affecting cytochrome P450 enzymes are among the most prominent in pharmacogenomics research to date, but effects on drug efficacy and toxicity are not limited to this group.

### **Barriers to implementation**

General barriers to clinical implementation of pharmacogenomics tests can be organised into those concerning evidence, finances, logistics and clinical knowledge (Johnson and Cavallari, 2013, Turner and Pirmohamed, 2014). Current methodological understanding supports of discovery, replication and validation of pharmacogenomic associations. However for pharmacogenomics evidence the traditional hierarchy, which values RCTs, needs to be intelligently and flexibly reinterpreted (Turner and Pirmohamed, 2014). There is increasing appreciation that RCTs may not be feasible or appropriate for all associations and the level of evidence required to drive change should reflect the potential for patient

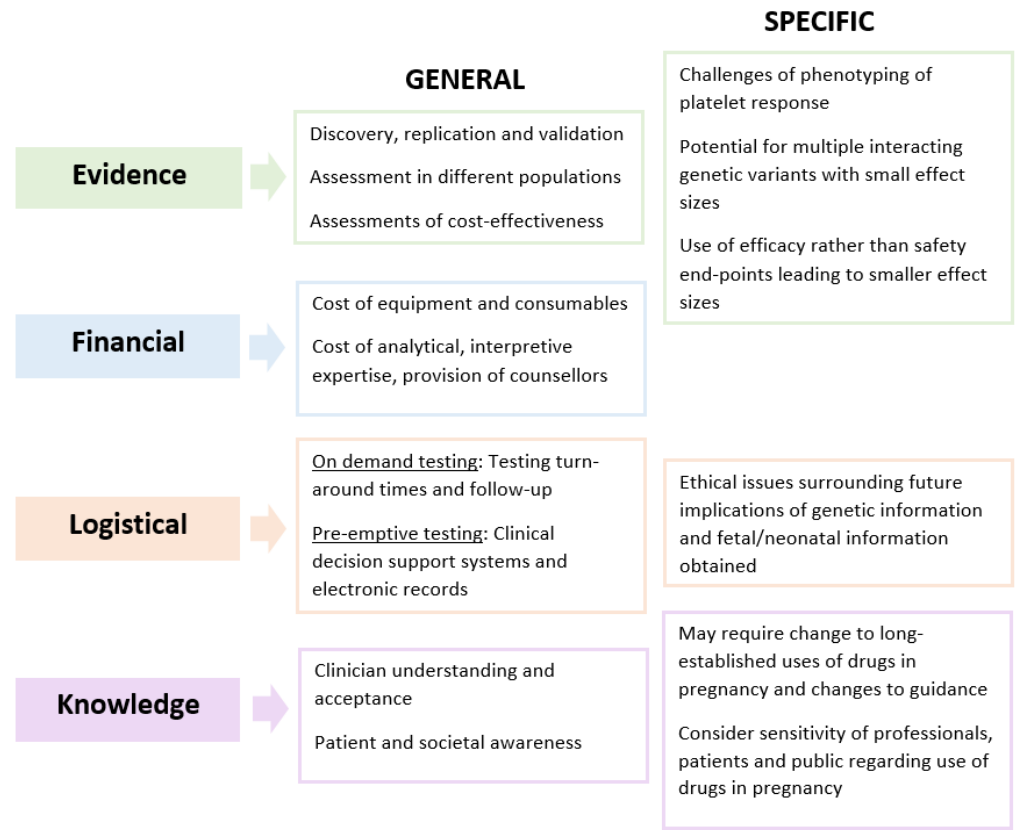
benefit in each situation (Manolio et al., 2013). Logistical and financial factors relating to the cost and feasibility of conducting tests in the clinical environment, including turn-around times and provision of facilities for interpretation and reporting are also influential. There is ongoing consideration of the potential benefits of pre-emptively embedding pharmacogenomic data into electronic patient records, versus an on demand approach to testing (Chisholm, 2013). The former option requires intensive financial and computational investment, with the aim of addressing many of the test-related logistical concerns discussed, and supporting a pre-emptive rather than reactionary approach to the use of pharmacogenomics information for the future. Ensuring clinicians understand and accept evidence underpinning use of pharmacogenomics tests and have the required knowledge to confidently interpret and apply results in their clinical area is vital. The scale of change required in this regard is likely to require specific educational programmes in addition to individual self-directed learning and motivation of healthcare professionals involved to make this investment. To facilitate incorporation of pharmacogenomics into clinical care, knowledge must reach patients and society at large. On an individual basis the counselling and informed consent process is central, but awareness will need to be raised within healthcare systems, patient groups and the media to ensure coverage. For the combination of reasons outlined there is currently a 15 to 20 year time lag between the discovery of a validated cardiovascular drug pharmacogenomic association and clinical implementation, as typified by warfarin and *VKORC1/CYP2C9* variants.

In the context of implementing pharmacogenomic testing for cardiovascular drugs, considering aspirin in particular, there are some unique challenges. Detailed phenotyping is considered key for all pharmacogenomics studies. For investigation of aspirin responsiveness, adherence must be established and response assessed using platelet function assays that better reflect pharmacodynamics to arrive at a meaningful classification of response to aspirin. Aspirin's relatively uncomplicated pharmacokinetics

do not involve highly polymorphic metabolic enzymes, such as the cytochrome P450s that have been implicated in variable response to warfarin and clopidogrel. It is more plausible that if variable response to aspirin is mediated by genetic factors, this is due to interplay of multiple variants, which may not be ideally suited to detection with GWAS and require fresh approaches.

In contrast to pharmacogenomics studies for other drug classes, most cardiovascular variants are assessed on efficacy rather than safety end-points. The fundamentally smaller effect sizes mean it is more difficult to definitively demonstrate associations. Additionally, most cardiovascular drugs investigated are licensed, off patent and in longstanding and widespread clinical use. Whilst there are benefits to clinicians having familiarity and appreciation of the importance of these drug responses, there is the considerable hurdle of changing established clinical practice on a large scale if associations are confirmed.





**Figure 9:** Barriers to implementation of pharmacogenomics of aspirin response in pregnancy. \*adapted from 'Cardiovascular Pharmacogenomics: Expectations and Practical Benefits (Turner and Pirmohamed, 2014).

### **1.5 Rationale for the thesis**

LDA at the current dose advised in the UK (75mg once daily) confers modest reduction in the risk of pre-eclampsia and associated IUGR, according to the best available data (Askie et al., 2007). Despite extensive basic science and clinical research, the causation and pathophysiology of pre-eclampsia remains incompletely understood. Additionally, significant questions remain unanswered: Which subgroups of women glean the most benefit from LDA, the optimum gestational age to commence LDA and how to dose pregnant women for optimal prevention. It is clear that inadequate placentation in early pregnancy is associated with pre-eclampsia, with evidence of prostaglandin imbalance and increased platelet activation detectable early in affected pregnancies. Aspirin non-responsiveness is a relatively new concept in pre-eclampsia research. Further investigation of platelet and clinical response to aspirin may provide fresh perspective on the efficacy of LDA, with potential applications for prediction and prevention of pre-eclampsia. There should be an emphasis on measuring platelet response to aspirin, using validated COX-specific assays, in the first trimester and early second trimester, when modification of preventative treatment may yield more benefit to the developing placenta.

#### **Aims**

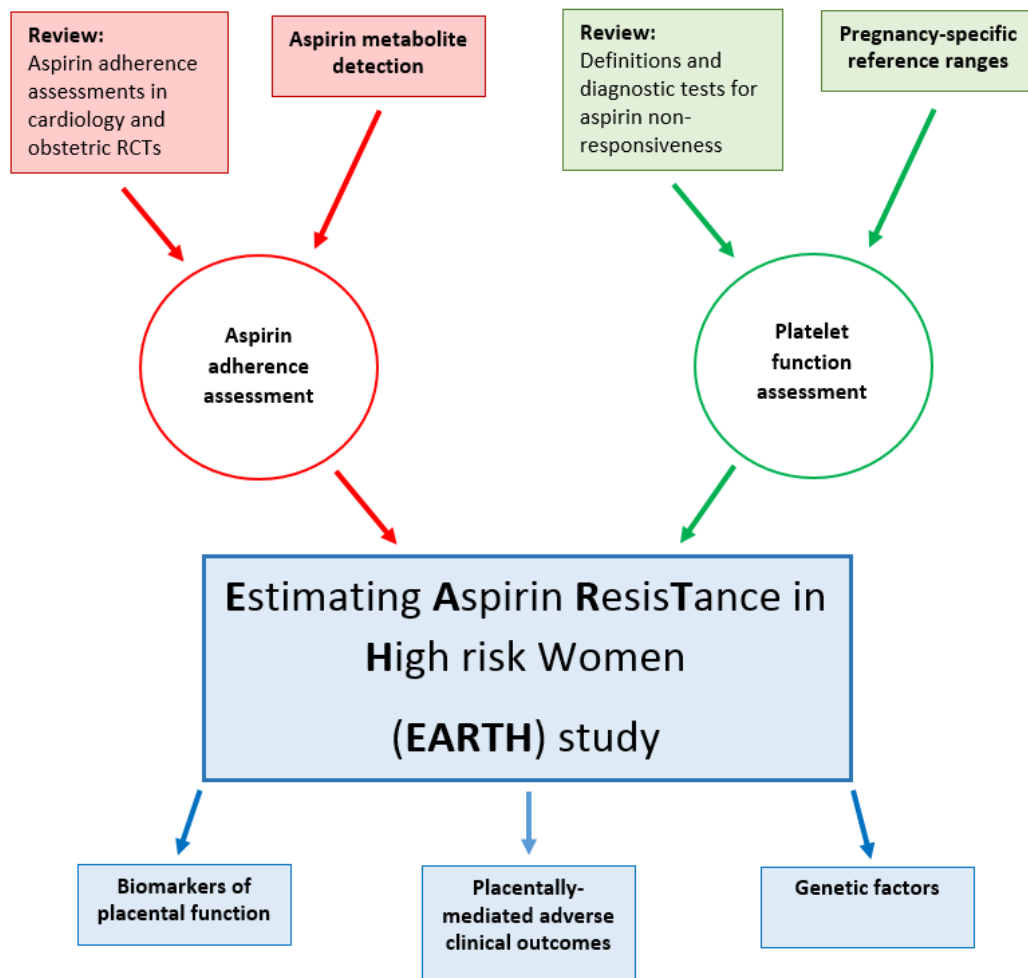
1. The primary aim of my thesis is to investigate whether variability in response to aspirin in pregnant women at high risk of pre-eclampsia exists.
2. In particular, I aim to investigate whether associations between individual platelet response to aspirin and placentally-mediated adverse pregnancy outcomes exist, and consider if this knowledge can be applied to personalise prevention of pre-eclampsia.

3. A further aim is to conduct an unbiased genome-wide assessment of genetic factors that may influence response to aspirin for prevention of pre-eclampsia in pregnant women at high risk of pre-eclampsia.

## **Objectives**

1. To review published reports of studies examining aspirin non-responsiveness across all speciality areas (Figure 10). In particular; to appraise the definitions of aspirin non-responsiveness described, diagnostic methods, incorporation of aspirin adherence assessments in study protocols and correlation of aspirin non-responsiveness with adverse clinical outcomes.
2. To review aspirin adherence assessments used in RCTs evaluating the efficacy of LDA (Figure 10). In particular, to compare long-established used of aspirin for secondary prevention of occlusive vascular events in cardiology RCTs with obstetric RCTs. To assess the proportion of RCTs considering aspirin adherence in their methodology, specific assessments described and reporting of aspirin adherence data.
3. To develop a reliable strategy to assess aspirin adherence in pregnant women (Figure 8).
  - a. Explore exact methods of drug and/or drug metabolite detection from biological samples that may easily be obtained and stored during clinical studies.
  - b. Include this adherence assessment strategy in the clinical research protocol and analyse clinical data obtained according to participants' aspirin adherence status.

4. To design and conduct a prospective cohort study to evaluate platelet response to aspirin in aspirin-treated pregnant women at high risk of pre-eclampsia, according to NICE criteria (Figure 10).
  - a. Perform longitudinal measurements of platelet response to aspirin using COX-specific validated platelet function assays assessed against the pregnancy-specific reference ranges described. Use this to define the proportion of women who are non-responsive to aspirin.
  - b. Assess if aspirin non-responsiveness is related to markers of declining placental function by assessing correlation with abnormal maternal and fetal Doppler ultrasound indices and third trimester assessment of PIGF and the sFlt-1/PIGF ratio.
  - c. Assess if aspirin non-responsiveness is related to placentally-mediated adverse clinical outcomes, including pre-eclampsia, IUGR and fetal loss.
5. Carry out a genome-wide assessment to investigate genetic factors that may correlate with response to aspirin in women at high risk of pre-eclampsia (Figure 10).
  - a. Collect maternal DNA samples during the clinical study to contribute to a case-control GWAS. Independently complete quality control and analysis of this data.
  - b. Establish collaborations with other research groups interested in aspirin response in pregnancy to ensure an appropriately powered case-control GWAS study can be undertaken.



**Figure 10:** Thesis structure.

## **CHAPTER 2: Literature review of diagnostic tests and definitions of aspirin non-responsiveness**

## **2.1 Rationale**

The purpose of the work in this Chapter was to review published reports of studies examining aspirin non-responsiveness completed across all speciality areas. The rationale was to provide a foundation of knowledge for selection of tests of aspirin response prior to undertaking a clinical study in the pregnant population (Chapter 1, Objective 1, Figure 10). In particular, I set out to appraise the definitions of aspirin non-responsiveness already described and gain a comprehensive view of diagnostic methods (Chapter 1, Objective 1, Figure 10). Additionally, I wanted to assess whether assessments of aspirin adherence had been incorporated in the protocols of studies assessing aspirin non-responsiveness and whether authors had assessed aspirin non-responsiveness in relation to adverse clinical outcomes (Chapter 1, Objective 1, Figure 10). This work was published as a review article in British Journal of Obstetrics and Gynaecology on the 1<sup>st</sup> of March 2016, the figures and text contained within this Chapter are reproduced from this article (Navaratnam et al., 2016).

## **2.2 Methods**

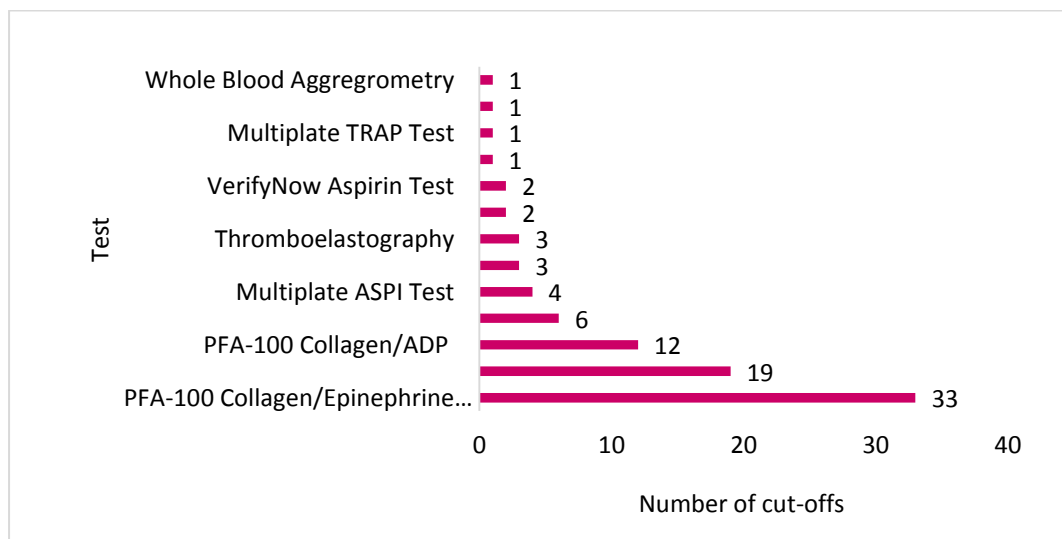
We searched MEDLINE, EMBASE and the Cochrane Library from 1957-28/02/15, limited to humans and English language. The search terms used were 'aspirin', 'acetylsalicylic acid' appearing adjacent to 'resistance', 'non-responsiveness', 'treatment failure' and pseudoresistance. All original articles were included, review articles were excluded. Additionally, we excluded articles relating to studies where participants received concomitant alternative antiplatelet agents or anticoagulants. This search yielded 492 articles, after abstract and full text reviews, 135 were included.

## **2.3 Results**

### **Tests and definitions**

There is now a broad range of experience with platelet function testing across speciality areas including; healthy individuals, cardiology, stroke, endocrinology, nephrology,

rheumatology, general medicine, general surgery, vascular surgery, paediatrics and high-risk obstetrics (Table 1). Our review identified thirteen platelet function assays, the three most commonly used were PFA-100™ collagen/epinephrine (Coll/Epi) cartridges, light transmission aggregometry (LTA) with arachidonic acid induction and PFA-100™ collagen/ADP (Coll/ADP) cartridges (Figure 1, expanded information in Table 1). In the examined literature, PFA-100™ collagen/epinephrine cartridges were associated with 33 different cut-offs, the commonest being a closure time of <165 seconds (Figure 1, Table 1). LTA had 19 proposed cut-offs, the commonest being mean aggregation of  $\geq 20\%$ . PFA-100™ Coll/Epi cartridges had 12 different cut-offs, the commonest being a closure time of <114 seconds (Figure 1, Table 1). The majority of studies refer to manufacturer's cut-offs, or have conducted in-house work to establish cut-offs from small numbers of predominantly male volunteers. Subsequently, there is limited applicability of these cut-offs to obstetric populations.



**Figure 1:** Platelet function assays reported with their number of different cut-offs.



Assay	No. of cut-offs	Specialities	No. of References
Flow Cytometry	3	Cardiology, Stroke Medicine/Vascular	3 (Macchi et al., 2002, Assadian et al., 2007, Hübl et al., 2007)
Ivy Bleeding Time	1	Cardiology	1 (Chakroun et al., 2004)
LTA/Optical Aggregometry	19	Vascular, Cardiology, General Medicine/Surgery, Stroke Medicine, Diabetes/Endocrine, Healthy, Paediatrics	17 (Linnemann et al., 2009, Lordkipanidzé et al., 2007, Schwartz et al., 2005, Renda et al., 2010, Harrison et al., 2008, Nielsen et al., 2008, Feher et al., 2008, Albert et al., 2005, Blais et al., 2009, Yee et al., 2008, Gum et al., 2003, Berrouschot et al., 2006, Guthikonda et al., 2008, Faraday et al., 2006, Sagdilek et al., 2011, Feher et al., 2006, Nidhinandana and Changchit, 2010)
Multiplate™ ASPI Test	4	Cardiology, Stroke Medicine, Rheumatology, Nephrology	4 (Jastrzębska et al., 2013, Petricević et al., 2013, Akdogan et al., 2013, Kilickesmez et al., 2012)
Multiplate™ TRAP Test	1	Rheumatology	1 (Akdogan et al., 2013)
PFA-100™ Collagen/ADP Closure Time	12	Cardiology, Stroke Medicine, General Medicine/Surgery, Vascular Surgery, Paediatric Cardiology	16 (Boncoraglio et al., 2009, Christiaens et al., 2008, McCabe et al., 2005, Chakroun et al., 2004, Christiaens et al., 2002, Gum et al., 2001, Hobikoglu et al., 2005, Pamukcu et al., 2007, Williams et al., 2006, Grundmann et al., 2003, Renda et al., 2010, Wong et al., 2006, Andersen et al., 2002, Pamukcu et al., 2008, Heistein et al., 2008, Jussen et al., 2013)
PFA-100™ Collagen/Epinephrine Closure Time	33	Cardiology, Stroke Medicine, Vascular Surgery, Diabetes/Endocrinology, General Medicine/Surgery, Healthy, Nephrology, Paediatric Cardiology, General Paediatrics	48 (Linnemann et al., 2009, Hobikoglu et al., 2007, Boncoraglio et al., 2009, Christiaens et al., 2008, Poulsen et al., 2007a, McCabe et al., 2005, Addad et al., 2010, Aydinalp et al., 2010, Chakroun et al., 2004, Christiaens et al., 2002, Coma-Canella et al., 2005, Faraday et al., 2006, Golanski et al., 2005, Gum et al., 2001, Hobikoglu et al., 2005, Lordkipanidzé et al., 2007, Macchi et al., 2002, Narvaez et al., 2007, Pamukcu et al., 2007, Williams et al., 2006, Grundmann et al., 2003, Fateh-Moghadam et al., 2005, Tasdemir et al., 2014, Renda et al.,

			2010, Golanski et al., 2004, Harrison et al., 2008, Assadian et al., 2007, Hübl et al., 2007, Wong et al., 2006, Muir et al., 2009, Andersen et al., 2002, Acikel et al., 2009, Chakroun et al., 2007, Pamukcu et al., 2008, Cohen et al., 2008, Kahraman et al., 2007, Bozic-Mijovski et al., 2008, Takahashi et al., 2007, Tschopp and Reinhart, 2008, Acikel et al., 2008, Akoglu et al., 2012, Postula et al., 2010)
Platelet Count Drop	1	Healthy	1 (Blais et al., 2009)
Serum Thromboxane B <sub>2</sub>	2	Cardiology, General Medicine/Surgery, Healthy	3 (Frelinger, 2006, Frelinger et al., 2008, Renda et al., 2010)
Thromboelastography	3	Healthy, Stroke Medicine, Cardiology	4 (Blais et al., 2009, Englyst et al., 2008, Cao et al., 2012, Tantry et al., 2005)
Urinary 11-Dehydrothromboxane B <sub>2</sub>	6	Cardiology, Diabetes/Endocrinology, Healthy, Paediatrics, Stroke Medicine, High-risk obstetrics	8 (Thomson et al., 2009, Faraday et al., 2006, Goncalves et al., 2011, Bordeaux et al., 2010, Eskandarian et al., 2012, Blais et al., 2009, Yee et al., 2008, Wojtowicz et al., 2011)
VerifyNow™ Aspirin Test	2	Cardiology, Healthy, Stroke Medicine, Diabetes/Endocrine, Nephrology	17 (Chen et al., 2007, Can et al., 2010, Lordkipanidzé et al., 2007, Mirkhel et al., 2006, Dyszkiewicz-Korpanty et al., 2007, Harrison et al., 2008, Kim et al., 2010, Lee et al., 2005, Nielsen et al., 2008, Yassine et al., 2010, Blais et al., 2009, Geara et al., 2012, Dawson et al., 2012, Seok et al., 2008, Dawson et al., 2011, Kim et al., 2009, Vaturi et al., 2013)
Whole Blood Aggregometry	1	Cardiology	2 (Golanski et al., 2005, Lordkipanidzé et al., 2007)

**Table 1:** Platelet function assays reported by speciality.

\*LTA=light transmission aggregometry, PFA-100=platelet function analyser

In total, eighty-eight different definitions of suboptimal platelet response to aspirin have been described, the vast majority utilise only laboratory-based parameters to delineate responsiveness to aspirin. One study defined suboptimal response clinically, as the occurrence of myocardial infarction in patients with stable coronary artery disease whilst on aspirin therapy (Neergaard-Petersen et al., 2013). Another described a combined definition, with laboratory assessment of 11-dehydrothromboxane B<sub>2</sub> (11-DHTXB<sub>2</sub>) and ischaemic cardiovascular or cerebrovascular events (Eikelboom et al., 2002). We identified 24 studies where suboptimal response to aspirin was linked to clinical outcomes, fourteen in cardiology, six in stroke, one in nephrology and three in obstetrics (Table 2).

Obstetric studies were carried out in pregnant women at high-risk of pre-eclampsia, including two prospective cohort studies, one case-control study and a dose escalation study from groups in the UK, Canada and Poland (Wojtowicz et al., 2011, Caron et al., 2009, Rey and Rivard, 2011, Sullivan and Elder, 1993) (Table 2). Two studies used PFA-100 Coll/Epi cartridges, with diagnostic cut-offs determined from the mixed adult population (Rey and Rivard, 2011, Caron et al., 2009). One study utilised 11-DHTXB<sub>2</sub>, using locally determined cut-offs (Wojtowicz et al., 2011), and one study used the LTA method (Sullivan and Elder, 1993). None of the obstetric studies defined pregnancy-specific reference ranges for platelet function with the assays they used (Caron et al., 2009, Rey and Rivard, 2011, Wojtowicz et al., 2011, Sullivan and Elder, 1993). In these high-risk obstetric populations, suboptimal platelet response to aspirin was identified in 29-39% of participants and associated with increased pre-eclampsia, preterm birth and delivery of small for gestational age infants (Caron et al., 2009, Rey and Rivard, 2011). Additionally, Rey et al assessed the impact of PFA-100<sup>TM</sup> guided aspirin dose escalation and determined that women requiring escalation had a higher risk of pre-eclampsia (11/43, 25.6% vs. 6/68, 8.8%, p = 0.03) (Caron et al., 2009).

Authors	Speciality	Study Design	Assay	No. Participants	Clinical outcomes reported
Chu 2010 (Chu et al., 2010)	Cardiology	Prospective dose escalation study	VerifyNow™ Aspirin Test	314	Acute MI (index admission), major adverse cardiac events including cardiovascular death or recurrent ACS requiring hospitalization within 6 months.
Poulsen 2007 (Poulsen et al., 2007b)	Cardiology	Prospective cohort study	PFA-100™ Coll/Epi cartridges	277	Acute MI at index admission.
Cotter 2004 (Cotter et al., 2004)	Cardiology	Prospective cohort Study	Plasma TXB <sub>2</sub>	73	Cardiovascular events, including death, reinfarction, and readmission for unstable angina, and cardiovascular readmission.
Glaser 2010 (Glaser et al., 2010)	Cardiology	Prospective cohort study	VerifyNow™ Aspirin Test	200	Composite end point; 30-day revisit, positive cardiac catheterization, or hospital length of stay longer than 3 days
Chen 2007 (Chen et al., 2007)	Cardiology	Prospective cohort study	VerifyNow™ Aspirin Test	468	Composite of cardiovascular death, myocardial infarction (MI), unstable angina requiring hospitalization, stroke, and transient ischemic attack.
Hobikoglu 2007 (Hobikoglu et al., 2007)	Cardiology	Prospective cohort Study	PFA-100™ Coll/Epi Cartridges	140	Composite of death, myocardial infarction, cerebrovascular accident and revascularization.
Cao 2012 (Cao et al., 2012)	Cardiology	Prospective cohort study	LTA, TEG	269	Composite of death, myocardial infarction, unstable angina, stroke and transient ischemic attack.
Christiaens 2008 (Christiaens et al., 2008)	Cardiology	Prospective cohort study	PFA-100™ Coll/Epi cartridges	97	Composite of death, myocardial infarction, and ischemic cerebral infarction or acute limb ischemia.
Poulsen 2007 (Poulsen et al., 2007a)	Cardiology	Prospective cohort study	PFA-100™ Coll/Epi cartridges	187	Death, admission with recurrent MI, stroke or mechanical revascularization of atherothrombotic disease.

Eikelboom 2002 (Eikelboom et al., 2002)	Cardiology	Nested case-control study	Urinary 11-DHTXB <sub>2</sub>	976 total (including 488 controls)	Composite outcome of MI, stroke, or cardiovascular death.
Addad 2010 (Addad et al., 2010)	Cardiology	Prospective cohort study	PFA-100™ Coll/Epi cartridges	204	Major Cardiac Adverse Events: cardiovascular death, MI, stroke or TIA. The secondary endpoint was the occurrence of Recurrent Acute Vascular Event; MI, stroke or TIA).
Pamukcu 2006 (Pamukcu et al., 2006)	Cardiology	Prospective cohort study	PFA-100™ Coll/Epi cartridges	105	MI, unstable Angina, Cardiac Death
Andersen 2002 (Andersen et al., 2002)	Cardiology	Randomised comparative study	PFA-100™ Coll/Epi cartridges	71 (taking aspirin alone)	Non-fatal reinfarction/MI and stroke, secondary endpoint of need for revascularisations (CABG or percutaneous coronary intervention (PCI)).
Gum 2003 (Gum et al., 2003)	Cardiology	Prospective cohort study	LTA	326	Composite of death, MI, or cerebrovascular accident.
Sullivan 1993 (Sullivan and Elder, 1993)	High-risk obstetrics	Prospective cohort study	LTA	6	Pre-eclampsia
Rey 2011 (Rey and Rivard, 2011)	High-risk obstetrics	Retrospective cohort study	PFA-100™ Coll/Epi cartridges	270	Pre-eclampsia, early-onset pre-eclampsia, severe pre-eclampsia
Wojtowicz 2011 (Wojtowicz et al., 2011)	High-risk obstetrics	Prospective cohort study	Urinary 11-DHTXB <sub>2</sub>	43 (taking aspirin)	Preterm birth, pre-eclampsia, SGA infants.

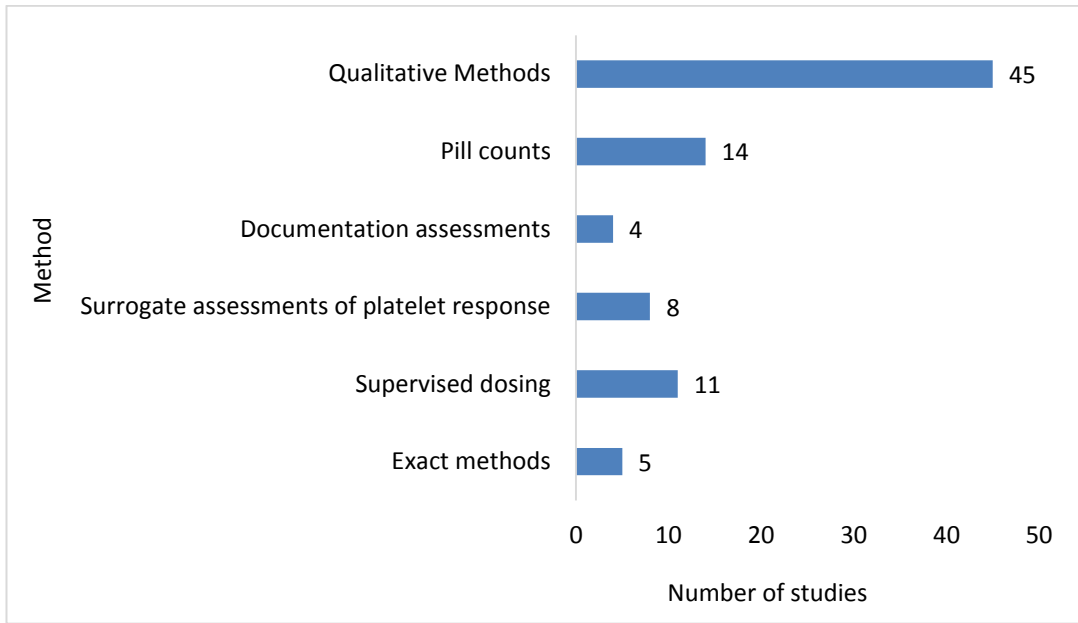
Kilickesmez 2013 (Kilickesmez et al., 2012)	Nephrology	Prospective Cohort Study	Multiplate™ ASPI Test	78	Composite of death, MI, unstable angina, or CVA. Secondary end points were the individual events of death, MI, unstable angina, and CVA.
Zheng 2013 (Zheng et al., 2013)	Stroke Medicine	Prospective cohort Study	VerifyNow™ Aspirin Test	90	Clinical stroke severity and infarct size.
Boncoraglio 2009 (Boncoraglio et al., 2009)	Stroke Medicine	Prospective cohort study	PFA-100™ Coll/Epi cartridges	129	Composite of stroke, TIA, myocardial infarction, and cardiovascular death.
Helgason 1994 (Helgason et al., 1994)	Stroke Medicine	Prospective dose escalation study	LTA	146	Cerebral ischaemic events.
McCabe 2005 (McCabe et al., 2005)	Stroke Medicine	Prospective cohort study	PFA-100™ Coll/Epi cartridges	103	Further stroke or TIA.
Lai 2012 (Lai et al., 2012)	Stroke Medicine	Prospective cohort study	PFA-100™ Coll/Epi cartridges	224	National Institutes of Health Stroke Scale and modified Rankin Scale scores
Berrouschott 2006 (Berrouschot et al., 2006)	Stroke Medicine	Prospective dose escalation study	LTA	291	Recurrent stroke.

**Table 2:** Clinical evaluation of aspirin non-responsiveness

\*PFA-100=platelet function analyser, Coll/Epi=collagen/epinephrine, Coll/ADP=collagen/ADP, TXB<sub>2</sub>=thromboxane B<sub>2</sub>, 11-DHTXB<sub>2</sub>=11-dehydrothromboxane B<sub>2</sub>, LTA=light transmission aggregometry, TEG=Thromboelastography.

### **Aspirin adherence testing**

To assess the clinical impact of suboptimal platelet response to aspirin, it is vital that true non-responsiveness can be differentiated from suboptimal adherence. In cardiovascular research patient non-adherence with medication is reported to be widespread, at 30-50% (Hankey and Eikelboom, 2006). The existing literature demonstrates that there is no consensus on robust assessment of aspirin adherence (Figure 2, expanded information in Table 1). Where researchers have endeavoured to quantify the impact of suboptimal adherence, most have relied on qualitative measures including patient enquiries, questionnaires and drug diaries which are likely to underestimate the true scale of the problem. In our review 5/87 (6%) relied on assessment of serum or urine thromboxane, both considered to be measures of platelet activation (Table 3). However, elevated thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and 11-DHTXB<sub>2</sub> may be secondary to non-platelet sources and therefore not a specific reflection of platelet COX inhibition by aspirin. Importantly, only 2/87 (2%) studies measured salicylate levels, both in patients taking aspirin for cardiovascular disease prevention (Table 3). Three out of four obstetric studies considered the issue of adherence, and undertook verbal enquiries at study visits (Caron et al., 2009, Rey and Rivard, 2011, Sullivan and Elder, 1993). Biochemical assessment of aspirin or aspirin metabolites has not been assessed in pregnant populations.



**Figure 2:** Aspirin adherence assessment methods.



Methods of adherence assessment	No. of studies	Citations
Case/prescription records	4	(Lee et al., 2005, Dawson et al., 2012, Harrison et al., 2008, McCabe et al., 2005)
Light Transmission Aggregometry	3	(Chakroun et al., 2004, Albert et al., 2005, Berrouschot et al., 2006)
Observed dosing/Inpatient population	11	(Glauser et al., 2010, Jastrzębska et al., 2013, Rajec et al., 2008, Schwartz et al., 2005, Williams et al., 2006, Grundmann et al., 2003, Kovacs et al., 2013, Yokoyama et al., 2014, Bennett et al., 2008, Berrouschot et al., 2006, Zheng et al., 2013)
Patient drug diary	1	(Shen et al., 2009)
Pill counts	14	(Abaci et al., 2006, Bordeaux et al., 2010, Faraday et al., 2006, Neergaard-Petersen et al., 2013, Nielsen et al., 2008, Blais et al., 2009, Kovacs et al., 2013, Santilli et al., 2009, Shen et al., 2009, Tschopp and Reinhart, 2008, Helgason et al., 1994, Helgason et al., 1993, Assadian et al., 2007, Wong et al., 2006)
Questionnaires	6	(Bordeaux et al., 2010, Faraday et al., 2006, Gum et al., 2001, Vaturi et al., 2013, Mehta et al., 2006, Seok et al., 2008)
Salicylic acid/aspirin metabolite quantification	2	(Dawson et al., 2011, Crowe et al., 2005)
Sample analysis with in vitro aspirin/salicylic acid	3	(Frelinger, 2006, Frelinger et al., 2008, Gonzalez-Conejero et al., 2005)
Serum, plasma or urine Thromboxane B2 quantification	5	(Andersen et al., 2002, Blann et al., 2013, Cotter et al., 2004, Neergaard-Petersen et al., 2013, Nielsen et al., 2008)
Verbal Enquiry/Interviews	38	(Acikel et al., 2009, Can et al., 2010, Blann et al., 2013, Chakroun et al., 2007, Christiaens et al., 2008, Christiaens et al., 2002, Cotter et al., 2004, Gulmez et al., 2007, Lee et al., 2005, Lordkipanidzé et al., 2007, Macchi et al., 2002, Muir et al., 2009, Narvaez et al., 2007, Neergaard-Petersen et al., 2013, Nielsen et al., 2008, Ozben et al., 2010, Poulsen et al., 2007b, Poulsen et al., 2007a, Tantry et al., 2005, Santos et al., 2008, Fateh-Moghadam et al., 2005, Blais et al., 2009, Ohmori et al., 2006, Caron et al., 2009, Rey and Rivard, 2011, Sullivan and Elder, 1993, Heistein et al., 2008, Boncoraglio et al., 2009, Bruno, 2004, Dawson et al., 2012, Englyst et al., 2008, Feher et al., 2006, Feher et al., 2008, Harrison et al., 2008, Helgason et al., 1994, McCabe et al., 2005, Linnemann et al., 2009, Wong et al., 2006)

**Table 3:** Aspirin adherence assessment methods in studies investigating aspirin non-responsiveness.

## 2.4 Discussion

### **Proposed causal mechanisms for aspirin non-responsiveness with confirmed adherence**

Where adherence is reliably confirmed, the remainder of individuals with persistent suboptimal suppression of platelet activation are likely to represent a heterogeneous group with interacting physiological, pharmacokinetic and pharmacodynamic factors.

In healthy adults 10% of circulating platelet numbers are replaced with aspirin naïve platelets daily. It is accepted that acetylsalicylic acid irreversibly inhibits cyclooxygenase 1 (COX-1) in exposed platelets and with regular dosing adequate global inhibition of platelet COX activity is maintained. Interestingly, recent work has demonstrated messenger RNA sequence coding for COX-1 within platelets, raising a possibility that generation of new COX-1, following inhibition of the native enzyme, may circumvent aspirin's antiplatelet action.

Patients may be insufficiently treated with aspirin for a variety of reasons including problems with total dose, inappropriate dosing intervals or dose delivery issues. Enteric coated aspirin in particular has been implicated in findings of suboptimal suppression of platelet activation as demonstrated by laboratory tests (Grosser et al., 2013). It is now clear that low-dose aspirin is equally effective for secondary prevention of ischaemic cardiovascular and cerebrovascular events when compared to high doses (ATT, 2002). However, there remain limitations in dose selection due to the range of low-doses currently manufactured (60-150mg). The standard low (75mg) daily dose recommended by NICE for secondary prevention of myocardial infarction and prevention of pre-eclampsia is more than double the dose required to maintain complete COX-1 inhibition in non-pregnant individuals.

Once daily dosing has become standard practice as the effects of aspirin are dependent of the rate of platelet turnover as opposed to the drug's half-life (Patrono and Patrono, 2015). However, incomplete maintenance of COX-1 suppression with once daily dosing has been described in association with states of increased platelet turnover in diabetes, obesity and postoperative states (Evangelista et al., 2007, Maree et al., 2005). Pregnancy is another state of enhanced platelet turnover for which, once daily dosing may be suboptimal. Daily low-doses of aspirin irreversibly inhibit COX-1 activity in platelets and impact COX-1 in megakaryocyte precursors, though the effect on megakaryocytes diminishes with accelerated platelet maturation (Patrono and Patrono, 2015). This is of particular relevance for obstetric populations where the increased platelet turnover results in more immature platelets within the circulation, which are prone to activate and aggregate readily.

Circadian timing of aspirin doses may also be of significance in pregnant women. Recently, evening dosing with LDA has been associated with beneficial effects on ambulatory blood pressure and decreased hazard ratios of a composite of serious adverse events in high-risk pregnant populations (Hermida et al., 1999, Ayala et al., 2013).

### **Implications for research in obstetrics**

It is vital that true suboptimal response to aspirin is distinguished from aspirin non-adherence. We firmly believe that adherence testing should be based on detection and quantification of aspirin metabolites from maternal blood or urine.

Suboptimal response to aspirin in adherent patients does not currently have a uniform definition, nor has an adequately sensitive, specific diagnostic test emerged and this should be prioritised. Tests aligned to the COX pathway are likely to be of greatest clinical utility, particularly if point-of-care use is feasible.

Platelet function should be measured against pregnancy-specific reference ranges, and if suboptimal response to aspirin is identified, its clinical significance must be judged in light of clinically important outcomes.

Accurate and user friendly stratification of pregnant women according to response to aspirin and adherence should encourage interventional studies of new treatments with alternative targets within platelet activation pathways or the coagulation cascade (Askie et al., 2007). For genuine non-responders to standard LDA, randomised comparisons with aspirin dose escalation and low-molecular-weight heparin should be carried out. The outcomes of interest should not be restricted to platelet response to aspirin, but also include placentally-mediated adverse outcomes (pre-eclampsia, IUGR, placental abruption and stillbirths). Heterogeneity in response to aspirin due to genetic factors has not yet been investigated in pregnancy, and may provide a unique early, discriminative means of risk stratification.

## **2.5 Conclusions**

The pathophysiology of pre-eclampsia remains incompletely elucidated and it is reasonable that increased understanding of its evolution is likely to be key in guiding screening and interventional approaches. Further investigation of platelet response to LDA in high-risk pregnant women represents a new avenue of investigation into the relatively modest risk reduction in placentally-mediated disease observed with current therapy. If women with suboptimal response to aspirin have a concentration of adverse clinical events, this may prove not only an important research area for preventative therapy, but a valuable opportunity to stratify maternal and fetal surveillance in the near future. With current debates regarding the potential utility and cost-effectiveness of no test/treat all approaches to pre-eclampsia prevention, acceptability of such approaches to women and adherence with aspirin will be key issues (Hyde and Thornton, 2013, Werner

et al., 2015). Additionally, recent signals of increased risk of placental abruption will need to be rigorously assessed (Xu et al., 2015).

## **CHAPTER 3: Literature review of aspirin adherence assessments in randomised controlled trials**

### **3.1 Rationale**

The purpose of the work presented in this chapter was to review adherence assessments used in both cardiology and obstetric randomised controlled trials (RCTs) evaluating the efficacy of low-dose aspirin (LDA) (Chapter 1, Objective 2, Figure 10). This was done to establish knowledge of available methods to assess adherence prior to defining the strategy to assess aspirin adherence in a clinical study in pregnancy. I aimed to compare long-established use of aspirin for secondary prevention of occlusive vascular events in cardiology RCTs with more recent use in obstetric RCTs. Specifically, I set out to assess the proportion of RCTs in both disciplines that considered aspirin adherence in their methodology, the specific assessments described and whether and how RCTs had reported their aspirin adherence data (Chapter 1, Objective 2, Figure 10).

### **3.2 Aspirin adherence**

The importance of adherence with long-term medications for the effectiveness of healthcare systems has been recognised by the World Health Organisation (WHO), and influential factors relating to the patient, condition, therapy, socioeconomic status, and health system have been outlined (WHO, 2003). Aspirin non-responsiveness has been linked to patient characteristics such as sex, age, ethnicity, disease severity and inflammation resulting in the production of non-platelet thromboxane A<sub>2</sub> (TXA<sub>2</sub>).

Drug factors have also been highlighted including suboptimal dosing, drug delivery issues, and drug-drug interactions (Fitzgerald and Pirmohamed, 2011). Several studies have demonstrated that where platelet function assays indicate potential aspirin non-responsiveness, this is largely explained by suboptimal adherence (von Pape et al., 2005, Schwartz et al., 2008). Following interventions to target adherence, including assessment after supervised dosing or spiking *ex vivo* biological samples with acetylsalicylic acid (ASA), an appropriate platelet response to aspirin can usually be demonstrated (von Pape et al., 2005, Schwartz et al., 2008).

Drug efficacy describes the maximum achievable drug response and the capacity for therapeutic effect. In the context of clinical research it is usually effectiveness rather than efficacy that is assessed. Effectiveness incorporates efficacy and adherence with therapy to provide a more real-world estimate of clinical benefit. Adherence refers to whether medication is taken at the indicated intensity, at correct quantities and dose intervals and whether treatment persists for the intended duration (Ho et al., 2009). Suboptimal adherence is common, tends to increase with polypharmacy and is independently associated with increased morbidity and mortality (Ho et al., 2006). Disregarding adherence with the assigned medication in the context of clinical trials will cause the true effectiveness of the intervention to be underestimated.

Adherence assessments have been proposed as ‘the next frontier in quality improvement’, with many indirect and direct methods reported (Heidenreich et al., 2004). We divided methods for assessment of adherence to aspirin into quantitative, semi-quantitative and qualitative, as shown in Tables 1- 3.



QUANTITATIVE METHOD	ADVANTAGES	DISADVANTAGES
Measurement of ASA metabolites (using several methods including, LC: MS and NMR)	<ul style="list-style-type: none"> <li>• Direct assessment that allows detection and quantification of ASA metabolites</li> <li>• Robust and reproducible</li> <li>• May offer additional information regarding dosing</li> <li>• Potential for non-invasive sampling of urine</li> </ul>	<ul style="list-style-type: none"> <li>• Requires biological sampling</li> <li>• Requires laboratory assessment</li> <li>• Influenced by individual pharmacokinetic variability</li> <li>• Comparatively expensive</li> </ul>
Measurement of platelet response COX-specific assays should be favoured, including; serum TXA <sub>2</sub> and its stable metabolites. These can be measured by immunoassay, LTA, Multiplate™ and VerifyNow™	<ul style="list-style-type: none"> <li>• Illustrates downstream effects of ASA e.g. indirect assessment of impact on COX-dependent production of TXA<sub>2</sub></li> <li>• Point-of-care assays</li> <li>• Reference ranges available</li> <li>• Can be performed non-invasively, using urine samples</li> <li>• Correlated with clinical outcomes</li> </ul>	<ul style="list-style-type: none"> <li>• Not a true direct assessment</li> <li>• May be confounded by alternative platelet pathways, depending on the assay</li> <li>• Requires biological samples to be used and may require laboratory assessment e.g. immunoassays</li> <li>• Comparatively expensive</li> </ul>

**Table 1:** Advantages and disadvantages of quantitative adherence assessments.

\*ASA=acetylsalicylic acid, LC: MS=liquid chromatography mass spectrometry, NMR=nuclear magnetic resonance, COX=cyclooxygenase, TXA<sub>2</sub>= thromboxane A<sub>2</sub>, LTA=light transmission aggregometry.

SEMI-QUANTITATIVE METHOD	ADVANTAGES	DISADVANTAGES
Observed dosing	<ul style="list-style-type: none"> <li>• Allows reasonable assessment of number of pills ingested</li> <li>• Easy to perform and can be integrated with follow-up visits</li> </ul>	<ul style="list-style-type: none"> <li>• Requires attendance at clinic/research unit</li> <li>• Difficult for participants and staff to sustain if longitudinal data required</li> <li>• Not a true direct assessment as pills may not be swallowed</li> </ul>
Pill counts, medication weighing, blister pack inspection	<ul style="list-style-type: none"> <li>• Allows reasonable assessment of number of pills likely to have been ingested</li> <li>• Easy to perform and can be integrated with follow-up visits</li> <li>• Accuracy improved with electronic counters or weights</li> </ul>	<ul style="list-style-type: none"> <li>• Not true direct assessments</li> <li>• Do not capture information for timing of doses and dose intervals</li> <li>• Manual counts remain prone to counting errors</li> <li>• Vulnerable to tampering and pill dumping</li> </ul>
Examination of prescription records	<ul style="list-style-type: none"> <li>• Correlates well with number of doses ingested</li> <li>• Easy to obtain from electronic records</li> <li>• Validated methods (medication possession ratio, proportion of days covered)</li> <li>• Accepted definitions of adherence</li> </ul>	<ul style="list-style-type: none"> <li>• Not true direct assessments</li> <li>• Requires collaboration with pharmacy and or general practitioner</li> <li>• Do not capture information for timing of doses and dose intervals</li> <li>• Vulnerable to manipulation via pill dumping or storing</li> </ul>

**Table 2:** Advantages and disadvantages of semi-quantitative adherence assessments.

QUALITATIVE METHOD	ADVANTAGES	DISADVANTAGES
Adherence questionnaires	<ul style="list-style-type: none"> <li>• Generally acceptable to participants</li> <li>• Can be completed face to face, via telephone or post</li> <li>• Validated questionnaires (Morisky Scale)</li> <li>• Correlated with clinical outcomes</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect assessments</li> <li>• Vulnerable to recall problems if not completed contemporaneously</li> <li>• May be adversely impacted by dishonesty and over-optimistic reporting due to social desirability</li> </ul>
Interviews	<ul style="list-style-type: none"> <li>• Acceptable to participants</li> <li>• Can be integrated with follow-up visits, face-face or via telephone</li> <li>• Structure and detail can be tailored</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect assessments</li> <li>• Vulnerable to recall problems</li> <li>• May be adversely impacted by dishonesty and over-optimistic reporting due to social desirability</li> <li>• May be informal</li> </ul>
Verbal enquiries	<ul style="list-style-type: none"> <li>• Acceptable to participants</li> <li>• Can be integrated with follow-up visits, face-face or via telephone</li> <li>• Structure and detail can be tailored</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect assessments</li> <li>• Informal</li> <li>• Vulnerable to recall problems</li> <li>• May be adversely impacted by dishonesty and over-optimistic reporting due to social desirability</li> </ul>
Self-reporting	<ul style="list-style-type: none"> <li>• Acceptable to participants and participant led</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect assessments</li> <li>• Informal</li> <li>• Very vulnerable to recall problems and dishonesty</li> </ul>

**Table 3:** Advantages and disadvantages of qualitative adherence assessments.

### **3.3 Literature review search strategy**

We set out to identify methods for assessment of aspirin adherence, plus corresponding definitions of appropriate adherence in randomised studies of aspirin for prevention of myocardial infarction and prevention of adverse outcomes in pregnancy (including pregnancy loss, pre-eclampsia, intrauterine fetal growth restriction and preterm birth).

Due to the large number of trials investigating aspirin in cardiology we limited the search to reports published in the preceding three years and to myocardial infarction. The purpose was to provide a focussed, contemporaneous view of adherence issues.

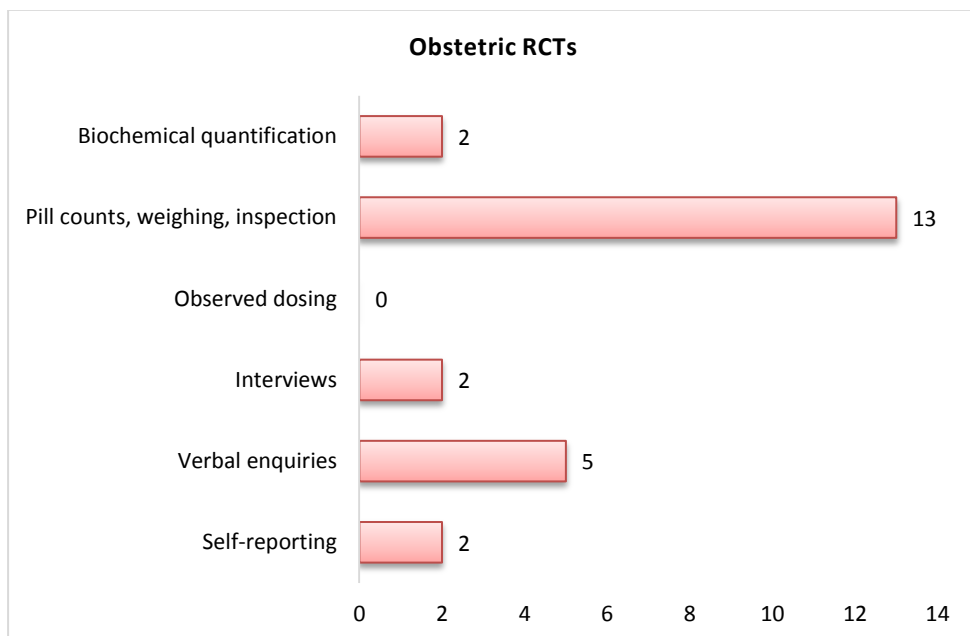
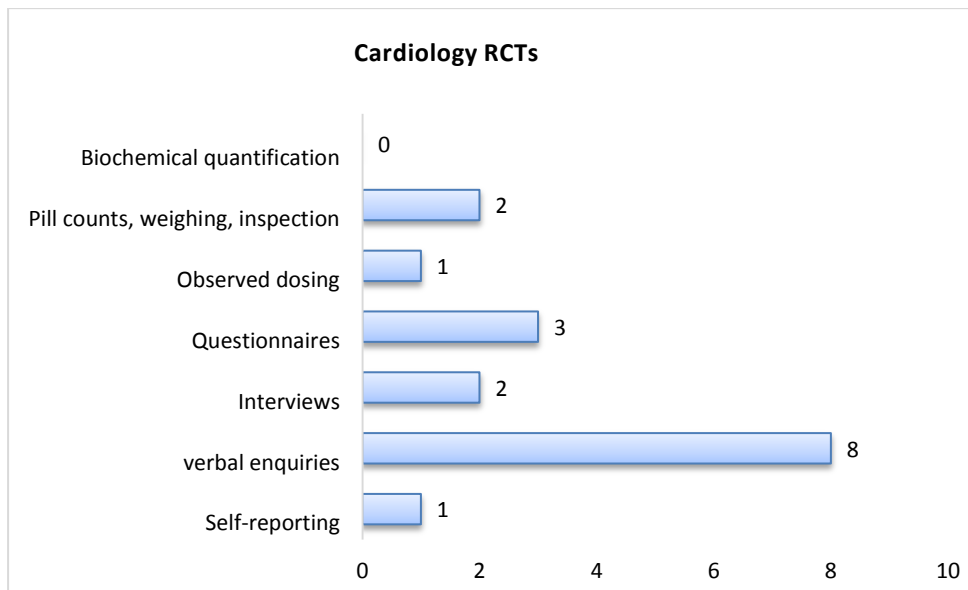
We searched MEDLINE, EMBASE and the Cochrane Library 14/03/13-13/03/16 for cardiology RCTs and from 1957 to 13/03/16 for obstetric RCTs. Both searches were limited to randomised trials, humans and English language. The search terms used were 'aspirin', 'acetylsalicylic acid' appearing adjacent to 'myocardial infarction' and 'pregnancy', 'pregnant', 'obstetric'. We included original articles describing randomised trials with aspirin as an intervention and myocardial infarction as an outcome or conducted in pregnant populations. Review articles and quasi-randomised studies were excluded. Studies where aspirin was used prior to trial enrolment and studies where participants were not pregnant at the time of enrolment were excluded.

### **3.4 Results**

Our search yielded 167 obstetric articles and 181 cardiology articles. Following abstract review, full text assessment was carried out for 82 cardiology articles and 70 obstetric articles. Sixty-five obstetric RCTs and 62 cardiology RCTs were analysed in detail and included in the review. The total population studied in the included RCTs was 63,943 participants in cardiology and 17,791 obstetric participants recruited from Europe, America, South America and Far East Asia, Australasia, Africa and the Middle East.

### **Aspirin adherence assessments and definitions of adherence**

We found that only 32% (20/62) of cardiology trials and 37% (24/65) of obstetric trials referred to aspirin adherence in their reports (Supplementary Tables 1 and 2, Appendices 1a and b). Of all trials where adherence was assessed, details of the specific methods of adherence assessment used were provided in 76% (15/20) of cardiology and 83% (20/24) of obstetric trials (Table 4). Aspirin adherence was poorly described and a threshold for acceptable adherence was included in the methods section in 29% (6/21) of trials in cardiology and 21% (5/24) in obstetrics (Castellano et al., 2014, Basili et al., 2014, Dangas et al., 2013b, Park et al., 2013a, Kim et al., 2011, Borghi et al., 2012, Hermida et al., 1997, Ayala et al., 2013, Souza et al., 2014, Byaruhanga et al., 1998, Caritis et al., 1998).



**Figure 1:** Aspirin adherence assessment used in obstetric and cardiology randomised controlled trials.

Methods	Cardiology Trials	Obstetric Trials
<b>Quantitative</b>		
Serum Thromboxane B <sub>2</sub> immunoassay	0	1 (McCowan et al., 1999)
Serum Salicylate spectrophotometry	0	1 (Martin et al., 1996)
<b>Semi-quantitative Methods</b>		
Observed dosing	1 (Hermanides et al., 2012)	0
Pill counts, blister pack inspection, medication weighing	2 (Kim et al., 2011, Castellano et al., 2014)	13 (Bower et al., 1996, Byaruhanga et al., 1998, Grab et al., 2000, Harrington et al., 2000, Hermida et al., 1999, Hermida et al., 2003, Morris et al., 1996, Regan et al., 1998, Rogers et al., 1999, Yu et al., 2003, Ayala et al., 2013, Caritis et al., 1998, Souza et al., 2014)
<b>Qualitative Methods</b>		
Questionnaires	4 (Lee et al., 2011, Park et al., 2013b, Kim et al., 2011, Castellano et al., 2014)	0
Verbal enquiries/interviews	10 (Valgimigli et al., 2012, Dangas et al., 2013a, Gwon et al., 2012, Lee et al., 2014, Mahaffey et al., 2014, Weimar et al., 2013, Youn et al., 2014, Kim et al., 2011, Basili et al., 2014, Borghi et al., 2012)	7 (Byaruhanga et al., 1998, Yu et al., 2003, Asemi et al., 2012, Goffinet et al., 1996, Khazardoost et al., 2014, Rotchell et al., 1998, Caritis et al., 1998)
Self-reporting	1 (Ikeda et al., 2014)	2 (Morris et al., 1996, Laskin et al., 2009)

**Table 4:** Methods of adherence assessment in RCTs.

#### **Qualitative methods**

Qualitative methods indirectly assess aspirin adherence. In the trials reviewed, we identified four qualitative methods: self-reporting, verbal questioning at enrolment and/or follow-up visits, interviews and use of adherence questionnaires. Interestingly, medication diaries were not used in any of the trials we reviewed. Qualitative methods were the most frequently used methods in cardiology trials, with at least one qualitative method incorporated in 93% (14/15) of protocols (Figure 1, Table 4). Of these, verbal enquiries at enrolment and/or follow-up visits were the commonest methods (57%; 8/14)

(Figure 1). Appropriate aspirin adherence ranged from 75 to 100%. One study specified that adherence should be confirmed over a minimum of 7 days prior to primary coronary intervention and another defined aspirin adherence of <80% as a protocol violation (Appendix 1a) (Basili et al., 2014, Park et al., 2013a).

By contrast, 45% (9/20) of obstetric trials detailed qualitative measures of adherence (Figure 1, Table 4). Qualitative methods were the second most frequently described methods in the obstetric literature, after semi-quantitative methods (Figure 1). Similarly to the cardiology literature, the predominant qualitative method described was verbal enquiry made prior to enrolment or at follow-up visits (78% 7/9). In addition, reports described single, regular and random questioning conducted face-face or via telephone calls.

#### **Semi-quantitative and quantitative methods**

Semi-quantitative assessments were used relatively infrequently in cardiology trials (13%; 2/15) (Table 4). In the obstetric literature, all definitions of appropriate adherence currently detailed relate to semi-quantitative or quantitative methods (Appendix 1b). In obstetric trials semi-quantitative assessments including pill counts, medication weighing and blister pack inspection were the most frequently used methods overall, described in 65% (13/20) of reports (Figure 1, Table 4).

Interestingly, no biochemical assessments were undertaken in cardiology trials, though one trial described observed dosing (Figure 1, Table 4). Quantitative methods were used in two obstetric trials. One study measured maternal serum thromboxane B<sub>2</sub> (TXB<sub>2</sub>), the stable metabolite of Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) (McCowan et al., 1999). TXB<sub>2</sub> was measured by immunoassay two weeks after recruitment and revealed near complete suppression of TXB<sub>2</sub> in aspirin-treated women. One study measured maternal serum salicylic acid (SA), a stable ASA metabolite (McCowan et al., 1999, Martin et al., 1996) using Trindor



spectrophotometry at three time-points (prior to, and 4 hours and 7 days, post placebo, 20, 40 and 80mg aspirin). A further obstetric trial presented data demonstrating detectable cord blood salicylates measured by gas chromatography mass spectrometry, though this was not identified as a measure of adherence (Regan et al., 1998).

The small number of cardiology trials using semi-quantitative adherence assessment methods describe acceptable adherence in the range of 80-100%; by contrast, in obstetrics the comparable threshold was 50% or more (Appendices 1a and 1b) (Castellano et al., 2014, Kim et al., 2011, Ayala et al., 2013, Souza et al., 2014, Byaruhanga et al., 1998, Caritis et al., 1998, Hauth et al., 1998). One cardiology study combined a Morisky-Green medication adherence questionnaire score of 16 or more with a pill count of 80-110%, and specified acceptable adherence as both components being fulfilled (Appendix 1a) (Castellano et al., 2014). One trial in cardiology and one in obstetrics stratified participants on the basis of adherence level using information volunteered at interview and derived from pill counts, respectively (Basili et al., 2014, Souza et al., 2014). Three categories were described at interview; fully compliant, not faithfully compliant, and poorly compliant (Basili et al., 2014). Similarly, pill counts were labelled as <50% ('poor compliance'), 50-69% ('regular use'), ≥70% ('good compliance') (Souza et al., 2014).

### **Prevalence of suboptimal aspirin adherence**

Amongst 20 cardiology trials describing methods of adherence assessment, 70% (14/20) reported their adherence data (Appendix 1a). Both cardiology and obstetric studies reported a broad range of suboptimal adherence, in cardiology trials the prevalence of suboptimal adherence was 0.3 to 28.2%. In obstetrics, 58% (14/24) of studies reported adherence data and the prevalence of suboptimal adherence ranged from 2.8 to 37.02%. In the obstetric study, measurement of serum SA concentrations using Trindor spectrophotometry (Martin et al., 1996), showed that all 12 participants had undetectable

SA levels, which may be related to lower SA concentrations in serum with levels falling below the lower limit of quantification of 1.0mg/DL that was applied (Appendix 1b) (McCabe et al., 2005).

### **3.5 Discussion**

#### **What this review contributes to current knowledge**

Despite clear associations between suboptimal aspirin adherence and adverse clinical outcomes, this review illustrates that there is currently low emphasis on assessment of adherence. To our knowledge, this is the only review addressing the scope and quality of aspirin adherence assessments in RCTs and has allowed comparisons to be drawn between long-established use of aspirin in cardiology and more contemporary use in obstetrics.

We have demonstrated that in both cardiology and obstetric RCTs, aspirin adherence is incompletely considered, with two thirds of trials not reporting any method of assessment. There is currently over-reliance on qualitative and semi-quantitative assessments which provide indirect measures of adherence. Whilst suboptimal adherence 'revealed' by qualitative or semi-quantitative measures does correlate with worse clinical outcomes, due to the nature of the methods this will represent the tip of the iceberg (Shalansky et al., 2004, Gehi et al., 2007). Though qualitative and semi-quantitative methods have high acceptability for incorporation into protocols, their inherent vulnerability to bias limits their value (Tables 2 and 3).

There is currently poor agreement on what constitutes acceptable aspirin adherence. It is important to consider the concept of forgiveness when determining clinically meaningful adherence parameters. Forgiveness of a drug accounts for post-dose duration of action minus actual dosing interval (Osterberg et al., 2010). For ambulatory individuals, dosing interruptions are significant with varying frequency and durations and forgiveness of

aspirin is highly relevant. Though aspirin has a short half-life, which can render many drugs less forgiving, with ASA's irreversible inhibition of COX and resultant sustained platelet inhibition, forgiveness is much improved. However, to date the acceptable number of missed doses defined in trial reports use arbitrary cut-offs, not underpinned by a pharmacological basis. Pragmatic translation of aspirin's forgiveness including acceptable dose intervals and number of missed doses to maintain therapeutic effect has not been ventured. There are significant disparities in the reported lower thresholds for acceptable adherence between cardiology (75% doses) and obstetric populations (50% doses). There may be strong rationale for alternative dosing in the pregnant population. There are significant progestogenic effects which may impact on gastric absorption and circulatory expansion. Platelet turnover is enhanced with uteroplacental sequestration resulting in higher proportions of immature platelets released from bone marrow, prone to activate and aggregate with lower stimuli (Fay et al., 1983, Ahmed et al., 1993). However, these factors indicate that more stringent adherence is required in this population rather than the reverse. Unfortunately, in both cardiology and obstetrics there is consistent under-reporting of adherence data. Three quarters of studies undertaking adherence assessments did not report any data.

Where obstetricians have measured adherence, suboptimal adherence has been reported to be up to 37%, 9% higher than in comparative cardiology trial populations. Due to weaknesses in prevalent methods, this is likely to be an underestimate of the true prevalence in this population. Additionally, a conservative estimate of suboptimal adherence of 37% indicates that aspirin adherence issues in pregnant populations are likely to be sufficiently prevalent to impact effectiveness.

Variation in aspirin adherence has been proposed as a key reason for apparent aspirin non-responsiveness detected using several platelet function assays and by recurrence of

adverse clinical outcomes (Fitzgerald and Pirmohamed, 2011). Despite this, in our recent review, we identified that 36% of studies specifically investigating aspirin non-responsiveness had not assessed adherence (Navaratnam et al., 2016). On comparing the aspirin non-responsiveness literature more broadly with RCTs in the same speciality areas, twice the number of RCTs designed to assess aspirin effectiveness had not considered aspirin adherence in their methodology.

#### **How should aspirin adherence assessment be approached in research?**

Assessment of adherence should be included in trial protocols and trialists should report suboptimal adherence as an outcome variable. This has previously been fraught with ambiguity due to the plethora of language used to describe adherence. A recent systematic review proposed a new taxonomy of terminology to promote consistency and support conduct, analysis and reporting of medication adherence (Vrijens et al., 2012). Adherence to medications is the process of taking medications as prescribed, divided into initiation, implementation and discontinuation phases (Vrijens et al., 2012). Future studies should report adherence using this taxonomy, and describe the rationale for the method in detail, including a pre-specified threshold for appropriate adherence. Adherence data should be reported in full as stratification of cohorts according to these data may add analytical value.

Measurement of aspirin metabolites from biofluids	Due to the short half-life of ASA direct assessment of drug levels is difficult, though stable predominant metabolites can be detected even at low doses. It is feasible to measure SA in capillary blood, plasma and urine and SUA can be measured in urine using LC: MS and NMR, respectively. These methods may be amenable for incorporation into clinical care.
Addition of biomarkers to pills	Aspirin tablets can be tagged with detectable biomarkers, quantifiable either from venous blood or urine, or primed to produce a characteristic colour change in urine. These methods increased the cost of aspirin use, and are more suitable in research environments.
Chip technology and electronic tagging of pills	A patch applied to an individual's skin can detect of tagged pills as they pass into the stomach. This technology allows automatic transfer of data and secure remote storage. These novel methods are currently limited to research environments

**Table 5:** Proposed quantitative adherence assessments.

\*ASA=acetylsalicylic acid, SA=salicylic acid SUA=salicyluric acid, LC: MS=liquid chromatography mass spectrometry, NMR=nuclear magnetic resonance.

There are clear limitations in the information that can be gleaned from traditional qualitative and semi-quantitative assessments and we advise focusing on quantitative assessment methods (Tables 1-3 and 5). However, direct detection and quantification of metabolites should be favoured over assessment of downstream effects which remain prone to confounding. Technology such as liquid chromatography mass spectrometry (LC: MS) and nuclear magnetic resonance (NMR) should be fully exploited to quantify ASA metabolites from standard biofluids and develop adherence assessments with a robust pharmacokinetic basis (Table 5). In parallel, there is a need to further investigate the forgiveness of aspirin, considering its sustained pharmacodynamic actions. This will provide pragmatic information on number of doses needed to maintain platelet suppression to translate into clinical settings. Maintenance of appropriate adherence requires collaboration between trial participants or patients and clinicians and is a continuous process. Provision of information on treatment rationale, safety and open communication around adherence are essential to implement all adherence improvement strategies (Table 6).

Collaborative prescribing	<p>Achieving optimal adherence requires a partnership between the individual and their clinician. Provide clear information on the reason for aspirin, pitched at the appropriate level for the individual. Be aware of socio-cultural and personal barriers and respond flexibly to concerns that are expressed.</p> <p>In pregnancy, it is important to discuss the safety of aspirin for the fetus and that a considerable amount of research supports this judgement.</p>
Simplify dosing regimen	<p>Most preventative regimens require once daily dosing, which can be linked to a daily activity for recall.</p> <p>In pregnancy, guidelines advise aspirin from 12 weeks as there were no trial data prior to this gestation. There is now considerable experience with aspirin at all gestations and no evidence of adverse effects. To simplify the regimen aspirin can be advised from the first consultation until delivery.</p>
Behavioural changes	<p>Forgetfulness has been identified as a major factor in suboptimal adherence. Work with individuals to generate ideas and implement them. This may include setting smart-phone reminders, using apps, aspirin calendars, charts and pill boxes to modify adherence behaviour.</p>
Evaluate adherence	<p>Aspirin adherence, particularly with long term regimens is multifaceted and requires continuous effort from both the individual and their clinician. Strive for an atmosphere of honesty and support in interactions, use agreed means to monitor and discuss adherence and be prepared to constructively address any issues that arise.</p>

**Table 6:** Strategies to improve aspirin adherence.

### **3.6 Conclusions**

Aspirin has proven efficacy, but reliable adherence information is required for accurate assessment of effectiveness in research and clinical contexts. Medication adherence issues are widespread, difficult to accurately quantify with traditional methods and associated with worse clinical outcomes in cardiology. No such data are currently available for pregnant populations. Previous RCTs relied heavily on qualitative and semi-quantitative adherence measures, with fundamental limitations. Researchers should choose quantitative assessments and prioritise development of methods to quantify metabolites that reflect both pharmacokinetics and pharmacodynamics. Future studies should select thresholds for appropriate adherence based on pharmacological evidence or desirable clinical effects. Robust adherence information should be regarded as an important pre-requisite for reliable assessment of aspirin's true effectiveness. However, significant challenges will remain in designing interventions to improve adherence in clinical practice for specific populations.

## **CHAPTER 4: Assessment of aspirin adherence**



#### **4.1 Background to development of adherence methods**

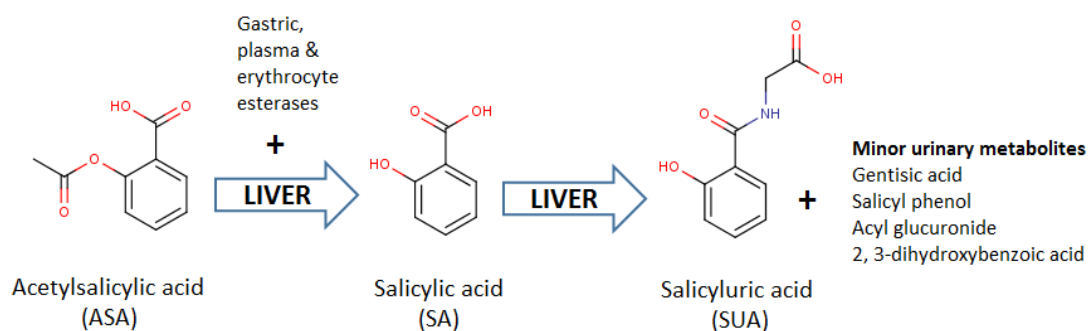
Frequently used qualitative and semi-quantitative methods to assess drug adherence, described in detail in Chapter 3, have inherent vulnerabilities. This limits the quality of information they may provide. The focus of this chapter was to explore options for detection of aspirin metabolites, taking into account low-dose aspirin's (LDA) pharmacokinetics. The most abundant circulating aspirin (ASA) metabolite is salicylic acid (SA) and the primary urinary metabolite is salicyluric acid (SUA), a conjugate of salicylic acid and glycine (detailed in Chapter 1). The work in this chapter supports the primary aim of my thesis, to investigate whether variable response to aspirin exists in pregnant women at high risk of pre-eclampsia. This rests on ensuring a robust assessment and interpretation of participants' aspirin adherence and subsequent platelet exposure to aspirin (Chapter 1). This chapter addresses Objectives 3a; to explore exact methods of aspirin metabolite detection from biological samples that may be easily obtained and stored during clinical studies (Chapter 1) and 3b; to include this adherence assessment strategy in the clinical research protocol and to analyse the clinical data obtained according to aspirin adherence status (Chapter 1).

The aspirin metabolite detection methods described in this chapter benefitted from two collaborations with experts in nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LC: MS) at the University of Liverpool. This work was supported by matched funding from the University of Liverpool Technology Directorate. The analyses described were carried out in the NMR Centre for Structural Biology and the Bioanalytical Facility, University of Liverpool. The NMR Centre for structural biology has state-of-the-art facilities for high-field NMR metabolomics and a focus on translational collaborations. There is considerable expertise in non-destructive metabolite characterisation and analysis. The NMR Centre houses 600, 700 and 800MHz solution-state spectrometers capable of providing high-quality reproducible data from a variety of

sample types and a dedicated computing laboratory. Similarly, the Bioanalytical Facility, a Good Clinical Practice Laboratory (GCP-L) accredited laboratory, currently houses six triple quadruple mass spectrometers. Much of the expertise underpinning the facility is in high-precision accurate assessment of drug concentrations and metabolites, with the aim of optimising drug safety and effectiveness

#### **4.2 Nuclear magnetic resonance detection of aspirin metabolites**

The aim of the NMR analysis was to determine if the currently available technology can be used to correctly identify aspirin metabolites. We focus on assessment of aspirin metabolites present in urine, as experience assessing NMR spectra revealed that serum and plasma samples would be complex to analyse for this purpose and more likely to result in limited sensitivity of detection. This is due to a lack of physical separation of the molecules in an NMR experiment. As such, the protein component in blood serum or plasma will bind and mask aspirin metabolites (such as SA) leaving smaller amounts of unbound metabolites observable amongst the vast array of metabolites in the spectra from these sample types. Conversely, urinary spectra do not have a protein component and as such the metabolites are unbound (and unmasked) and thus may be observed in an area of the spectrum that is less densely populated. The chemical structure of ASA and its metabolites, result in their signals occurring in the relatively uncrowded aromatic spectral region (Figures 1 and 2).



**Figure 1:** Molecular structure of aspirin and its principal circulating and urinary aspirin metabolites.

### Aspirin Metabolites in Healthy Volunteers study

Before analysing samples from pregnant women we first conducted a small study to assess detection of key aspirin metabolites before, and at intervals following LDA dosing. We intended to construct dose-sampling time-course curves for detection of aspirin metabolites. The Aspirin Metabolites in Healthy Volunteers (AMV) study was a clinical study time-limited to 3 months. The AMV study was approved by the University of Liverpool Health and Life Sciences Committee on Research Ethics (reference no. 1138, Appendix 2a) and sponsored by the University of Liverpool. All procedures referred to in this chapter are contained in the study protocol (Appendix 2b).

### Methods

University staff and students were invited to take part in the study via email. Further verbal and written information (Appendix 2c) was given to individuals expressing an interest and their eligibility confirmed according to the criteria detailed in Table 1. All women opting to participate provide informed written consent (Appendix 2d). Assessment of eligibility, consent, enrolment and provision of LDA was conducted in the Centre for Women’s Health Research, University of Liverpool.

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> <li>• Female</li> <li>• Not currently pregnant</li> <li>• Aged 18-45 years old</li> <li>• Has not reached menopause (has a menstrual cycle)</li> <li>• BMI less than 30</li> </ul>	<ul style="list-style-type: none"> <li>• Pre-existing medical conditions including; asthma, diabetes, high blood pressure, autoimmune diseases, inflammatory bowel disease, gastritis, gastro-oesophageal reflux disease.</li> <li>• Already taking aspirin, other antiplatelet agents (e.g. dipyridamole), NSAIDs, steroids (including prednisolone and steroid inhalers) or taking any anticoagulant medication.</li> <li>• Any known adverse reactions or allergies to aspirin or other NSAIDs.</li> </ul>

**Table 1:** Inclusion and exclusion criteria for the AMV study.

\*NSAID=non-steroidal anti-inflammatory drugs.

### Clinical study procedures

Following enrolment, two convenient non-consecutive days for sample acquisition were agreed between participants and the research team. No dietary or lifestyle restrictions were imposed on participants during the study. In all cases aspirin dosing took place in the morning (prior to 1200).

All participants were provided with packs of 10ml, sterile, universal containers in which to provide urine samples. The containers were labelled with unique barcodes for each participant. No record was kept to link participants' identifying information with the barcodes issued and from the point of samples being deposited to the laboratory by participants samples were considered fully anonymised. On sampling days, participants were asked to record the date and timing of their samples on the containers in twenty-four hour clock. A '0 hours', aspirin naïve urine sample was provided, participants were then directly observed taking 75mg dispersible aspirin, dissolved in 100ml water, orally. Participants were then advised to follow the schedules detailed in Table 2 when providing further urine samples. However, if they were unable to complete both days or had omitted samples within the day's schedule, the remaining samples were analysed and contributed to the time-course curves produced.

Urine sample no.	Day 1 sampling schedule (hours)	Day 2 sampling schedule (hours)
1	0	0
2	1	2
3	3	4
4	5	6
5	7	8
6	9	10
7	11	12
8	24	24

**Table 2:** Sampling schedule for urine sample acquisition in the AMV study.

#### Sample preparation

On receipt, urine samples were securely stored at  $-80^{\circ}\text{C}$  in the NMR Centre for Structural Biology laboratory prior to processing in batches. On the day of analysis samples were thawed and centrifuged (21500 g,  $4^{\circ}\text{C}$  for 5 minutes) to remove precipitant. Samples were then prepared to a final composition of 50% urine, 10%  $^2\text{H}_2\text{O}$ , 500mM sodium phosphate buffer (pH 7.4), 100 $\mu\text{M}$  trimethylsilyl propionate (TSP) and 0.1% sodium azide, following established protocols (Beckonert et al., 2007). 600 $\mu\text{L}$  aliquots were transferred into 5mm outer diameter NMR tubes.

#### NMR acquisition

Spectra were acquired on a 700MHz Bruker spectrometer equipped with a 5mm triple resonance (TCI) cryoprobe and a chilled Samplejet Autosampler, using a 1H 1D NOE pulse sequence (96000 complex points, 2048 transients, 4s interscan delay) at  $27^{\circ}\text{C}$ . Spectral acquisition was automated using iconNMR (Bruker), following calibration of the spectrometer via methanol thermometer and optimisation of the offset for water suppression on a single representative urine sample. Processing was also automated, using a standard Bruker script implementing an exponential window function, 0.3Hz line broadening, chemical shift referencing to TSP (as the internal standard), baseline correction and a single zerofill. No downstream processing was required.

### Quality control and metabolite identification

The quality of individual spectra generated was appraised according to three criteria;

- Low and reproducible TSP line width
- Flat and consistent baseline
- Residual water evident in the spectrum distorts less than 0.4ppm.

The data described for both the AMV and EARTH cohorts were generated from spectra that met these criteria and were subsequently normalised to the spectra's creatinine signal. The peaks identified were integrated with respect to the reference signal, TSP. This produced signal intensities representative of the effective concentration of the sample. Urine, as a waste product does not have a fixed dilution. Subsequently, normalisation of these values was conducted with respect to the total peak volume (in the absence of residual water contribution) observed in the samples and to creatinine (Figure 3).

The presence of ASA metabolites were identified in a semi-automated manner using Chenomx™ pattern recognition software (Chenomx, Canada). The characteristic signals, associated with salicylates that arise in the aromatic region (Figure 2) can be searched and matched within the software's library of over 350 human and drug metabolites. This software contains a peak fitting algorithm that enables rapid, automated appraisal of the NMR spectra to obtain a maximal fit between library peaks and experimental data signals. We supported this preliminary Chenomx™ review with expert review of each individual spectrum acquired, to validate metabolite identification. This was necessary to eliminate false positive findings as a result of low aspirin metabolite levels and signal overlap with unidentified (mostly dietary) compounds not present in the library.

## Results

### Aspirin Metabolites in Healthy Volunteers study

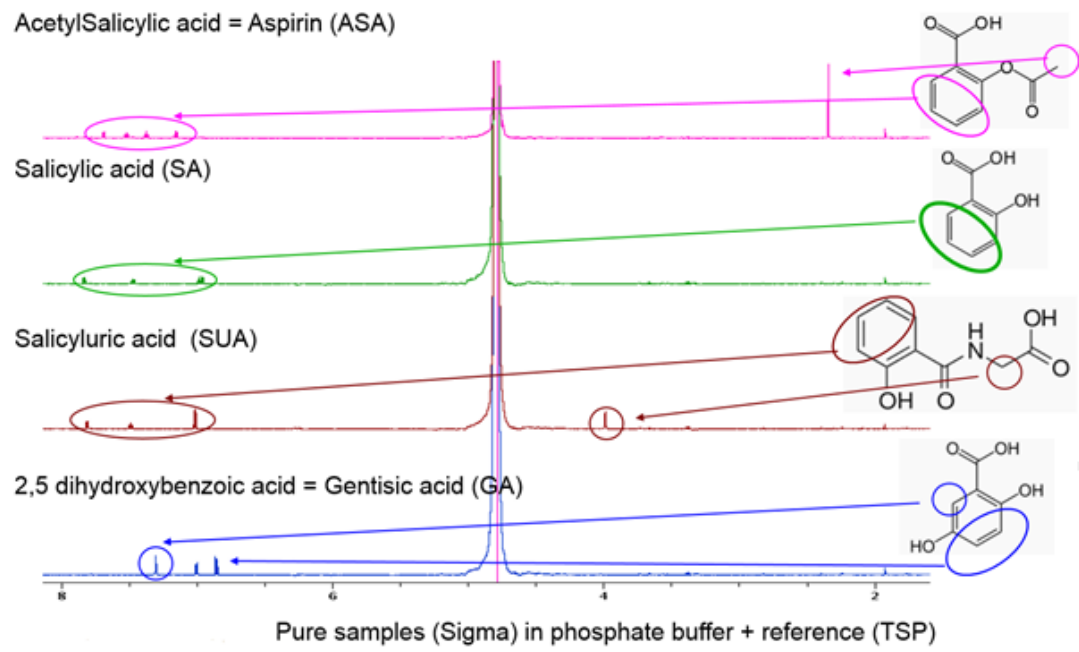
20 participants were recruited to the AMV study and provided a total of 289 samples over 37 participant sampling days (Table 3).

<b>Total no. of participants</b>	<b>20</b>
completing both sampling days	17
completing single sampling day	3
<b>Total no. sampling days</b>	<b>37</b>
with 8 time-points	32
with 7 time-points	3
with 6 time-points	2
Average no. samples per time-point (excluding 0 & 24 hours)	17.5
Least sampled time-point (hours post aspirin dose)	11 (15 samples)
Most sampled time-points (hours post aspirin dose)	2, 4, 6, 8, (20 samples each)

**Table 3:** AMV study participants and samples collected.

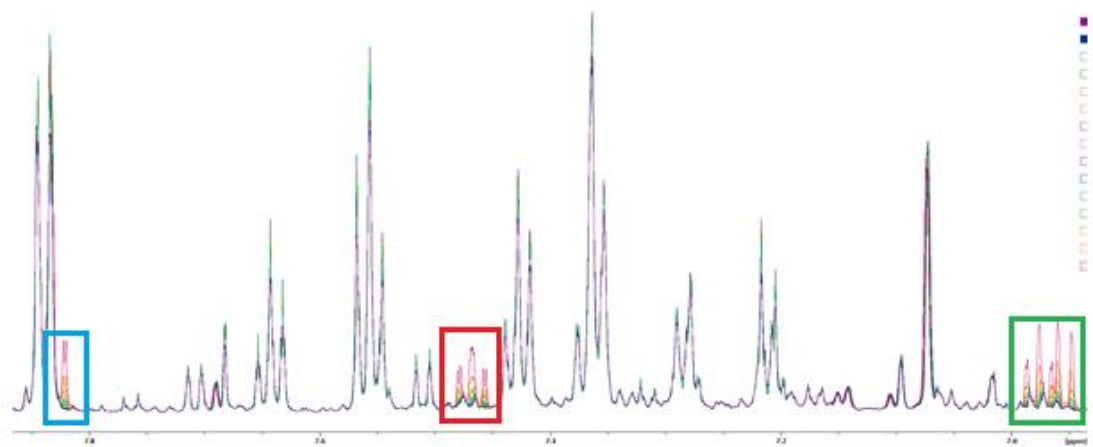
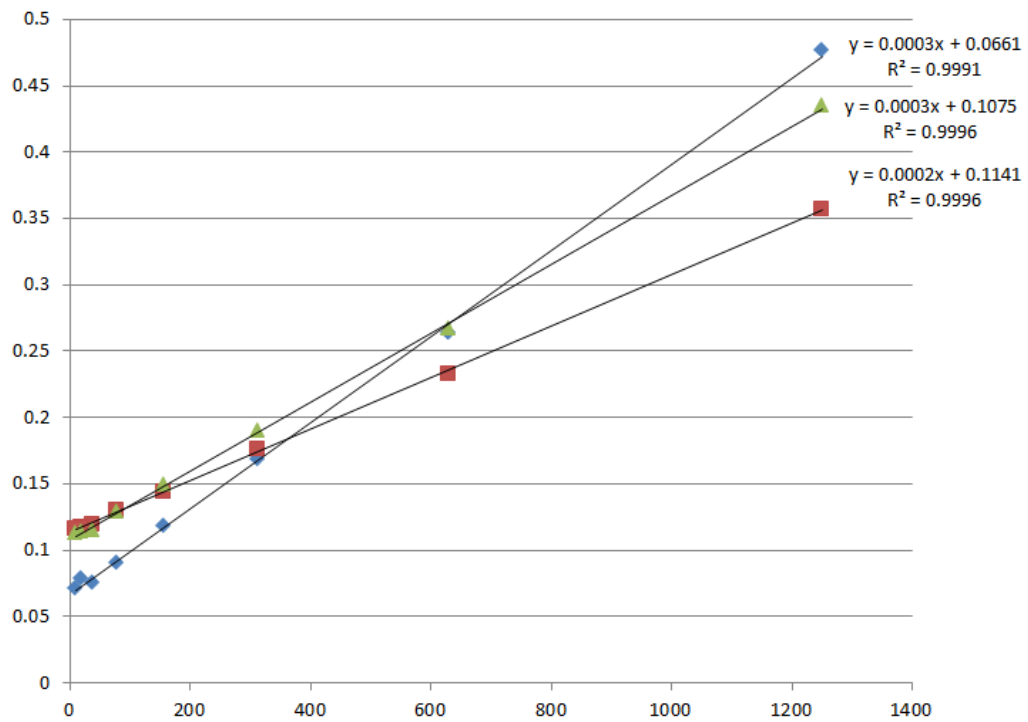
Prior to analysing samples obtained post LDA, we sought to confirm the signal appearances due to ASA and its metabolites, SUA, SA and gentisic acid (GA) and their spectral position. We also assessed the stability of the most abundant urinary metabolite, SUA, by repeating spectral acquisition following successive freeze-thaw cycles, to a total of three cycles. These investigations were completed by spiking aspirin naïve urine aliquots from three healthy volunteers enrolled on the first day of the AMV study with the metabolites described, purchased from Sigma (Sigma-Aldrich, St Louis, MO).

The spectral appearances of these urinary metabolites are shown in Figure 2. Using the samples, we then focussed on SUA and spiked the aspirin naïve urine samples with successive concentrations of SUA and monitored integration. In the absence of the effects of dilution, this signal integration was successful to a level of 9.8µg/L with an R<sup>2</sup> value of 0.999 for all three signals due to SUA (depicted in Figure 3). The appearance of NMR signals from SUA was unaffected by successive freeze thaw cycles or after storage at room temperature for at least 48 hours.



**Figure 2:** Aromatic region of  $^1\text{H}$  nuclear magnetic resonance spectra of acetylsalicylic acid, salicylic acid, salicyluric acid and gentisic acid.  
 \*Molecular structures and atomic assignment of each resonance are depicted to the right.



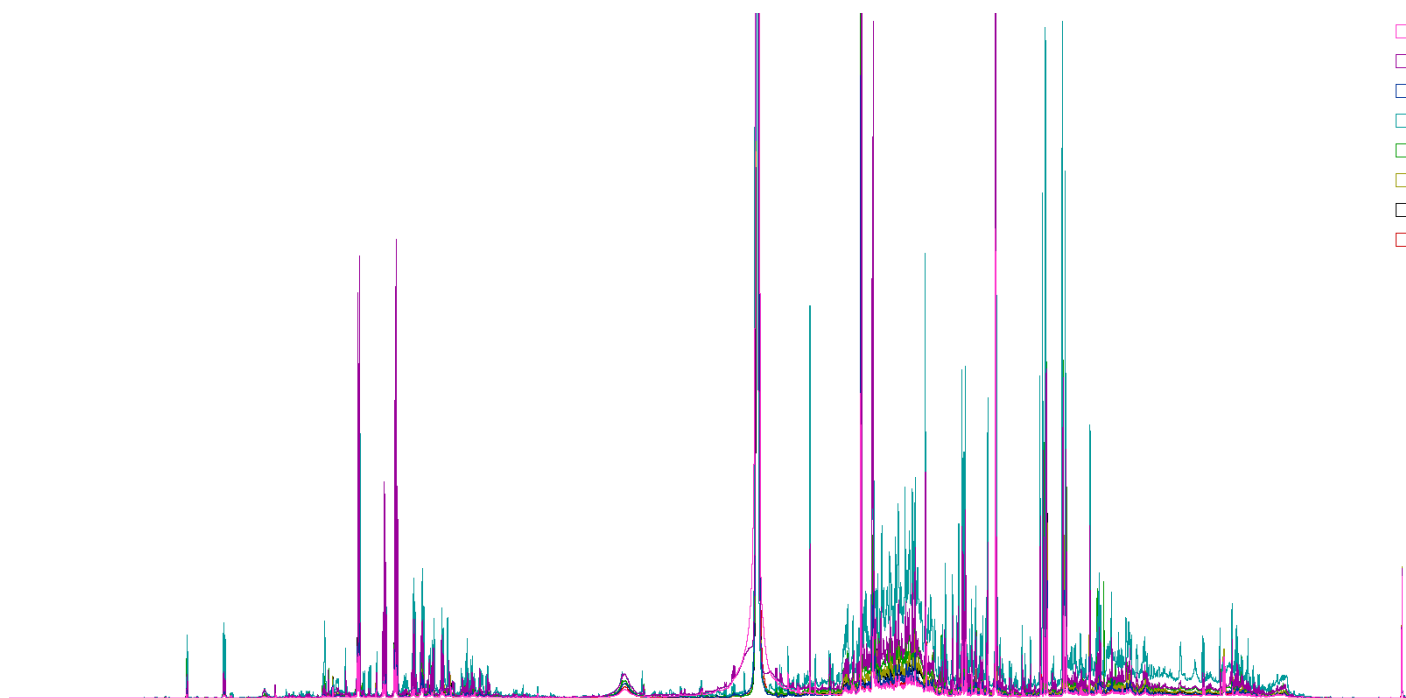


**Figure 3:** Salicyluric acid concentration relationship determined from aspirin naïve urine samples from healthy female volunteers spiked with salicyluric acid.

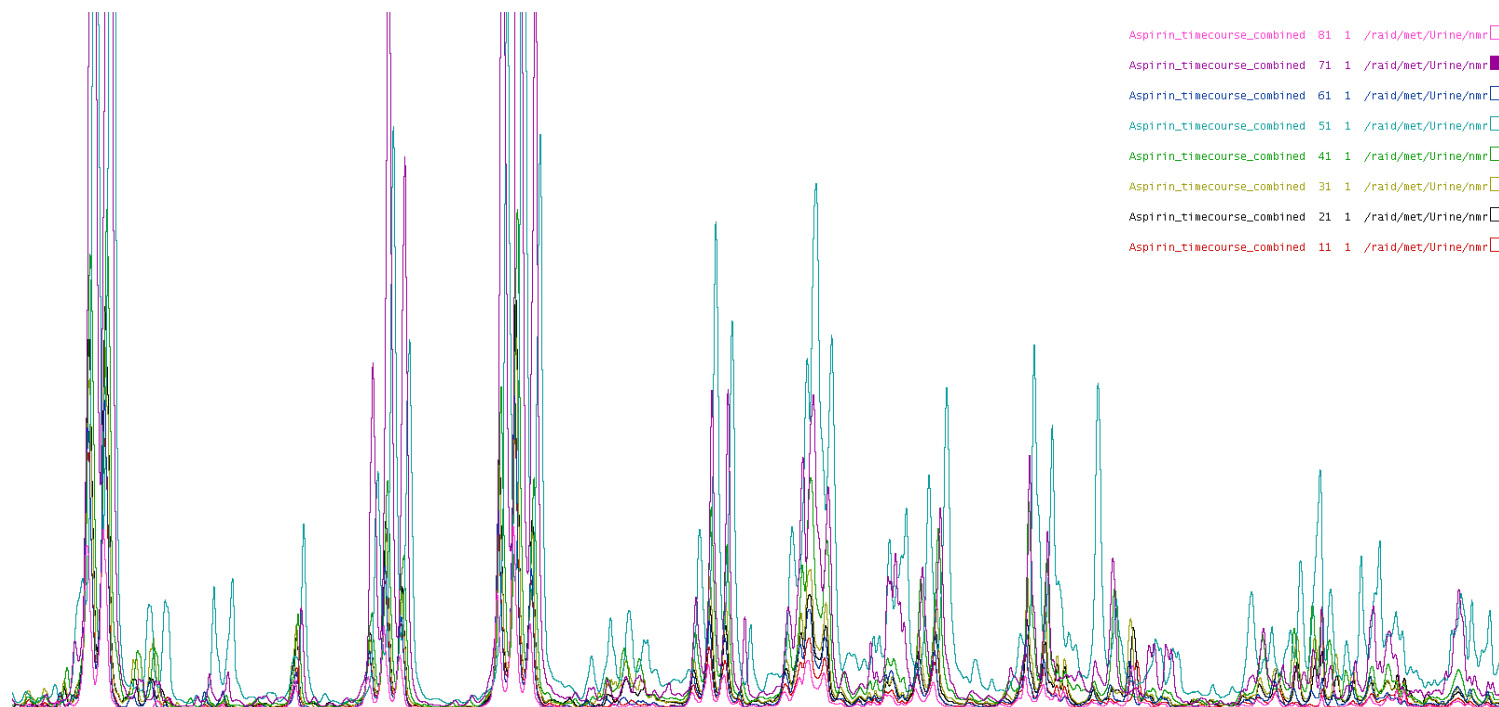
Signal integration: Concentration  
 Relationship is linear to a  
 concentration of 9.8 µg/L  
 R2 = 0.999 for all three signals

Spectra obtained from AMV samples following LDA exhibited SUA signals in the aromatic region, comparable to the appearances of spiked urine samples examined in the preliminary assessment (Figure 4). Integrals of the signals were obtained for three distinct regions; centred around 7.8, 7.5 and 7.0 ppm, respectively. Peaks observed at 7.8 ppm (double doublet) and 7.5 ppm (double triplet), correspond to one H atom each whereas the peak at 7.0ppm corresponds to an overlap of triplet and doublet of the remaining two H atoms on the aromatic ring. Integrals of these peaks exhibit classic 1:1:2 ratio. The build-up and decay of metabolites were plotted by combining data from both sampling days for each participant (Figures 5 and 6). These time-course curves constructed illustrate the time interval from LDA dose to urine sampling to detect SUA by NMR in healthy women (Figures 5 and 6). Combined data indicated a reliable dose to sampling interval of 1 hour to 8 hours. However, for several participants SUA remained detectable above baseline for up to 10 hours post-dose (Figure 5). Peak detection of SUA in the cohort occurred at 3 hours post-dose (Figure 6). These intervals were used to inform analysis of samples obtained from pregnant women in the Estimating Aspirin ResisTance in High risk women (EARTH) study.

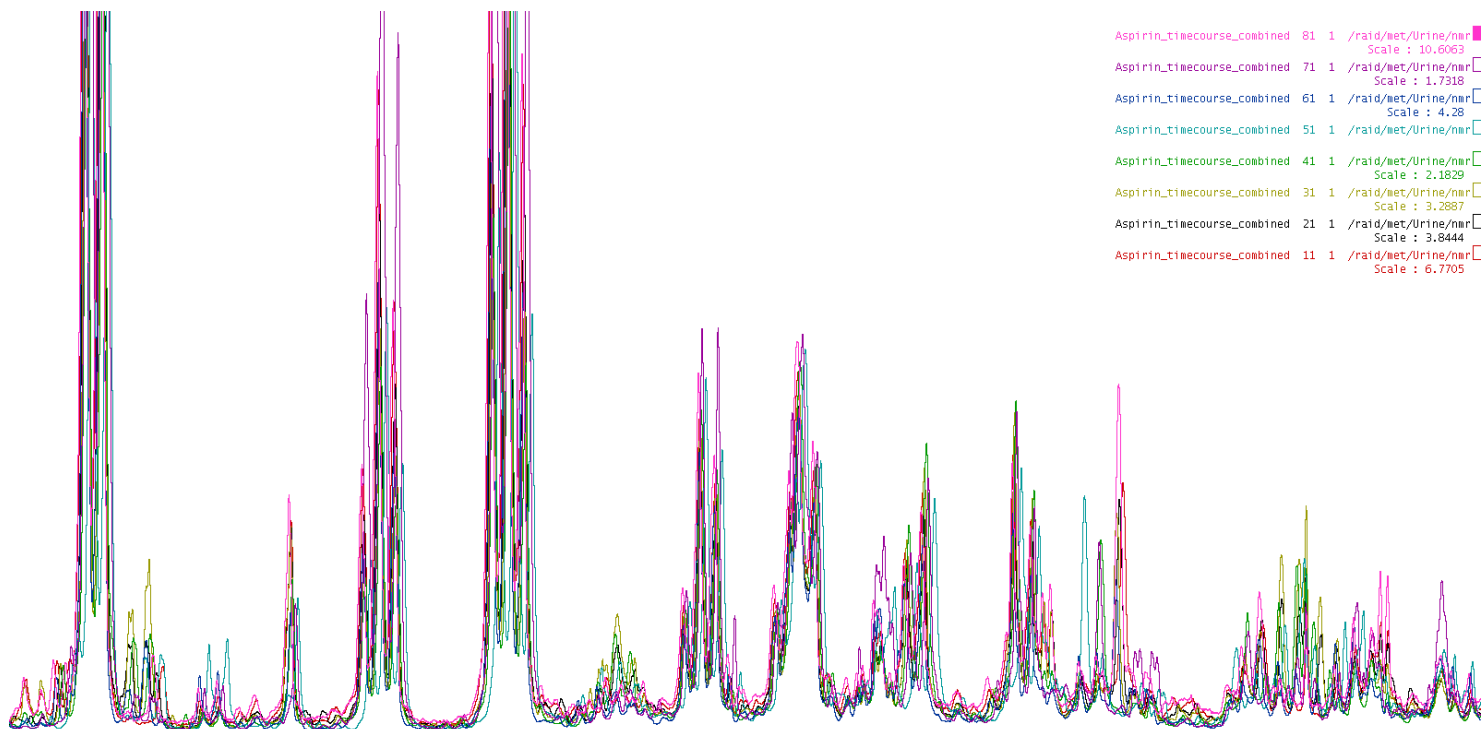
a)



b)



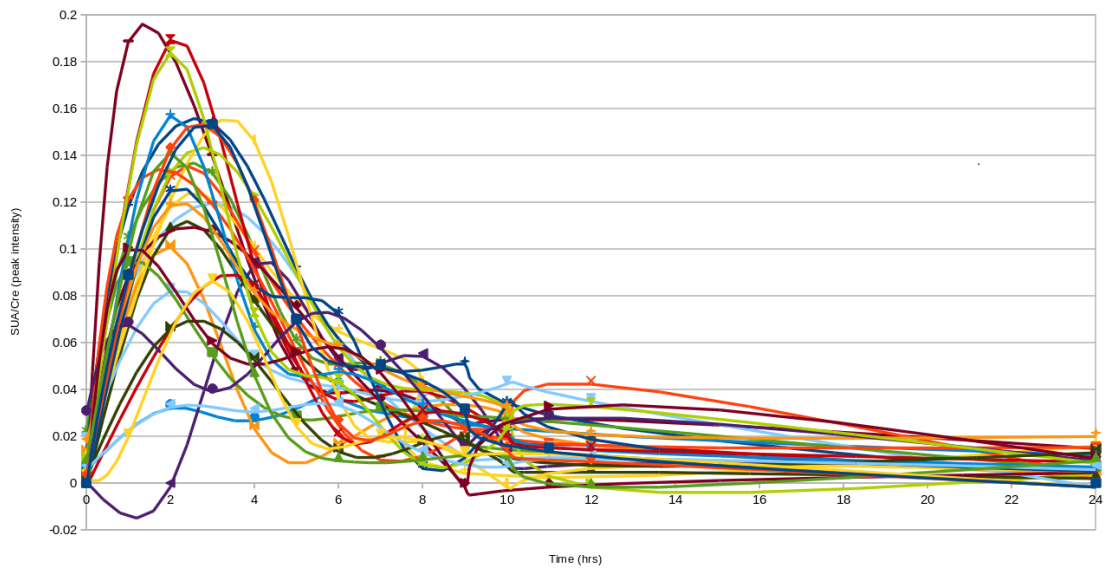
c)



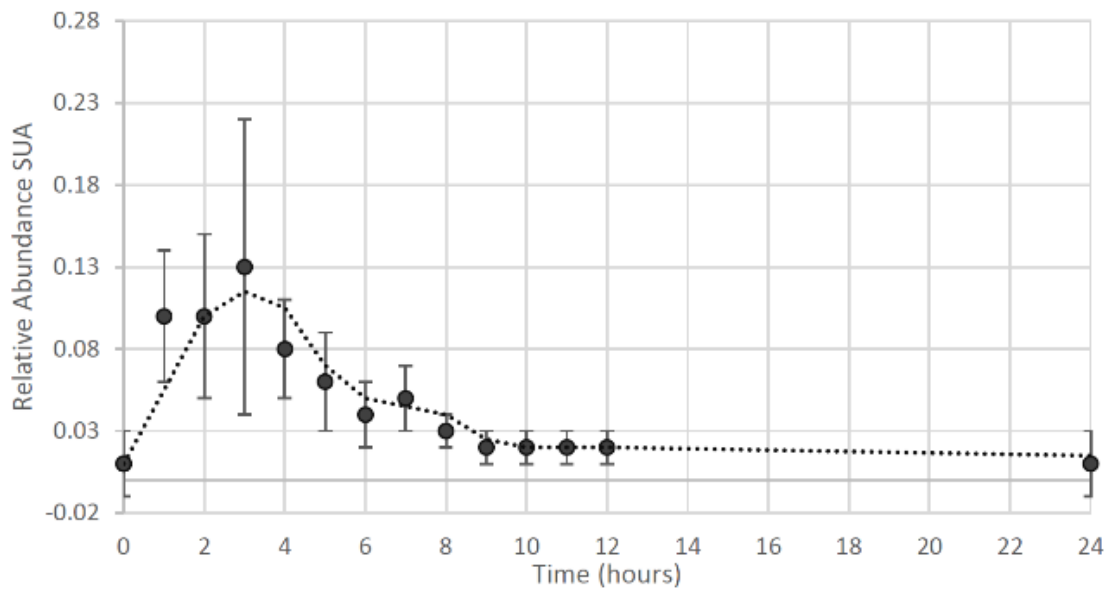
**Figure 4:** Overlay of  $^1\text{H}$  nuclear magnetic resonance spectra from AMV study participants from samples at the specified intervals following low-dose aspirin.

\*Aspirin naive spectrum shown in red, post dose time-points: 1 hour (black), 2 hours (yellow), 3 hours (green), 4 hours (cyan), 5 hours (blue), 6 hours (purple) and 7 hours (magenta).

- Full  $^1\text{H}$  NMR spectrum frequencies of urinary metabolites observed between 0 and 10 ppm.
- Aromatic frequency region 7.9-6.9 ppm. Zoomed (x4).
- Aromatic frequency region 7.9-6.9 ppm. Abundances (y axis) normalised to creatinine.



**Figure 5:** Time-course curves indicating build-up and decay of salicylic acid signals for AMV study participants.



**Figure 6:** Salicylic acid metabolite level observed in AMV study participants.

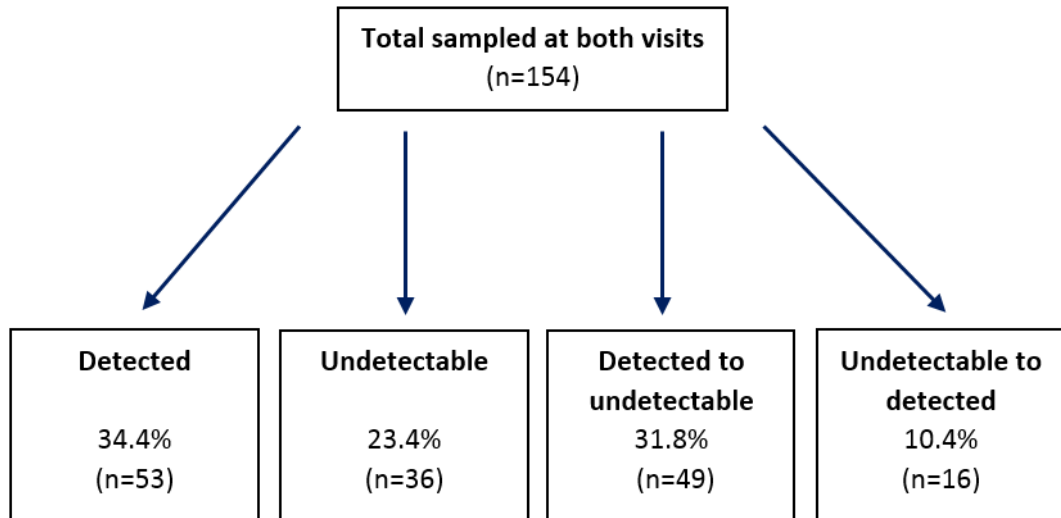
\*Each point is average relative abundance of salicylic acid (normalised against creatinine) with error bars indicating the standard deviation of participant levels. The trend line is based upon average position (2 points).

### Estimating Aspirin Resistance in High-risk women study

EARTH study procedures are described in full in Chapter 5, all study procedures are contained in the study protocol (Appendix 2c). Briefly, women participating in the study were prescribed 75mg dispersible aspirin daily. Maternal blood and urine samples were obtained at two time-points during the study: the first visit (5<sup>+0</sup> to 20<sup>+6</sup> weeks) and the third visit (33<sup>+0</sup> to 35<sup>+6</sup> week's gestation).

At the first visit, 178 participants of a total of 180 initially recruited provided urine samples for NMR. Using the methods described above, signals due to SUA were detected in the urinary spectra of 65.2% (n=116) participants. For 34.8% (n=62), SUA was undetectable. At the third visit, 156 of a total of 160 participants who remained under follow-up provided urine samples for NMR. Signals due to SUA were detected in 44.2% (n=69). SUA was undetectable in 55.1% (n=86) of participants.

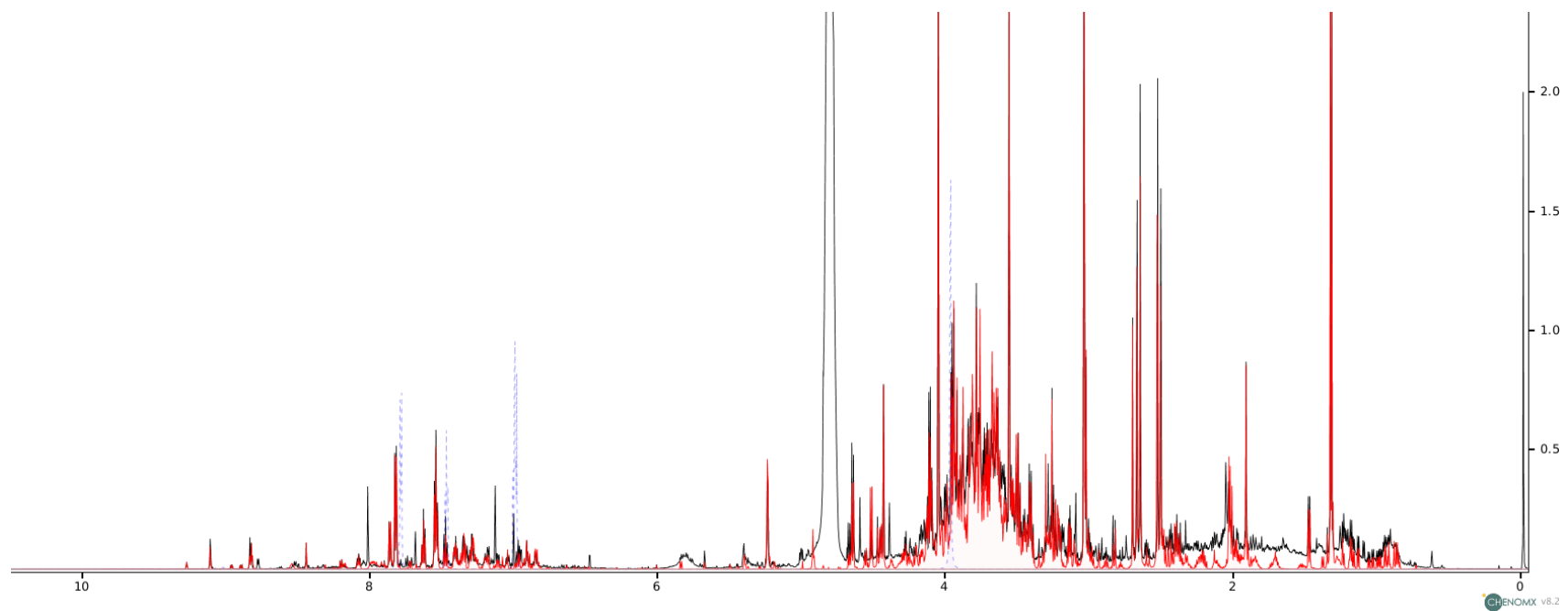
Identification of SUA was also interpreted longitudinally, by participants' status at each study visit (Figure 7). 57.8% (n=89) participants demonstrated consistent presence or absence of urinary aspirin metabolites throughout follow-up, whereas in 42.2% (n=65) presence or absence of metabolites varied across visits.



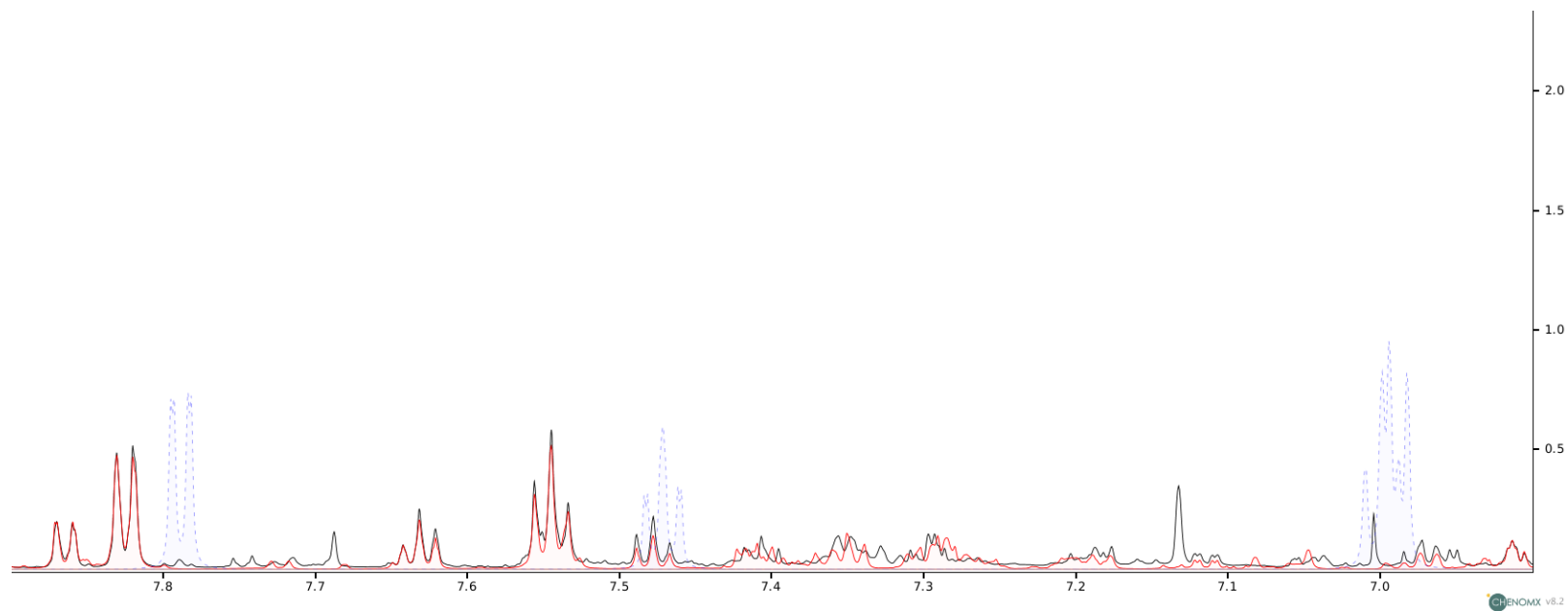
**Figure 7:** Longitudinal detection of salicylic acid in maternal urine by nuclear magnetic resonance from 154 EARTH study participants.



a)



b)

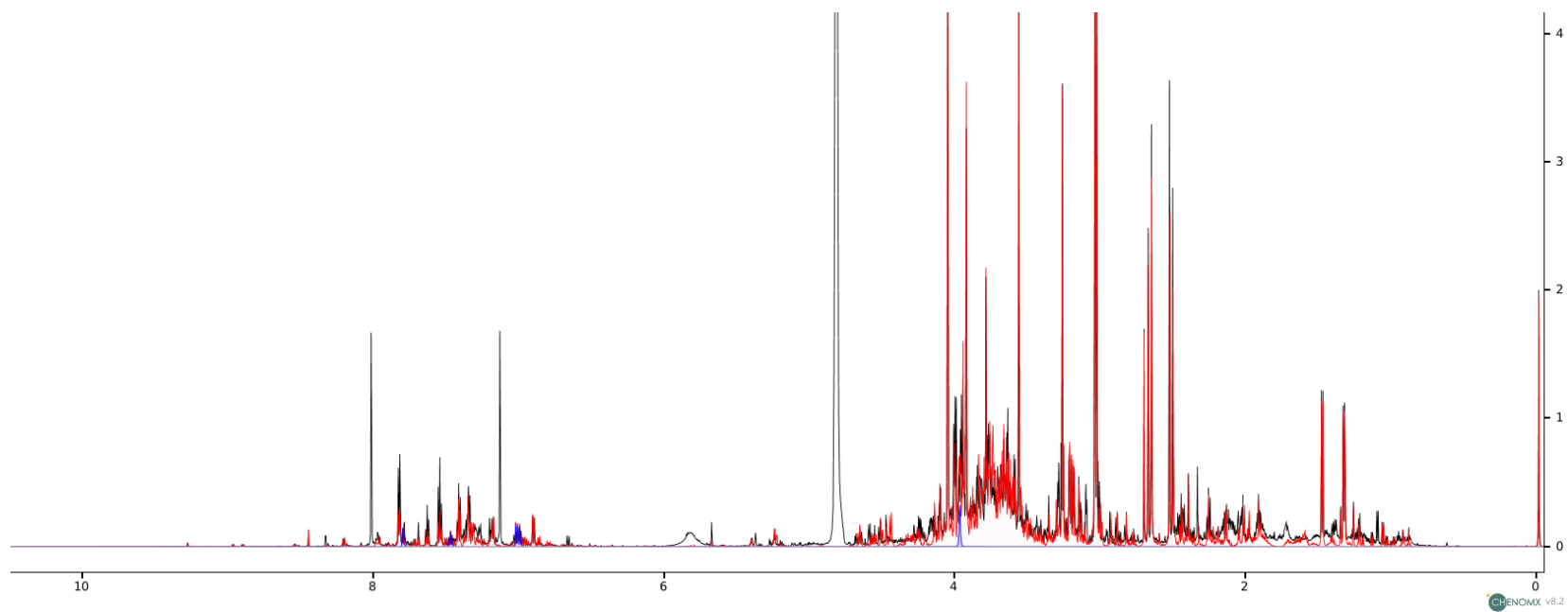


**Figure 8:** EARTH study example spectra negative for salicylic acid signals.

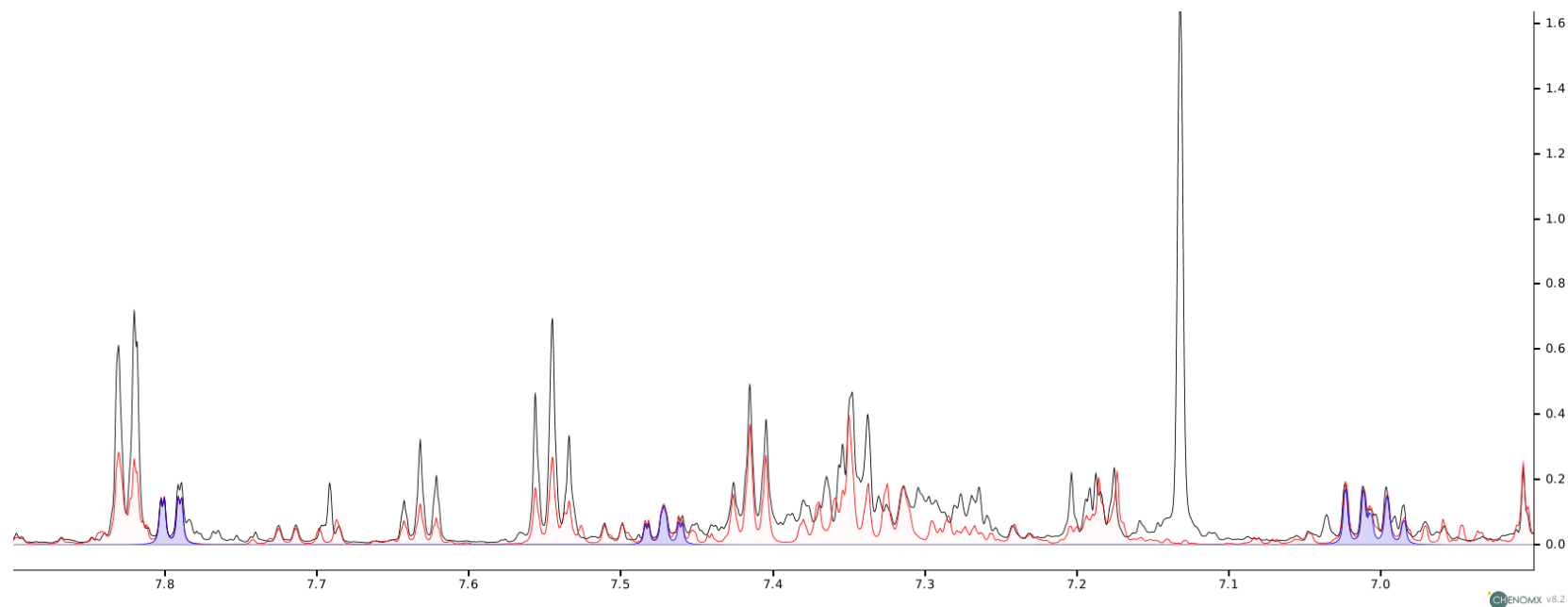
\*Chenomx™ expected position of signals due to salicylic acid shown in blue dashes. For clarity size of signals have been shown at higher than expected abundance to clearly indicate regions where signals are expected.

- a) Full spectrum.
- b) Magnified aromatic spectral region.

a)



b)



**Figure 9:** EARTH study example spectra positive for salicylic acid signals.

\*Chenomx™ automated fitting of urinary metabolites shown in red overlaid with the sample spectrum in black, the match of signals due to SUA shown in blue. For clarity size of signals have been shown at higher than expected abundance to clearly indicate regions where signals are expected.

- a) Full <sup>1</sup>H NMR spectrum frequencies of urinary metabolites observed between 0 and 10 ppm.
- b) Magnified aromatic spectral region between 7.9 and 6.9 ppm.

### **4.3 Liquid chromatography mass spectrometry detection of aspirin metabolites**

Plasma ASA and SA can be determined by high performance liquid chromatography (HPLC) interfaced with a triple quadruple mass spectrometer. The primary aim of the LC: MS analysis was to determine if ASA and its more stable plasma metabolite SA could be detected in samples from EARTH study participants (pregnant women) prescribed LDA. The laboratory methods described below are taken from the Bioanalytical Facility Protocol (Analysis of Salicylic acid and Acetylsalicylic Acid by LC/MS-MS, version 1.0).

#### **Methods**

##### Chemicals and reagents

Reference standards of ASA and SA (assay purity 98%) were obtained from Sigma-Aldrich, UK. Ammonium acetate and formic acid (FA) were obtained from VWS international, UK. Acetonitrile (LC: MS grade) was obtained from Fischer Scientific, UK, and methanol from VWR laboratory supplies, UK. Deionised water was obtained from an Elga Option-S water purification unit and further purified to 18.2 MΩ using the Purelab Ultra.

##### Samples required

A minimum volume of 1ml plasma is required for this method. Citrate plasma was collected from EARTH participants, sodium fluoride and ammonium oxalate samples are also suitable.

##### Equipment

The HPLC system included a variable loop Accela autosampler and an Accela pump (Thermo Scientific, Hemel Hempstead, UK). The compounds were chromatographically resolved onto a reverse-phase Waters Atlantis C18 column (3.0 µm: 100 mm x 2.1 mm) set at an oven temperature of 40°C and interfaced with a 2 µm C18 Quest column saver (Thermo Scientific, Hemel Hempstead, UK). Quantification was performed using a Thermo Quantum Access triple quadruple mass spectrometer interfaced with a heated

electrospray ionisation (H-ESI) source (Thermo Fisher Scientific, Hemel Hempstead, UK). TSQ Tune software was used for tuning and optimizing the analytes and data acquisition and processing was performed using LC Quan™ software (version 2.7, Thermo Scientific, Hemel Hempstead, UK).

#### Preparation of calibrators, quality controls and internal standard

Stock solutions of 1mg/mL of both ASA and SA were prepared in 0.25% FA in acetonitrile and these were subsequently diluted in 0.25% FA in acetonitrile to yield intermediate solutions of 250, 25 and 5 µg/mL concentrations of both the analytes. Both the stock and intermediate solutions were stored at -40°C. Working plasma calibration standards (of 100, 500, and 5000 ng/mL) were prepared by spiking (2% of total plasma volume) drug-free plasma with the intermediate solutions. These standards were prepared in bulk, aliquoted out and stored at -40°C. On the day of analysis, the standards were diluted (in duplicate) with drug-free plasma (100µl per calibrator level) to yield a concentration curve ranging from 50 to 5000 ng/mL for each analyte. The intervals in this 8-point calibration curve were 50, 100, 250, 500, 1250, 2500, 4000 and 5000ng/mL, respectively.

Internal quality control samples were prepared at low, medium and high (LQC, MQC, HQC) with the following nominal concentration levels: 126 ng/mL (LQC; within 3 times of the assay lower limit of quantification; LLQ), 630 ng/mL (MQC), and 4200 ng/mL (HQC).

Stock and intermediate solutions of each internal standard were prepared separately in acetonitrile and stored at 4°C. The intermediate solutions were then combined to create a pre-mixed working internal standard solution (in 0.25% FA in acetonitrile) containing 1µg/mL of ASA d7 and SA d4.

### Sample pre-treatment

100µL of plasma sample was added to clean glass tubes followed by precipitation with 300µl of acetonitrile (0.2%FA) containing ASA-d3 and SA-d7 (1µg/ml) of working internal standard. The tubes were vortex mixed for 5 seconds and centrifuged at 4000 rpm for 10 min. 150µl of supernatant was transferred into clean labelled 5ml glass tube and this fraction was evaporated to dryness using the nitrogen drier. The samples were reconstituted in 100 µL of reconstitution solution (90:10 v/v 2mM ammonium acetate containing 0.1% formic acid in water and Acetonitrile).

### Separation and mass spectrometry

This method presents a very challenging assay for the retention of two polar analytes. The Atlantis dc18 reverse phase column was used for retention and separation of these polar compounds. The optimal ligand density of Atlantis dC18 columns exhibits strong retention of polar compounds without excessive retention of nonpolar compounds.

Mobile phase A contained 2mM ammonium acetate in deionised water and mobile phase B contained acetonitrile. A chromatographic gradient at a flow rate of 400 µL/min was used. The Initial gradient was started with 95% mobile phase A and held for 0.2 minutes and then decreased to 70% mobile phase A for 4.0 minutes, during which the analytes were eluted. The column was re-equilibrated to original conditions for 2 minutes giving a total run time of 6.0 minutes. Extracted samples were injected (10 µL) on to the column and the needle was washed with a mixture of acetonitrile and water (90:10 v/v) between injections.

The mass spectrometer was operated in negative ion electrospray ionisation mode using selective reaction monitoring (SRM). The electrospray voltage was 3.5Kv, the capillary temperature and vaporiser temperature were set at 270°C and 350°C, respectively. The

sheath and auxiliary gas pressures were set of 50 and 15 arbitrary units. Argon, used as the collision gas, was delivered at a pressure of 1.5 mTorr.

The m/z transitions for all analytes and deuterated internal standards, as well as optimal collision energies (CE) and tube lens (TL) parameters, are summarised in Table 4. The scan width was set at 0.01 and the scan time at 0.1s. The peak width settings for Q1 and Q3 were set at unit resolution (0.7).

	Q1	Q3	CE	TL
<b>ASA</b>	178.9	138.7	12	32
<b>ASA-d7</b>	185.8	143.5	14	50
<b>SA</b>	137.1	96.3	18	54
<b>SA-d4</b>	141.2	100.7	19	52

**Table 4:** Mass transitions and instrument parameters.

\*CE=collision energy, TL=tube lens, d7 and d4=deuterated internal standards.

#### Validation

A partial validation of the method consisting of precision and accuracy batches (n=5), recovery and matrix effect were conducted before analysing the patient samples. Calibration curves, accuracy and precision data can be seen in Tables 5a and b. The method was linear with weighing factor ( $1/x^2$ ) in the range of 50-5000 ng/ml for both ASA and SA with accuracy and precision within the acceptance criteria as per US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines (Tables 5a and b). The mean regression coefficient was >0.99 for both the analytes.



Standards	Acetylsalicylic acid			Salicylic acid		
	Mean (ng/ml)	Precision (% CV)	Accuracy (% bias)	Mean (ng/ml)	Precision (ng/ml)	Accuracy (% bias)
50	53.4	8.3	2.3	50.5	4.3	0.9
100	90.7	7.2	-5.1	100.5	5.0	0.5
250	250.4	9.2	0.2	247.6	2.3	-1.0
500	488.1	8.5	-2.4	484.4	5.3	-3.1
1250	1296.0	7.4	3.7	1278.3	4.1	2.3
2500	2553.1	5.4	2.1	2593.4	3.7	3.7
4000	4086.0	4.5	2.2	3930.6	2.2	-1.7
5000	5158.1	5.6	3.2	4928.3	5.5	-1.4

**Table 5a:** Concentration-response linearity data for acetylsalicylic acid and salicylic acid.

\*CV=coefficient of variation.

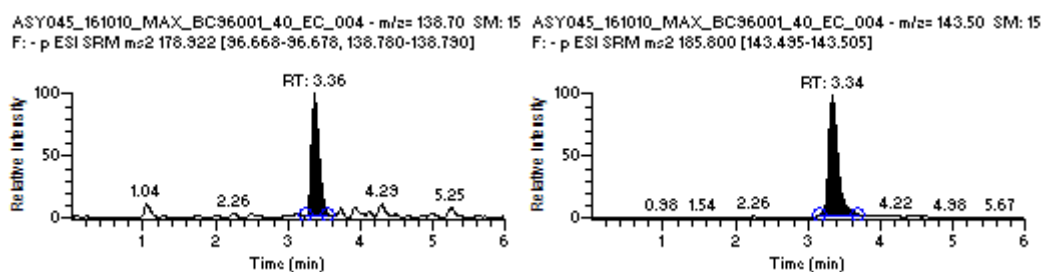
QC Parameters	Acetylsalicylic acid			Salicylic acid		
	LQC	MQC	HQC	LQC	MQC	HQC
Nominal Concentration (ng/ml)	126.0	630.0	4200	126.0	630.0	4200
Mean concentration (ng/ml)	121.1	609.6	4168.9	113.8	573.4	3991.6
% CV: <i>inter-day</i>	8.6	8.8	7.2	3.6	6.5	5.1
% CV: <i>intra-day</i>	3.0	4.5	3.3	3.6	0.8	1.6
% Accuracy: <i>inter-day</i>	-3.9	-3.2	-0.74	-9.7	-9.0	-5.0
% Accuracy: <i>intra-day</i>	-11.4	-7.9	-5.3	-8.3	-9.6	-1.6

**Table 5b:** Inter- and Intra- day Precision (%CV) and accuracy (% bias) data for acetylsalicylic acid and salicylic acid.

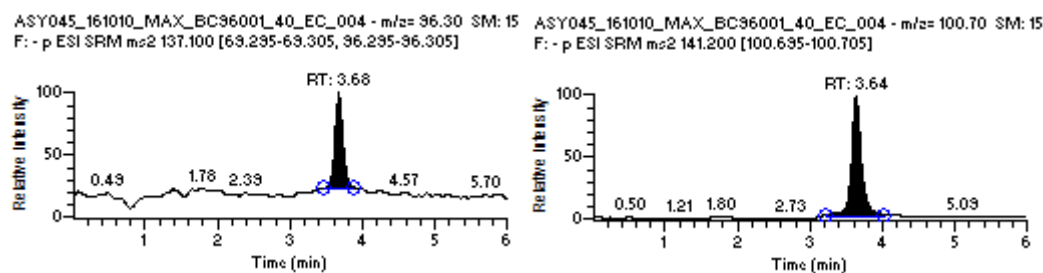
\*CV=coefficient of variation, LQC=low quality control, MQC=medium quality control, HQC=high quality control.

## Recovery and matrix effect

The mean overall recovery for ASA was 77% with a CV% of 14.0. The mean recovery of SA was 85.1% with CV% of 1.07. There was no significant matrix effect for both the analytes.



**Figure 10a:** Chromatograms of acetylsalicylic acid (left) and acetylsalicylic acid-D7 (right) at low quality control level.

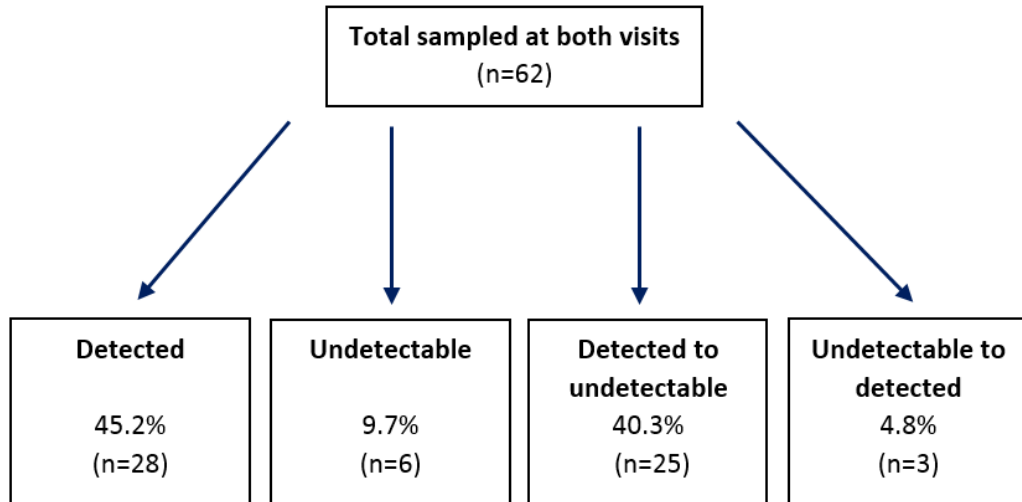


**Figure 10b:** Chromatograms of salicylic acid (left) and salicylic acid-D4 (right) at low quality control level.

## Results

At the first visit, 75 of 180 participants initially recruited had sufficient volumes of citrate plasma to allow LC: MS assessment of SA. Using the methods described above SA was detected above the lower limit of quantification in 84% (n=63) of samples. SA could not be detected in the remaining 16% (n=12) of samples. SA was not detected in the aspirin naïve (pre-dose) samples obtained from participants from the observed dosing subgroup, described in Chapter 5. At the third visit, of 160 participants who remained under follow-up at this visit, 109 provided citrate plasma samples for LC: MS analysis. SA was detected in 48.6% (n=53) of samples but could not be detected in 51.4% (n=56).

In the same manner as the analysis of NMR identification of SUA, Identification of SA was also interpreted longitudinally (Figure 11).



**Figure 11:** Longitudinal detection of salicylic acid in maternal plasma by liquid chromatography mass spectrometry from 62 EARTH study participants.

#### 4.4 Comparison of adherence assessment methods

At the first visit, 75 participants provided both urine and citrate plasma samples, to allow simultaneous assessment of aspirin metabolites by NMR and LC: MS, respectively. At the third visit, 106 participants provided samples allowing analysis by both NMR and LC: MS. Positive (PPA), negative (NPA) and overall percent agreement (OPA) for the two methods of aspirin metabolite identification are detailed in Table 6. There was a high level of OPA between the two methods (Table 6). There were six samples and 10 samples with discrepant results between methods at the first and third visits, respectively. This subgroup of conflicting results comprised predominantly samples where SA was detectable in maternal plasma by LC: MS, but urinary SUA was undetectable with NMR. At each visit there was just one sample where urinary SUA was positively identified with NMR, but plasma SA was undetectable with LC: MS.

a)

		LC: MS		PPA 58/(58+5) = 92.1% NPA 11/(11+1) = 91.7%
		+	-	
NMR	+	58	1	
	-	5	11	
		NMR		PPA 58/(58+1) = 98.3% NPA 11/(11+5) = 68.8%
		+	-	
LC: MS	+	58	5	
	-	1	11	
				OPA 58+11/(58+11+5+1) = 92.0%

b)

		LC: MS		PPA 43/(43+9) = 84.3% NPA 53/(53+1) = 98.1%
		+	-	
NMR	+	43	1	
	-	9	53	
		NMR		PPA 43/(43+1) = 97.7% NPA 53/(53+9) = 86.9%
		+	-	
LC: MS	+	43	9	
	-	1	53	
				OPA (43+53)/(43+53+9+1) = 86.9%

**Table 6:** Positive (PPA), negative (NPA) and overall agreement (OPA) for identification of salicylic acid by nuclear magnetic resonance (NMR) and identification of salicylic acid by liquid chromatography mass spectrometry (LC: MS) in the EARTH study.

a) First visit (5+<sup>0</sup>-20+<sup>6</sup> weeks).

b) Third visit (33+<sup>0</sup>-35+<sup>6</sup> weeks).

Samples from a total of 16 different participants were found to have conflicting results, 6 at the first visit and 10 at the third visit (Tables 6 and 7). Urinary spectra, Chenomx™ hits and LC: MS detection of ASA and SA were re-examined in these cases. We also undertook a rapid, semi-quantitative LC: MS analysis of urinary SA and SUA on samples from these individuals.

The second review of NMR spectra revealed for urine samples where SA and SUA were deemed not to be detected (n=14), 50% (n=7) had neither SA nor SUA Chenomx™ hits and were verified to be negative for ASA metabolites following expert review. The remaining 50% (n=7) did have Chenomx™ hits for SA and/or SUA. However these hits occurred below baseline with overlapping signals and were subsequently deemed to be negative following expert review. The two samples where NMR was deemed to have detected SUA, but LC: MS did not detect plasma ASA or SA both had Chenomx™ hits for SA and SUA.

These hits occurred above baseline, without overlap and were confirmed as positive results following expert review.

The review of LC: MS data verified the initial findings. Additionally, both SA and SUA were detected in the urine of all 16 participants with conflicting NMR and LC: MS results following a rapid analysis by LC: MS.

a)

Participant no.	Dose-sampling interval (minutes)	LC:MS ASA (+/-)	LC:MS SA (+/-)	NMR SA (+/-)	NMR SUA (+/-)
1	60	+	+	-	-
2	55	+	+	-	-
3	60	+	+	-	-
4	475	-	+	-	-
5	60	+	+	-	-
6	57	-	-	-	+

b)

Participant no.	Dose-sampling interval (minutes)	LC:MS ASA (+/-)	LC:MS SA (+/-)	NMR SA (+/-)	NMR SUA (+/-)
7	Not stated	-	+	-	-
8	725	-	+	-	-
9	1435	-	+	-	-
10	1420	+	+	-	-
11	Not stated	-	+	-	-
12	65	+	+	-	-
13	10	-	+	-	-
14	Not stated	-	+	-	-
15	Not stated	-	+	-	-
16	8640	-	-	-	+

**Table 6:** EARTH study samples with conflicting Liquid chromatography mass spectrometry (LC: MS) and nuclear magnetic resonance (NMR) results per visit.

- a) First visit (5<sup>0</sup>-20<sup>6</sup> weeks).
- b) Third visit (33<sup>0</sup>-35<sup>6</sup> weeks).

#### **4.5 Aspirin diaries**

At the first visit 178 of 180 participants recruited recalled the timing of their most recent LDA. This allowed calculation of their dose-sampling intervals for samples provided. The median dose-sampling interval for participants at this visit was 2 hours 50 minutes (range 30 minutes to 42 hours 30 minutes).

7 days prior to the third visit participants were asked to complete an aspirin diary (Appendices 3c and 3f). Of 160 participants who remained under follow-up at this visit, 83.1% (n=133) returned their aspirin diary and 72.9% (n=97) of these diaries were completed in full. 12.8% (n=17) participants reported omitting at least one LDA in the previous 7 day period, with a median of 1 dose omitted (range 1-6). One participant recorded that she had completely stopped taking LDA from 28 week's gestation. Of participants who recorded the timing of their last LDA prior to maternal blood and urine sampling the median dose-sampling interval was 4 hours 20 minutes (range 10 minutes to 144 hours).

Data collated from the aspirin diaries and detection of aspirin metabolites by NMR and LC: MS for participants providing sufficient samples at the third visit (n=159) are summarised in Table 7. Plasma SA was detectable within 24 hours of the last LDA, and detected as early as 10 minutes post-dose. After excluding one outlier with SUA detected by NMR only at 144 hours post dose (Tables 6b and 7) urinary SUA was detectable within 14 hours following LDA and detectable from 60 minutes post-dose. There was no difference in the number of omitted doses disclosed between groups, but an overall longer median dose-sampling interval (13:20 vs. maximum of 07:08) was observed when samples with undetectable aspirin metabolites were compared to those with metabolites detected by either method.

	Aspirin metabolites detected			Aspirin metabolites not detected (n=80)
	NMR (n=26)	LC: MS (n=10)	Both (n=43)	
<b>Aspirin diary returned</b>	(n=18)	(n=10)	(n=39)	(n=66)
<b>Median doses omitted</b> max. 7 doses (range)	0 (0-6)	0 (0-0)	0 (0-2)	0 (0-6)
<b>Dose-sampling interval</b> hours: minutes (range)	02:46 (01:00-14:00)**	07:08 (00:10-23:55)	02:43 (01:20-15:50)	13:20 (01:30-96:00)

**Table 7:** Aspirin diary data and detection of aspirin metabolites at the third EARTH study visit (33<sup>0</sup>-35<sup>6</sup> weeks).

\*NMR=nuclear magnetic resonance, LC: MS=liquid chromatography mass spectrometry.

\*\*One outlier detected with NMR only at 144 hours post dose.

#### 4.5 Discussion

Qualitative and semi-quantitative methods of aspirin adherence assessment are somewhat limited in reliability (Chapter 3). Additionally the short half-life and rapid clearance of ASA make detection and quantification of drug levels in maternal biofluids impractical for clinical research purposes and applicability to clinical practice (Ali et al., 1980, Bochner et al., 1988). It is ASA itself that exerts the principal antiplatelet action, the SA produced following this action has no antiplatelet activity but is responsible for aspirin's anti-inflammatory effects. However, the plasma (SA) and urinary metabolites (SUA, SA and a number of minor metabolites) of ASA are more stable and sustained in body fluids making them more amenable to detection. The work in this chapter demonstrates that these principal metabolites can be reliably detected from sample types (maternal blood and urine) routinely obtained, both in clinical research and clinical obstetrics. Use of maternal blood for this purpose requires some preparation, centrifugation and extraction of plasma which must be preserved at -80 degrees. However, urine samples suitable for NMR assessment of SUA do not require any specific preparation at the point of sampling and SUA is stable both at room temperature and through successive freeze-thaw cycles. These specific factors make NMR an attractive

method for assessment of drug adherence in clinical studies, where samples could be postal-shipped and processed in batches. We have also demonstrated the utility of the Chenomx™ package for identification of ASA metabolites, used as a first-pass review of a large number of spectra. A negative to NMR identification of aspirin metabolites is the high-level of expertise required to verify signals identified at Chenomx™ review of spectra. There is a very limited pool of experts in this field and the review process is time-consuming. This may limit the practicality of NMR for large cohorts or cohorts with longitudinal samples.

LC: MS is a highly sensitive technique for the identification of aspirin metabolites. There is also the capacity for a truly quantitative assessment of LDA metabolites both from plasma and urine with this method. As with NMR, this method is more amenable to batch processing and analysis, which favours use in research contexts. In contrast to NMR, with development and validation of an appropriate method, LC: MS data generated do not require the level of scrutiny necessary to verify results. A negative aspect of LC: MS is also related to its high sensitivity. The sensitivity of LC: MS introduces the risk of identifying low, background levels of dietary salicylates and producing false positive results. As such, it would be informative to establish baseline salicylate metabolites in maternal blood and urine and examine the effects of dietary constituents known to influence salicylates (Lawrence et al., 2003, Blacklock et al., 2001), to set a threshold for positive identification of metabolites due to LDA.

We found a high degree of agreement between identification of aspirin metabolites with NMR and LC: MS, despite the methods operating with different media. The side-by-side interpretation of NMR and LC: MS data in the EARTH cohort was particularly valuable when determining adherence to LDA. However, we accept that both methods are limited to some extent by the rate of clearance of the metabolites following dosing. Due to this, a



more holistic interpretation of adherence to LDA that combines measures of metabolites with platelet response, reflecting both short and medium term exposure to LDA, respectively was decided on for analysis of the EARTH cohort (results displayed in Chapter 5).

#### **4.6 Conclusion**

From experience with the AMV and EARTH cohorts LDA metabolites can be detected in both maternal plasma and urine. The AMV study established that urinary SUA can be reliably detected from one to eight hours post-dose. However, there are specific limitations and cautions to be observed both with NMR and LC: MS and these data should not solely determine aspirin adherence status assigned for analysis. Consideration of LDA adherence in the EARTH cohort benefitted from parallel assessments using both NMR and LC: MS. Taking into account the LDA metabolite detection data generated for the cohort, the definition of response to aspirin applied for analysis of EARTH data incorporates both detection of aspirin metabolites and evidence of the expected platelet response to LDA.

## **CHAPTER 5: Platelet function testing in pregnancy**

## **5.1 Rationale**

The design and conduct of the Estimating Aspirin ResisTance in High risk women (EARTH) study is described in this chapter. The purpose of this clinical study was to investigate variable response to aspirin in pregnant women at high risk of pre-eclampsia and evaluate for associations between response to aspirin and placentally-mediated adverse outcomes. This work addresses Objective 4a; to conduct longitudinal assessments of aspirin response using cyclooxygenase (COX)-specific platelet function assays and define the proportion of women who are non-responsive to aspirin. Objective 4c is also addressed; to assess if aspirin non-responsiveness is related to placentally-mediated adverse clinical outcomes, including pre-eclampsia and a composite of placentally-mediated adverse outcomes (Chapter 1, Figure 10).

## **5.2 Selection of assays for the EARTH study**

The reference ranges for platelet activation in pregnancy study provided valuable insight when designing the EARTH study. We observed that platelet activation and function appears to be independent of gestational age across the late first trimester and early second trimester (until 20+<sup>6</sup> week's gestation). This work established pregnancy-specific reference ranges for tests to be used. We were also able to assess the pragmatic utility and clinical performance of a range of platelet activation and function tests using maternal whole blood, plasma and urine samples. Analysis of results from the study provided information about the ability of individual tests to discriminate between aspirin naïve and women prescribed aspirin, which was of key importance for the EARTH study.

### Included tests

Multiplate™ ASPI test and TXB Cardio™ were selected for inclusion. Both tests provided reproducible discrimination between aspirin naïve and aspirin dosed women. Multiplate™ proved an operator-friendly, point-of-care test that required

minimal training and rarely resulted in errors requiring repeated samples, with the additional benefit of targeting COX-specific platelet function. Urinary 11-dehydrothromboxane B<sub>2</sub> (urinary 11-DHTXB<sub>2</sub>), measured by TXB Cardio™ has been correlated with adverse outcomes in non-pregnant populations, with signals in pregnant populations. Urinary 11-DHTXB<sub>2</sub> was analysed externally (by Randox Laboratories, Ireland) using a validated method, following sample aliquoting no further preparation of samples was required. We also opted to assess the VerifyNow™ point-of-care system. The VerifyNow™ Aspirin test is COX-specific and based on the principles of light transmission aggregometry (LTA). VerifyNow™ Aspirin test and has been used in cardiovascular and stroke research (Lordkipanidzé et al., 2007). We had not been able to add this assay to the reference ranges for platelet activation in pregnancy study as the cost was prohibitive. We took the opportunity to compare VerifyNow™ Aspirin test against the other selected methods after the cost was reduced and we were awarded external funding (Wellbeing of Women, 2014, Research Training Fellowship 409).

#### Excluded tests

PFA-100™ collagen/epinephrine cartridges and Platelet Solutions™ were ruled out. The PFA-100™ test required frequent repeat samples to be analysed. This was principally due to the PFA-100™ system's sensitivity to micro clots and bubbles in whole blood samples and the test produced variable results. Unfortunately, after promising initial use, we encountered serious issues with quality control of the Platelet Solutions™ test, a novel test, centred on platelet-bound P-selectin. Quality control issues led to misidentified samples. Fortunately, due to our in-house sample tracking records we were subsequently able to salvage the correct data for the cohort.

### **5.3 Methods**

#### **Design of the Estimating Aspirin ResisTance in High risk women (EARTH) study**

This prospective cohort study was conducted at the Liverpool Women's Hospital NHS Foundation Trust. The study was approved by the Liverpool Research Ethics Committee (REC reference no. 13/NW/0764, Appendix 3a). The study was jointly sponsored by Liverpool Women's Hospital and the University of Liverpool and funded by Wellbeing of Women (Appendix 3b).

Screening for eligibility and recruitment took place in the antenatal clinics and ultrasound departments of Liverpool Women's Hospital. Written, informed consent was obtained for women between 5+<sup>0</sup> weeks and 20+<sup>6</sup> weeks (Appendices 3d and 3e). The procedures outlined below were contained in the study protocol (Appendix 3c).

#### **Inclusion Criteria**

To be eligible for inclusion pregnant women had to be deemed at high risk of pre-eclampsia, defined in accordance with National Institute for Health and Care Excellence (NICE) guidance, as having single high risk factor, or at least two moderate risk factors (Table 1) (NICE, 2010a). Women also had to have a viable single intrauterine pregnancy 5+<sup>0</sup>-20+<sup>6</sup> weeks with their expected date of delivery (EDD) calculated from crown-rump length (CRL) at a first trimester dating scan and be prescribed aspirin 75mg daily.

High risk factors	Moderate risk factors
<b>Previous hypertensive disease in pregnancy</b>	First pregnancy
<b>Chronic hypertension</b>	Maternal age >40 years
<b>Pre-existing type 1 or 2 diabetes</b>	BMI >35
<b>Chronic renal disease</b>	Pregnancy interval >10 years
<b>Autoimmune disease e.g. Systemic Lupus Erythematosus, Antiphospholipid Syndrome</b>	Family history of pre-eclampsia (first-degree relatives)

**Table 1:** High and moderate risk factors for pre-eclampsia (NICE, 2010a).

### **Exclusion Criteria**

Women were excluded if they were taking medication which could interfere with platelet function. This included aspirin doses higher than 75mg, and/or dosing more than once daily, anticoagulants (e.g. low molecular weight heparin, LMWH) or other antiplatelet agents (e.g. clopidogrel).

### **Study procedures**

Key study procedures at each visit are detailed in Tables 2-5. Consent, interviews and venous blood samples were taken by EARTH research staff. Point-of-care platelet function tests were performed within 3 hours of venepuncture. A subgroup of aspirin naïve participants underwent observed dosing of their first LDA with platelet function testing immediately prior to and 60 minutes following their initial LDA.

Platelet function tests were performed in the Centre for Women’s Health research laboratories. Results of Multiplate™ and VerifyNow™ test results were printed and securely stored by a trained laboratory technician for analysis by the research team on completion of the clinical study. Platelet function results were not disclosed to the clinical investigators or participants during the clinical study. Plasma and serum samples for placental growth factor (PIGF) and FMS-like soluble tyrosine kinase-1 (sFlt-1) measurements were initially stored at -80°C in the Centre for Women’s Health Research laboratories. The samples were processed following delivery of the final participant (described in detail in Chapter 7). Placental samples were also collected by EARTH

research staff, placental samples were prepared and biobanked for to allow future omics (proteomic and metabolomic) research. Placental samples were prepared and stored at -80°C in the in the Centre for Women's Health Research Laboratories. We collected, stored and extracted DNA from maternal blood samples (EDTA whole blood and whole blood blot cards) for pharmacogenetic work examining for key genetic factors in the mechanism of action and disposition of aspirin (described in detail in Chapter 6).

Ultrasound scans were coordinated with visits offered for standard antenatal care (20<sup>+0</sup>-23<sup>+6</sup>, and at 33<sup>+0</sup>-35<sup>+6</sup> weeks). Ultrasound scans were performed by myself and research colleagues, fully trained in fetal biometry and maternal-fetal Doppler assessments. The scan results were available to the Fetal Medicine team for review and clinical decision making. Non-invasive cardiac output monitoring (NICOM) and arteriography measurements were taken from a subgroup of women in the EARTH study to allow a future analysis of the cardiovascular profile of participants to be undertaken. Measurements were obtained at 20<sup>+0</sup>-23<sup>+6</sup> and 33<sup>+0</sup>-35<sup>+6</sup> weeks. It was stipulated that any clinically relevant results would be referred to the clinical care team, however this situation did not arise.

During the EARTH study, clinical recruitment was continuously monitored. The key laboratory methods used were standard and established within our laboratories, and we encountered no issues.

Interview  (coordinated with booking visit, serum screening, or anomaly scan)	To assess eligibility (gestational age calculated from early pregnancy ultrasound, prescribed aspirin).  Past medical, obstetric and family history.  Current medications.  Aspirin diary filed in patient's hand-held records for future use.
Consent	Written and verbal.
Blood and urine sampling	Venous blood sample (15ml).  3ml Hirudin (Multiplate™), 2ml Citrate (VerifyNow™), 3ml EDTA (DNA storage)  3ml Citrate (plasma for LC:MS)  4ml whole blood (serum biobanked).  *Subgroup observed taking 75mg dispersible aspirin.15ml venous blood sample repeated 1 hour following aspirin dose.  1-20ml urine sample (TXB Cardio™, urine for NMR).

**Table 2:** Study procedures 5+<sup>0</sup>-20+<sup>6</sup> visit.

\*NMR=nuclear magnetic resonance.

Obstetric ultrasound scan	Mean uterine artery Doppler PI.
NICOM/Arteriography	Non-invasive measurement of cardiac output, blood pressures and arteriography.

**Table 3:** Study procedures 20+<sup>0</sup>-23+<sup>6</sup> visit.

\*PI=pulsatility index.



Aspirin Diary	Participants reminded by telephone 14 days prior to visit to commence aspirin diary. Dates, times, missed doses and other medications during time period 7days prior to visit recorded and collected by EARTH research team.
Blood and urine sampling	Single venous blood sample (12ml total) 3ml EDTA (PIGF) 3ml Hirudin (Multiplate™), 2ml Citrate (VerifyNow™)  2ml whole blood (serum to assess PIGF, sFlt-1)  2ml Citrate (plasma for LC: MS)  1-20ml urine sample (TXB Cardio™, urine for NMR).
Obstetric ultrasound scan	Mean uterine artery Doppler PI  Fetal Doppler (Umbilical artery PI, Middle Cerebral Artery PI, venous Doppler)  Fetal growth measurements (bi-parietal diameter, head circumference, abdominal circumference, femur length), Liquor volume (Maximum vertical pool depth).
NICOM/Arteriography	Non-invasive measurement of cardiac output, blood pressures and arteriography.

**Table 4:** Study procedures 33+<sup>0</sup>-35+<sup>6</sup> visit.

\*EDTA=Ethylenediaminetetraacetic acid, LC: MS=liquid chromatography mass spectrometry,  
NMR=nuclear magnetic resonance, PI=pulsatility index

Placental Tissue	Assessment of placental vasculature, biomarkers for preeclampsia and placental disease, genomics, proteomics and metabolomics.
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**Table 5:** Study procedures following delivery.

## Laboratory techniques

**Multiplate™ Impedance aggregometry.** We opted to use Multiplate™ impedance aggregometry (Roche diagnostics international) provides a COX-1-specific assessment of platelet function. The instrument is for point-of-care use and provides a result within 10 minutes (Cobas, Toth et al., 2006). The instrument is semi-automated and detects the change in electrical impedance when platelets aggregate on the electrode sensors inside the test cuvette. The instrument has 5 channels that allow simultaneous assessment of different samples or assessment of aggregation stimulated by different agonists. Each test cuvette contains two sets of electrode sensors which provide an internal control for the measurement (accepted if correlation coefficient greater than 0.98 and difference of each curve from mean curve is less than 20%). Within each cuvette 300µl of hirudin anticoagulated whole blood is incubated for 3 minutes, saline diluted, then combined with the selected agonist, ASPI test (arachidonic acid, AA). The change in impedance, transformed to aggregation units (AU), is plotted against time at 0.5 second intervals over 6 minutes, allowing display of area under the curve (AUC).

**VerifyNow™ Aspirin Test (Elitech, UK)** a COX-1 specific point-of-care platelet function test, has been validated in cardiology populations, but not previously been assessed in pregnancy (Accumetrics, 2017). The agonist, arachidonic acid, is used to activate platelets. The instrument measures platelet function based upon the ability of activated platelets to bind fibrinogen. Fibrinogen-coated micro particles aggregate in whole blood in proportion to the number of unblocked platelet glycoprotein (GP) IIb/IIIa receptors. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads, measured as a change in optical signal.

This instrument is a closed point-of-care system that provides a result within 10 minutes. At venepuncture 2ml whole blood is drawn into a 3.2% sodium citrate coated tube and

inverted five times to ensure thorough mixing and incubated at room temperature for 30 minutes prior to analysis. The aspirin test cartridge and citrated whole blood tube are inserted into the instrument, when prompted, and the lid closed to allow accurate recording of the optical signal. The strength of the change in optical signal due to platelet aggregation within the cartridge is reported as aspirin reaction units (ARU). Less than 550 ARU is defined by the manufacturer as an appropriate response to aspirin.

**TXB Cardio™**, 1ml urine aliquots, intended for assessment of urinary 11-DHTXB<sub>2</sub> were stored at -80°C within 2-3 hours of collection. We opted to use the TXB Cardio™ test (Randox, UK) which is a validated latex enhanced immunoturbidimetric method, with a measuring range of 400-6000pg/ml (Randox, 2017). Samples were shipped on dry ice to Randox laboratories, Ireland, for analysis. The standardised results were reported by expressing the concentration of 11-DHTXB<sub>2</sub> in relation to the level of creatinine in the sample.

To allow development of the **NMR and LC: MS methods** used to detect of aspirin metabolites, described in full in Chapter 4 we obtained maternal plasma and urine samples. Maternal citrated whole blood samples were centrifuged at 2600 rpm for 10 minutes at 4 °C and plasma obtained aliquoted into 1ml volumes. We also used the stored 1ml maternal urine aliquots previously described during this method development. Both samples types were stored at -80°C, prior to transfer on dry ice to the NMR Centre and the Bioanalytical Facility for LC: MS (both University of Liverpool).

**PIGF and sFlt-1.** Citrated whole blood samples were centrifuged at 4 °C, 1500g for 10 minutes and care taken not to disturb the buffycoat to obtain plasma. Clot-activated whole blood samples were centrifuged at 4 °C, 1500g for 10 minutes to obtain serum. Maternal plasma and serum were divided into 1ml aliquots within 3 hours of venepuncture, securely stored at -80°C and subsequently thawed for analysis in batches.

250µL of EDTA plasma was tested for PIGF using the Alere Triage Meter-Pro™ point-of-care test in the Centre for Women's Health Research Laboratories. Additionally, PIGF and s-Flt-1 were determined from 1ml serum aliquots using the Elecsys® sFlt-1 and Elecsys® PIGF automated immunoassays in Royal Liverpool University Hospital Laboratory. PIGF was also measured from 1ml plasma aliquots using the DELFIA® time resolved fluorescence technique at Perkin Elmer laboratories. Specific PIGF and sFlt-1 test parameters and methods, for each test are described in detail in Chapter 7.

**Blood for DNA extraction:** Five millilitres of whole blood withdrawn in EDTA coated tubes was stored at -20°C in the Wolfson Centre for Personalised Medicine prior to DNA extraction. DNA samples were stored in a state-of-the-art DNA archive with integrated robotics, laboratory information management system (LIMS) and automated sample tracking with 2D bar-coding.

DNA was extracted using the Chemagen Chemagic Magnetic Separation module 1, according to the 1-5ml whole blood Chemagic Magnetic Separation Module 1 localised protocol version 1.0 2009, Wolfson Centre for Personalised Medicine. This module allows simultaneous extraction of DNA from 12 samples simultaneously.

Prior to extraction, EDTA whole blood samples were allowed to thaw completely for 45 minutes from storage at -20°C and vortexed for 5 seconds to ensure thorough mixing. 2ml EDTA whole blood samples were designated from each participant to enable a minimum of 1ml EDTA whole blood to be preserved for repeat extraction in the event of method failure. Samples were aliquoted into pre-labelled sterile tubes, 7.5ml lysis buffer and 20µL protease were added. Following sample preparation, the automated protocol A (lysate mixing) programme was completed over a period of ten minutes. On completion of protocol A, 19.5ml of binding buffer 2 and 500 µL magnetic beads were added to each of the tubes containing samples. Additionally, wash buffers 3-6 and elution buffer 7 were

added to the remaining racks, as detailed in the full protocol, to enable sequential elution. The automated protocol B (DNA blood elution) programme was then commenced, with a duration of 40 minutes. Following completion of protocol B, extracted DNA samples were carefully aliquoted into pre-labelled sterile 1.5ml epindorph tubes. These samples were visually inspected for signs of contamination with residual magnetic beads due to the extraction method. Any DNA samples discoloured by magnetic beads were centrifuged at 1500 g for 10 minutes and the supernatant was transferred to new sterile 1.5ml epindorph tubes. This procedure was repeated if necessary to ensure DNA samples appeared clean. Nanodrop quantification was carried out immediately following this to ascertain if extraction of sufficient had been carried out. Optimal ratios for samples were taken as A260/280: 1.65-2.1, 1260/230: 2.0-2.2, according to the full protocol. Subsequent quantification of DNA using the Picogreen method and preparation of samples for genome-wide analysis is described in detail in Chapter 6.

### **Clinical outcome measures**

#### MATERNAL OUTCOME MEASURES

**Response to aspirin** incorporated platelet response and LDA adherence. Platelet response was measured against the pregnancy specific reference ranges established for Multiplate™ and TXB Cardio™ tests, described in Chapter 1, Table 5. For VerifyNow™, the manufacturer's reference range (<550 ARU, considered to be a normal platelet response to aspirin) was used as no pregnancy-specific cut-off was available. To be considered as having an expected platelet response to aspirin participants were required to have 2/3 platelet function test results (Multiplate™, VerifyNow™ and/or TXB Cardio™) lying outside the reference range stipulated for each test.

Assignment of LDA adherence status took account of the best available LDA adherence data for the EARTH cohort, by incorporating detection of aspirin metabolites detailed in Chapter 4. Aspirin metabolites were detected in maternal urine (using NMR) and/or

maternal plasma (using LC: MS). Evidence of LDA adherence was defined as detection of aspirin metabolites using either method, or evidence of the expected platelet response to LDA (defined above).

- Responsive

Platelets responsive with or without detectable aspirin metabolites.

- Non-responsive

Platelets non-responsive with detectable aspirin metabolites.

- Indeterminate response

Platelets non-responsive and aspirin metabolites not detected.

**Abnormal uterine artery Doppler** was defined as a mean pulsatility index (PI) of 1.45 or greater at 20<sup>+</sup><sup>0</sup>-23<sup>+</sup><sup>6</sup> weeks.

**Preeclampsia** was defined as proteinuric hypertension (with new onset after 20 weeks). Systolic blood pressure (BP)  $\geq 140$  mmHg and/or diastolic BP  $\geq 90$  mmHg (Korotkoff V) on at least 2 occasions 4h apart, with proteinuria (spot urine  $\geq 2+$ , protein: creatinine ratio  $\geq 30$  mg/mmol, or  $\geq 300$  mg/24h (using a validated 24 hour collection method) (NICE, 2010a). Severity was graded, mild (140-149/90-99mmHg), moderate (150-159/100-109) or severe ( $>160/110$  mmHg) in accordance with NICE guidelines (NICE, 2010a).

**Gestational hypertension** was defined as non-proteinuric hypertension (with new onset after 20 weeks). Systolic blood pressure (BP)  $\geq 140$  mmHg and/or diastolic BP  $\geq 90$  mmHg (Korotkoff V) on at least 2 occasions 4h apart, with proteinuria (NICE, 2010a). Severity was graded, mild (140-149/90-99mmHg), moderate (150-159/100-109) or severe ( $>160/110$  mmHg) in accordance with NICE guidelines (NICE, 2010a).

**Placental abruption** was defined as clinical evidence of placental abruption at the time of delivery, on examining the placenta, or from placental histology diagnosis.

## FETAL AND NEONATAL OUTCOME MEASURES

**Intrauterine fetal growth restriction (IUGR).** The estimated fetal weight at 33+<sup>0</sup>-35+<sup>6</sup>, and birthweight were assessed against customised centiles, adjusted for gestation, maternal weight, height, parity and ethnicity (GROW, 2016b). IUGR was defined as less than 5<sup>th</sup> customised birthweight centile with abnormal fetal Doppler indices including; umbilical artery Doppler (PI>95<sup>th</sup> centile, reduced, absent, or reversed end diastolic flow), evidence of brain-sparing in the middle cerebral artery, absent a-wave in the ductus venosus, or pulsatile umbilical vein.

**Small for gestational age** was defined as customised birthweight <5<sup>th</sup> or <10<sup>th</sup> centile, with no evidence of abnormal Doppler indices (described above) during antenatal assessments.

**Admission (and duration of admission) to neonatal unit** was recorded.

**Neonatal morbidity**, including respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular haemorrhage, periventricular leukomalacia, seizures, hypoxic ischaemic encephalopathy, need for cooling therapy, necrotising enterocolitis, retinopathy of prematurity. These diagnoses were taken from electronic neonatal records and death certificates.

**Perinatal mortality.** We included intrauterine fetal deaths (deaths of structurally normal fetuses occurring after 24+<sup>0</sup> weeks), and neonatal deaths up to seven completed days after birth.

**Neonatal mortality**, deaths occurring after 7 completed days of life, up to 28 completed days of life.

## COMPOSITE ADVERSE PLACENTALLY-MEDIATED OUTCOME

Any, or a combination of pre-eclampsia, placental abruption, IUGR and perinatal mortality.

### **Statistical analysis**

#### Sample size calculation

The reference ranges for platelet activation in pregnancy study demonstrated that 2-3 high risk women can be recruited per week. Therefore, up to 200 high risk women could feasibly be recruited during a 2 year study period. The expected frequency of aspirin non-responders in this cohort was unknown since this has not been reliably measured in pregnancy. Assuming a conservative proportion of aspirin non-responsiveness of 20% (Caron N, 2009), a sample size of 100 would have good estimated precision (i.e. 95% Confidence Intervals from 13%-29%) to detect this. The frequency of pre-eclampsia in high risk women taking LDA is 15% overall (Roberge et al., 2013). As NICE also recommends LDA for women with more than one moderate risk factor, a realistic estimate is 12% (12 cases expected from the sample). We hypothesised that approximately 40% of aspirin non-responders would develop pre-eclampsia, compared with less than 10% of responders. A sample size of 100 women would have adequate power (power of 80% with alpha error of 5%) to test this hypothesis, assuming that 15-20% of our cohort would be non-responsive to aspirin. We estimated that 180 total participants would be required to ensure 100 women appropriately adherent to LDA would be included in the total cohort (Ho et al., 2006).

For genetic analyses, with the envisaged number of 100 appropriately adherent participants, we would have 80% power at 5% significance level to detect medium to large effects (Odds Ratio 4-5) for variants of medium to high frequency (>10%).

SPSS v.24 (IBM Corporation, USA) was used for statistical analysis of EARTH study data.

Descriptive statistics for the cohort, including mean median and mode values for



characteristics of maternal age, BMI, ethnicity, gravidity, parity and proportions of risk factors for pre-eclampsia were calculated.

Overall percent agreement (OPA) was calculated for the three comparisons of the platelet function assays used in EARTH; i) VerifyNow™ Aspirin test and Multiplate™ ASPI test ii) VerifyNow™ Aspirin test and TXB Cardio™ and iii) Multiplate™ ASPI test and TXB Cardio™. OPA was calculated as the sum of positive and negative results with both tests, divided by the total tested. Positive (PPA) and negative percent agreement (NPA) were calculated for these three groups in both directions (VerifyNow™ to Multiplate™, Multiplate™ to VerifyNow™, VerifyNow™ to TXB Cardio™, TXB Cardio™ to VerifyNow™, Multiplate™ to TXB Cardio™ and TXB Cardio™ to Multiplate™). PPA was calculated by the number of agreed positives between the tests, divided by the agreed positives plus negatives for the test under comparison only. Similarly, NPA was calculated as the agreed negatives, divided by agreed negatives plus negatives for the test under comparison only.

Proportions of participants in the following groups; i) aspirin responders, ii) aspirin non-responders and ii) indeterminate aspirin response were calculated per visit. Longitudinal aspirin response status was also determined for participants sampled at both visits, in addition to the groups described above we calculated the proportion of participants with variable aspirin response status (changing between groups) between visits. Percentages of all maternal, fetal and neonatal outcome measures were calculated for comparison in the groups outlined. Pearson's chi-square test was used to assess for associations between longitudinal aspirin response status and i) any pre-eclampsia ii) composite adverse placentally-mediated outcome (previously defined). A Bonferroni correction was applied to the p value of 0.05, to correct for the effect of these multiple analyses between groups, resulting in a new p value for these analyses of 0.025.

Analysis of metabolite detection data is described in full in Chapter 4. PlGF and sFlt-1/PlGF ratio data were analysed using SPSS v.24 (IBM Corporation, USA), the methods for these analyses are detailed in Chapter 7. GWAS data was quality controlled and analysed using Plink v.1.07 (Purcell et al., 2007). Manhattan plots were produced with the genetics package of R v.3.1.1 (R, 2017), regional plots were produced with LocusZoom (Pruim et al., 2010). Complete methods for GWAS quality control and analysis are described in Chapter 6.

#### **5.4 Results: Description of the cohort and clinical outcomes**

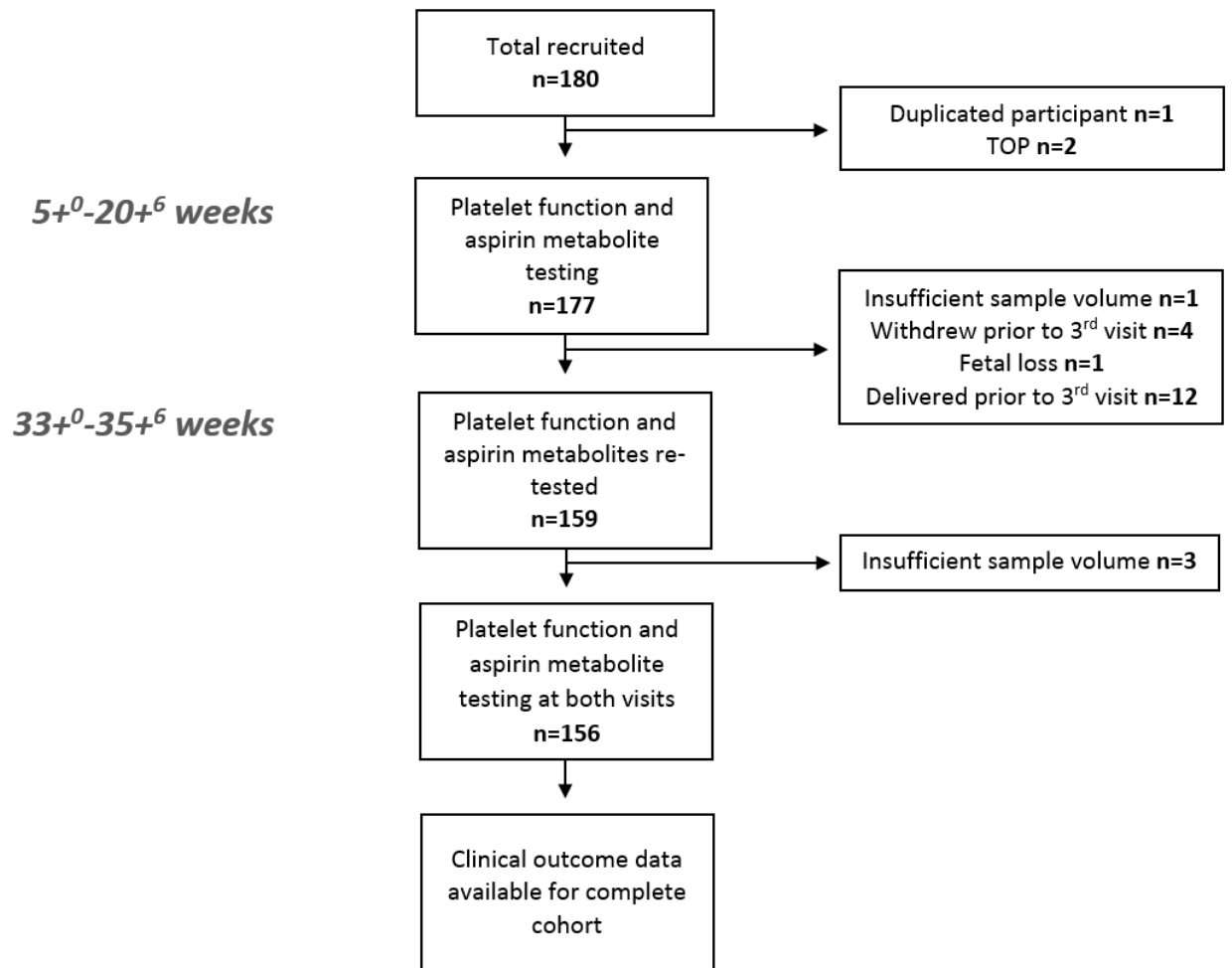
The EARTH study was conducted in the antenatal clinics at Liverpool Women's Hospital between 17<sup>th</sup> January 2014 and 31<sup>st</sup> March 2016, according to the previously described protocol (Appendix 3c). 180 women at high risk of pre-eclampsia and prescribed 75mg aspirin were recruited to the study and followed longitudinally as specified (Figure 1). All women were recruited between 5+<sup>0</sup> weeks and 20+<sup>6</sup> weeks and had commenced aspirin by 18+<sup>0</sup> weeks. A subgroup of 53 aspirin naïve women were recruited and sampled prior to, and 60 minutes following the first 75mg aspirin dose (Appendix-3c). The clinical study completed on 31/03/16.

One woman was enrolled twice, with consecutive pregnancies, her second participation was subsequently excluded from all analyses. Two participants underwent termination of pregnancy due to fetal abnormalities identified in the second trimester and were excluded from further analyses due to inability to determine true clinical outcomes.

Overall retention of participants and attendance at follow-up visits was good. There were four withdrawals from the study prior to collection of third trimester samples (Figure 1). All of the women who decided to withdraw did so prior to the third visit (33+<sup>0</sup>-35+<sup>6</sup> weeks) as they did not wish to provide further clinical samples or to have study examinations. All women who withdrew gave consent for their samples already donated

to be analysed and for their clinical outcome data to be collected and analysed. Demographic data and clinical outcomes for the EARTH cohort are shown in Table 6.

In total 13 participants delivered during the course of the clinical study (Table 7). Two women suffered fetal losses due to placental insufficiency, one combined with placental abruption and severe antepartum haemorrhage, necessitating hysterotomy at 22<sup>0</sup> weeks. The other had an IUFD at 25<sup>0</sup> week's gestation. A further 11 women had livebirths prior to the completion of EARTH follow-up visits (Table 6). Of these thirteen participants delivered during the course of the study, a total of 69.2% (n=9) were responsive to aspirin (Table 7). 61.5% (n=8) had a composite placentally-mediated adverse outcome and of these 87.5% (n=7) were responsive to aspirin (Table 7).



**Figure 1:** EARTH study flow diagram.

<b>MATERNAL DEMOGRAPHIC CHARACTERISTICS</b>	<b>Total (n=177)</b>
Mean maternal age (SD)	31.2 (5.3)
Mean body mass index, BMI (SD)	31.6 (7.1)
Nulliparous	23.2% (n=41)
<b>Antenatal care</b>	
Median gestational age of commencing LDA (range)	10 (0-18)
Antihypertensive treatment	32.8% (n=58)
Admissions (pre-eclampsia, gestational hypertension, IUGR)	16.9% (n=30)
Median length of admission (range)	2 (1-13)
<b>Intrapartum care</b>	
Admissions to high dependency/intensive therapy unit (HDU/ITU)	7.9% (n=14)
Median length of HDU/ITU admission (range)	1 (1-1)
Acute antihypertensive treatment	15.3% (n=27)
Magnesium sulphate treatment	6.8% (n=12)
Spontaneous labour	15.3% (n=27)
Induction of labour	49.2% (n=87)
<b>Delivery</b>	
Spontaneous preterm birth <37 weeks	4.5% (n=8)
Caesarean section	46.3% (n=82)
<b>MATERNAL CLINICAL OUTCOMES</b>	
Pre-eclampsia	Total 16.4% (n=29)
≥37 weeks with appropriate growth for gestational age (AGA)	7.3% (n=13)
≥37 weeks with small for gestational age (SGA) 5-9 <sup>th</sup> centile	0.6% (n=1)
≥37 weeks with SGA <5 <sup>th</sup> centile	1.1% (n=2)
<37 weeks with AGA	2.8% (n=5)
<37 weeks with SGA 5-9 <sup>th</sup> centile	0.6% (n=1)
<37 weeks with SGA <5 <sup>th</sup> centile	1.7% (n=3)
<37 weeks with IUGR	2.3% (n=4)
Gestational Hypertension	5.1% (n=9)
Placental abruption	1.1% (n=2)
<b>FETAL AND NEONATAL OUTCOMES</b>	
IUFD	1.7% (n=3)
Isolated IUGR	2.8% (n=5)
Isolated SGA	Total 10.7% (n=19)
<5 <sup>th</sup> customised centile	3.4% (n=6)
5-9 <sup>th</sup> customised centile	7.3% (n=13)
Admissions to neonatal intensive care unit (NICU)	16.4% (n=29)
Median length of NICU admission (range)	5 (1-77)
RDS	10.7% (n=19)
Bronchopulmonary dysplasia	0.6% (n=1)
Intraventricular haemorrhage	0.6% (n=1)
Periventricular leukomalacia	0.6% (n=1)
Seizures	0.6% (n=1)
Hypoxic Ischaemic encephalopathy	0.6% (n=1)
Necrotising enterocolitis	0.6% (n=1)
Neonatal death	0.6% (n=1)

**Table 6:** Demographic data and clinical outcomes for the EARTH cohort.

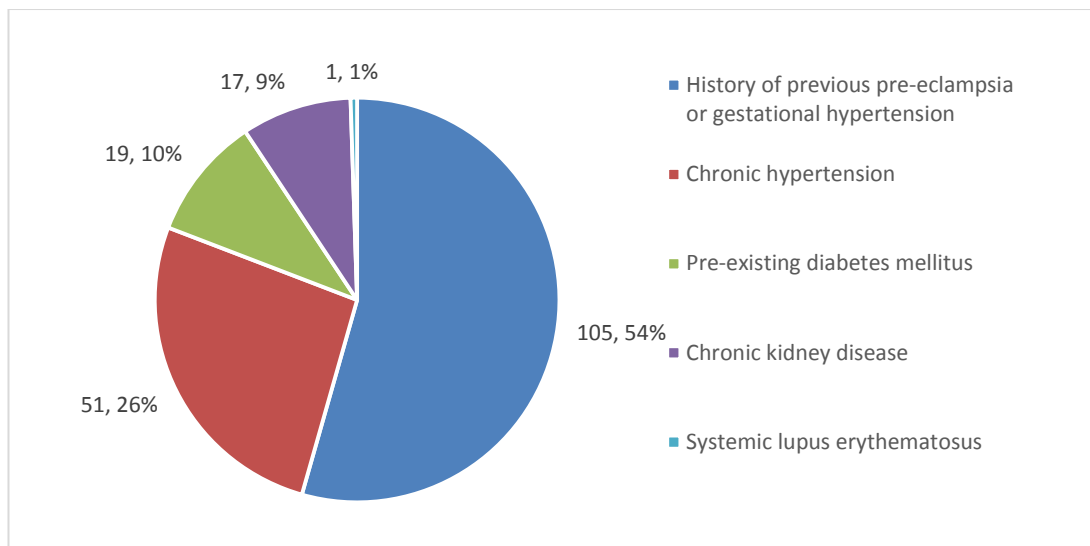
Case no.	Risk factors	Aspirin response (R/N/I)	Abnormal uterine artery Doppler? (Y/N)	Gestation ( delivery)	Clinical outcomes
1	Previous pre-eclampsia, family history of pre-eclampsia	R	N/A	22 <sup>0</sup>	Placental abruption, maternal collapse, hysterotomy, fetal loss.
2	Chronic hypertension, chronic kidney disease, nulliparous	R	N/A	25 <sup>0</sup>	IUFD, induction of labour, NVD, 1st centile.
3	Chronic hypertension, nulliparous	I	N	26 <sup>6</sup>	Spontaneous preterm birth, NVD, 19th centile. RDS, thrombocytopaenia, metabolic bone disease.
4	Chronic hypertension, chronic kidney disease, previous pre-eclampsia	R	N	29 <sup>0</sup>	Spontaneous preterm birth, 43rd centile, RDS, periventricular leukomalacia.
5	Chronic hypertension, previous pre-eclampsia, BMI>35	R	Y	31 <sup>1</sup>	Severe pre-eclampsia, EMCS, 0 centile, RDS, placenta-MVUP.
6	Chronic hypertension, previous pre-eclampsia	R	N	31 <sup>4</sup>	Severe pre-eclampsia, EMCS, 4th centile, RDS.
7	Chronic hypertension, nulliparous, family history of pre-eclampsia	N	N	32 <sup>1</sup>	Severe pre-eclampsia, EMCS, 7th centile, RDS, hypoglycaemia.
8	Previous pre-eclampsia, BMI>35	N	N	32 <sup>2</sup>	Spontaneous preterm birth, footling breech, EMCS, 18th centile, RDS.
9	Chronic hypertension, previous pre-eclampsia	R	N	32 <sup>4</sup>	Spontaneous preterm birth, EMCS, 64th centile.
10	Pre-existing diabetes, nulliparous, BMI>35	R	N	33 <sup>4</sup>	Moderate pre-eclampsia, EMCS 95th centile, RDS, HIE grade III, NEC, NND day 16 PN. Placenta-MVUP.
11	Pre-existing diabetes, nulliparous, BMI>35	R	Y	33 <sup>6</sup>	Moderate pre-eclampsia, EMCS 40th centile, RDS, thrombocytopaenia.
12	Previous pre-eclampsia	N	N/A	34 <sup>3</sup>	Fetal distress, fetal duodenal atresia, EMCS, 42nd centile.
13	Chronic kidney disease, nulliparous	R	N	35 <sup>4</sup>	Severe pre-eclampsia, IOL, NVD, 9th centile.

**Table 7:** Participants delivered prior to completion of EARTH study follow-up visits.

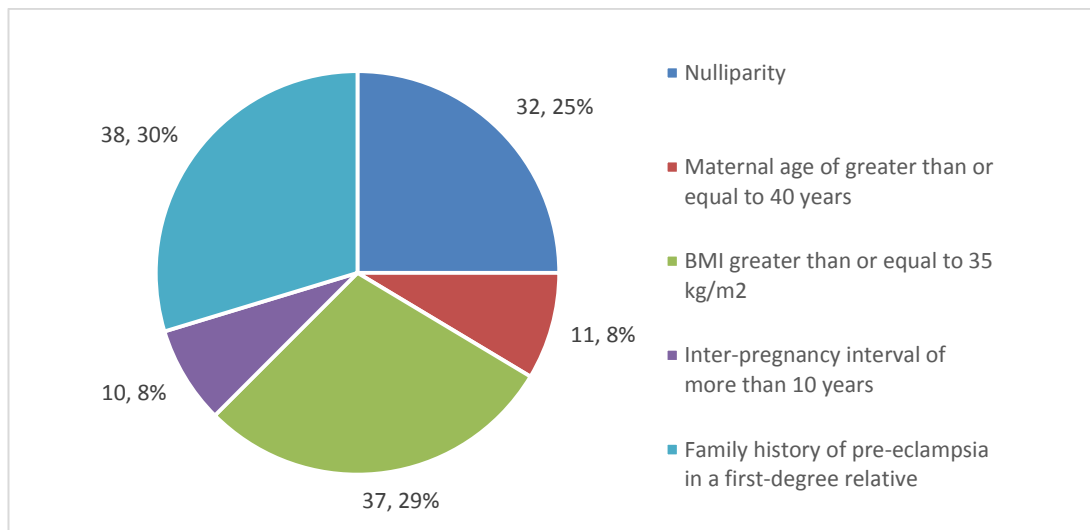
\*R=responder, N=Non-responder, I=Indeterminate response to aspirin, IUFD=intrauterine fetal death, NVD=normal vaginal delivery, RDS=respiratory distress syndrome, EMCS=emergency caesarean section, MVUP=maternal vascular under perfusion, HIE=hypoxic ischaemic encephalopathy.

### Risk factors of included participants

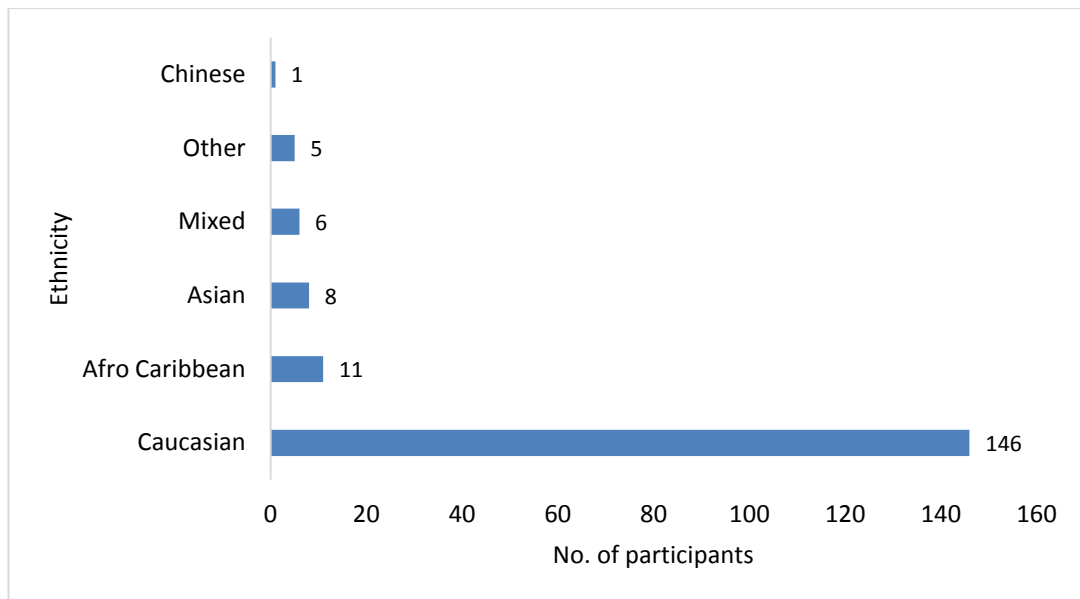
92.7% of women (n=164) had high risk factors for pre-eclampsia, according to NICE risk factor classification(NICE, 2010a). 14.1% of women in the cohort (n=25) had multiple high risk factors for preeclampsia. 54.8% of women (n=97) had moderate risk factors for pre-eclampsia. 19.8% (n=35) of women in the cohort had multiple moderate risk factors for pre-eclampsia.



**Figure 2:** High risk factors for pre-eclampsia in the EARTH cohort.



**Figure 3:** Moderate risk factors for pre-eclampsia in the EARTH cohort.



**Figure 4:** Ethnicity of EARTH participants

### 5.5 Results: Response to aspirin

#### Failed samples/analyses: Visit 1 (5<sup>0</sup>-20<sup>6</sup> weeks)

Of participants sampled at the first visit, 98.3% (n=174) participants had VerifyNow™ Aspirin test results, 98.3% (n=174) had TXB Cardio™ results and all participants had results for Multiplate™ ASPI test.

A subgroup of 53 participants provided samples for point-of-care platelet function tests (VerifyNow™ Aspirin test and Multiplate™ ASPI test) immediately prior to and 60 minutes following their first LDA. Within this observed dosing subgroup, 49 participants had paired VerifyNow™ Aspirin test results, and all participants had paired Multiplate™ ASPI test results.

Of 171 participants who had results for all three platelet function tests at the first visit 64.9% (n=111) had consistent results across all tests. 78.4% (n=87) demonstrated appropriate platelet response to aspirin and 21.6% (n=24) whose platelets appeared to be non-responsive. The remaining 35.1% (n=60) participants demonstrated inconsistent platelet response across the tests carried out.



According to the previously described definitions for determining platelet response, it was possible to ascribe platelet response for all participants at this visit. Similarly, after assigning LDA adherence status, it was possible to determine response to aspirin for 99.4% (n=176) of participants at the first visit.

**Failed samples/analyses: Visit 3 (33<sup>0</sup>-35<sup>6</sup>)**

Of participants sampled at the third visit, 95.0% (n=152) participants had VerifyNow™ Aspirin test results, 96.9% (n=155) had TXB Cardio™ results and 97.5% (n=156) had results for Multiplate™ ASPI test. Of 148 participants who had results for all three platelet function tests at this visit, 69.6% (n=103) had consistent results across all tests. 75.7% (n=78) demonstrated appropriate platelet response to aspirin and the platelets of 24.3% (n=25) appeared to be non-responsive. The remaining 30.4% (n=45) participants demonstrated inconsistent platelet response across the three tests.

It was possible to assign platelet response and after incorporating aspirin adherence status, to determine response to aspirin for 98.1% (n=157) participants sampled at this visit.

In total, 156 participants sampled longitudinally across both time-points, had sufficient platelet function and aspirin metabolite data to determine their response to aspirin (Figure 1).

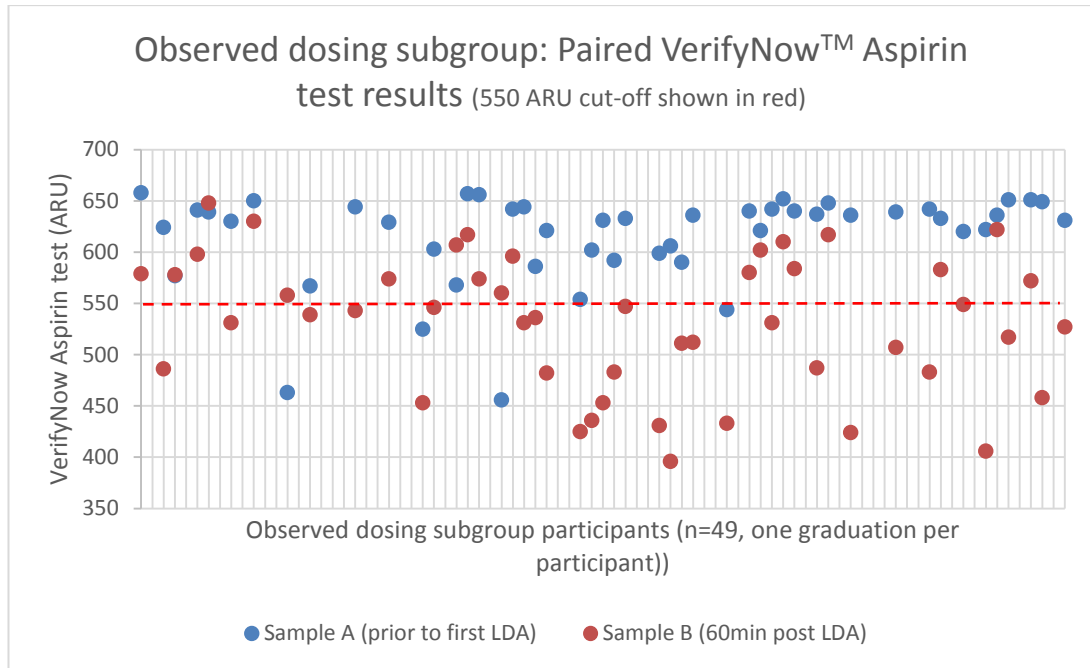
### **Platelet function tests**

Across all three platelet function tests a large proportion of results fell outside the range expected for aspirin-treated individuals (inside pregnancy-specific reference ranges for aspirin naïve women or above the manufacturer's cut-off for 'aspirin resistance' Figures 5-8). For VerifyNow™ Aspirin test 28.2% (n=49) were above the manufacturer's threshold at the first visit and 28.2% (n=43) at the third visit. For Multiplate™ 29.4% (n=52) fell within the pregnancy-specific reference range at the first visit and 30.1% (n=47) at the third visit. For TXB Cardio™ the percentage falling above the lower limit of the pregnancy-specific reference range fell by approximately 10% between the first and third visits (36.2% n=63 and 25.8% n=40, respectively).

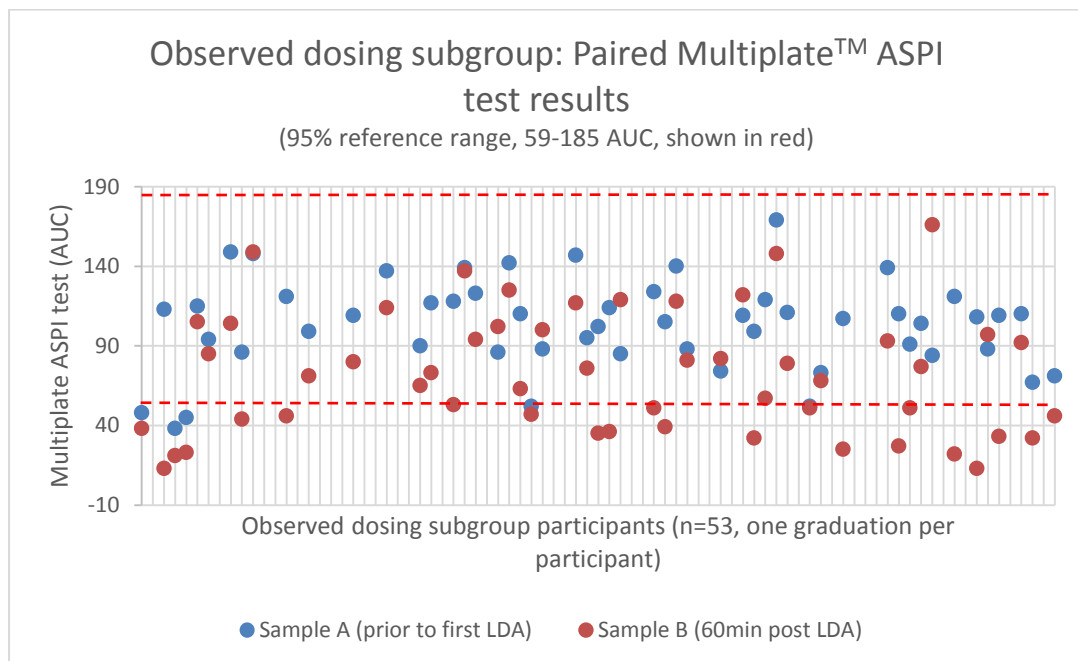
Results from the observed dosing subgroup demonstrate the consistent, measurable effect of a single 75mg dispersible aspirin on platelet function using both point-of care tests (Figure 5). However, the variability in the magnitude of response to a single dose between individuals is apparent when examining the paired data from this subgroup (Figure 5).

For VerifyNow™ Aspirin test and Multiplate™ ASPI test, two participants in each analysis had higher platelet activity after LDA as compared to before dosing, the opposite of the expected effect. One of these participants demonstrated this effect with both point-of-care assays. Source data from the assays and the laboratory book were examined to confirm the identity of the 'A' (prior to the first LDA) and 'B' samples (obtained 60 minutes following the first LDA) and were found to be correct. Additionally, NMR and LC: MS data for all three participants indicated undetectable aspirin metabolites in 'A' samples and detectable aspirin metabolites in 'B' samples.

a)



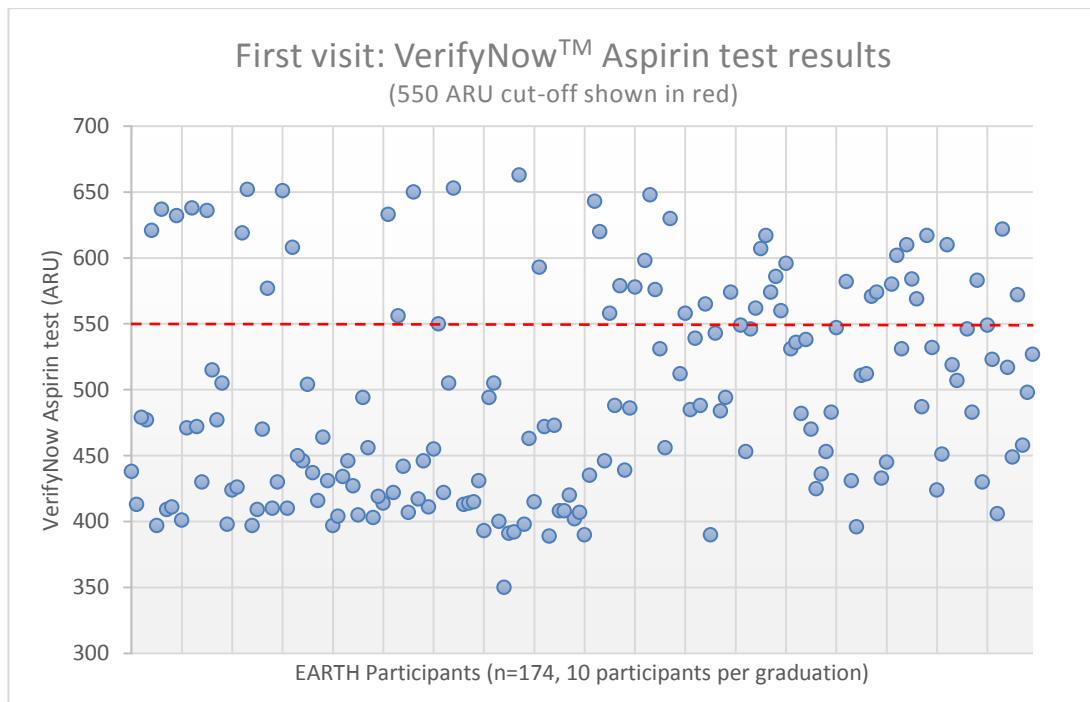
b)



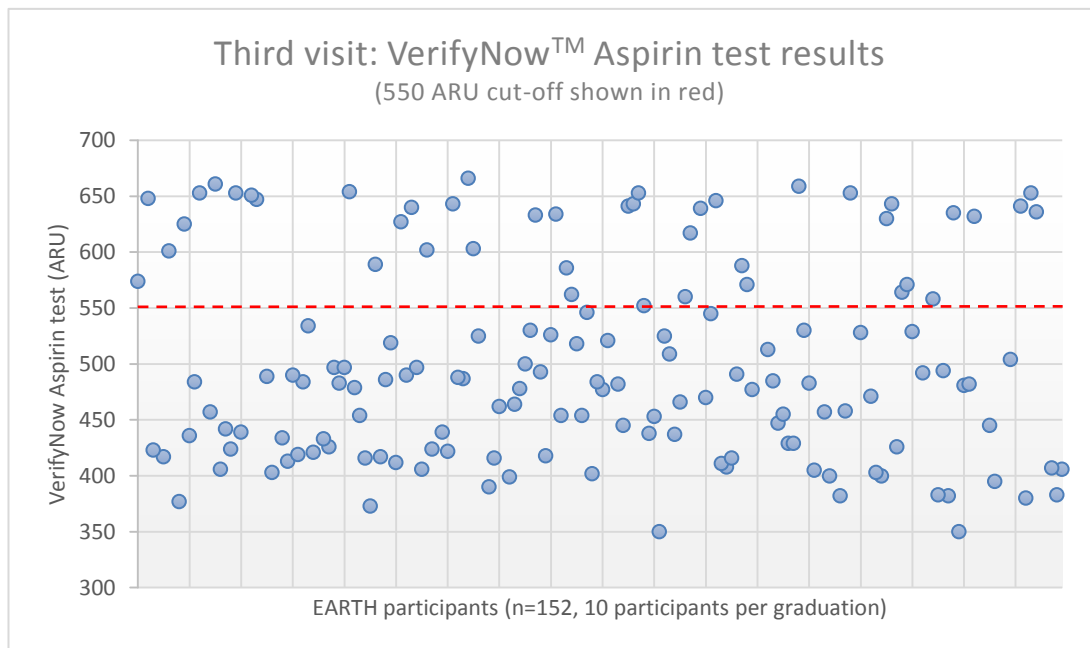
**Figure 5:** Observed dosing subgroup, paired point-of-care platelet function results

- a) VerifyNow™ Aspirin test
- b) Multiplate™ ASPI test™

a)



b)

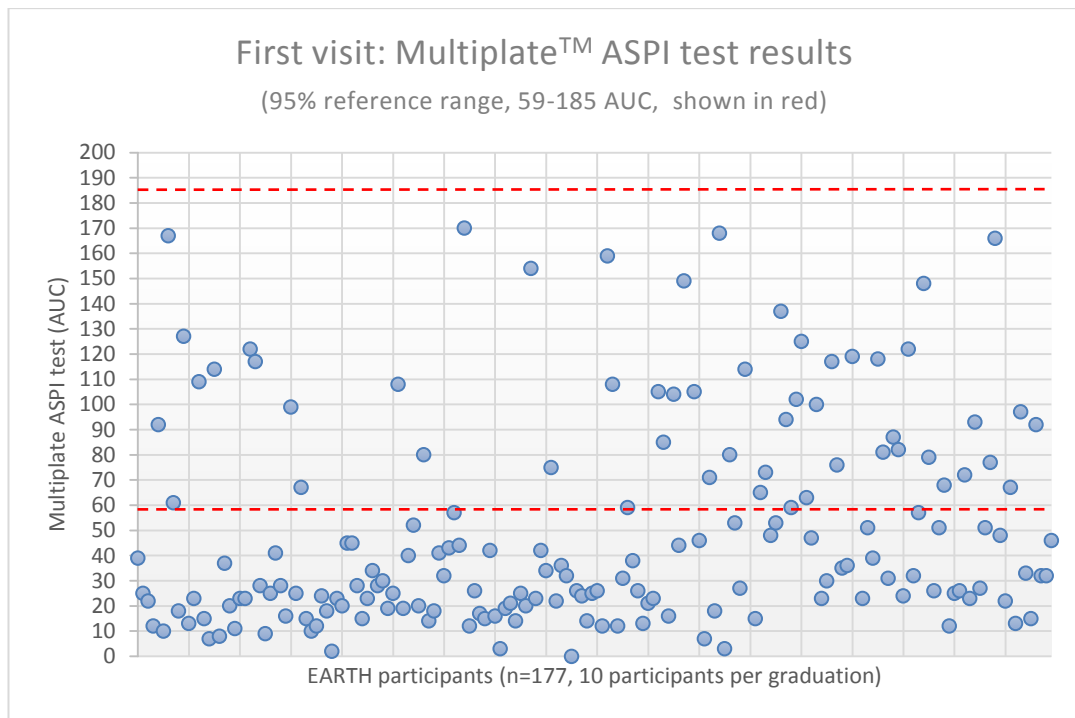


**Figure 6:** VerifyNow™ Aspirin test results (550 ARU cut-off shown in red)

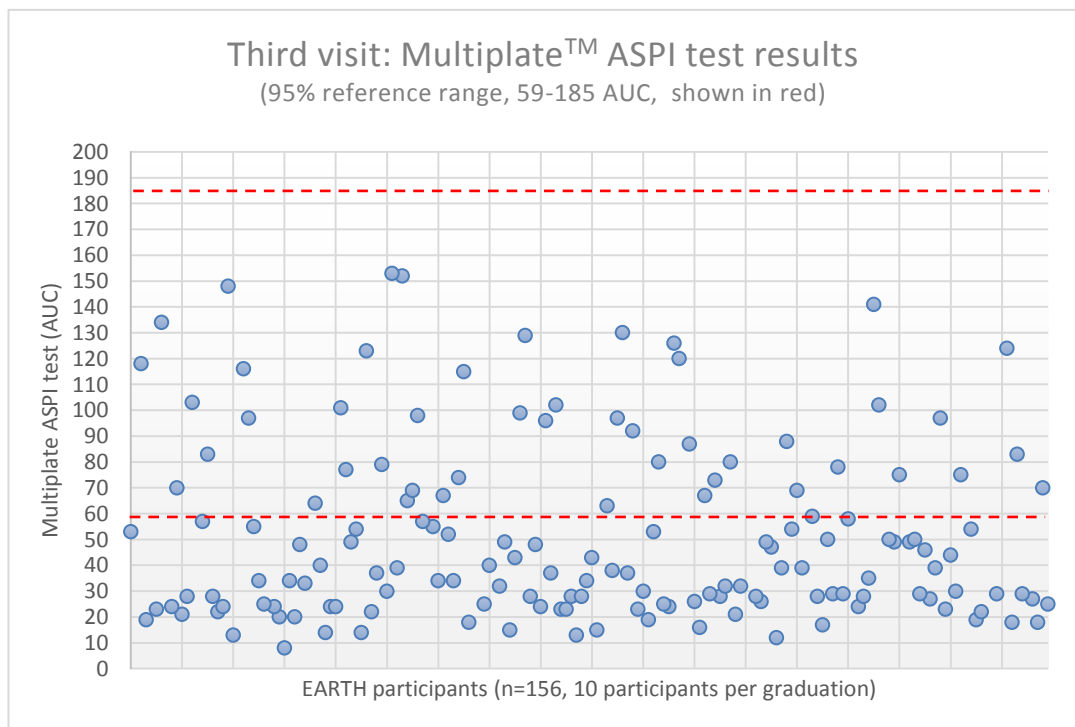
a) first visit

b) third visit

a)



b)

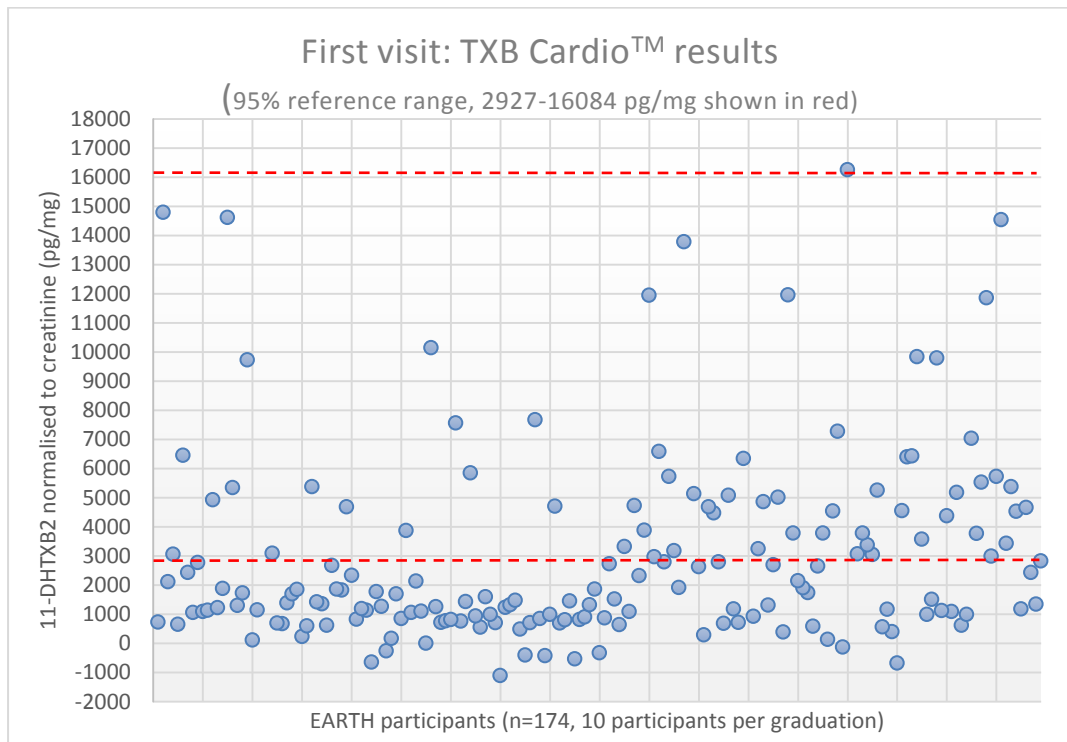


**Figure 7:** Multiplate™ ASPI test results (59-185 AUC, 95% reference range shown in red)

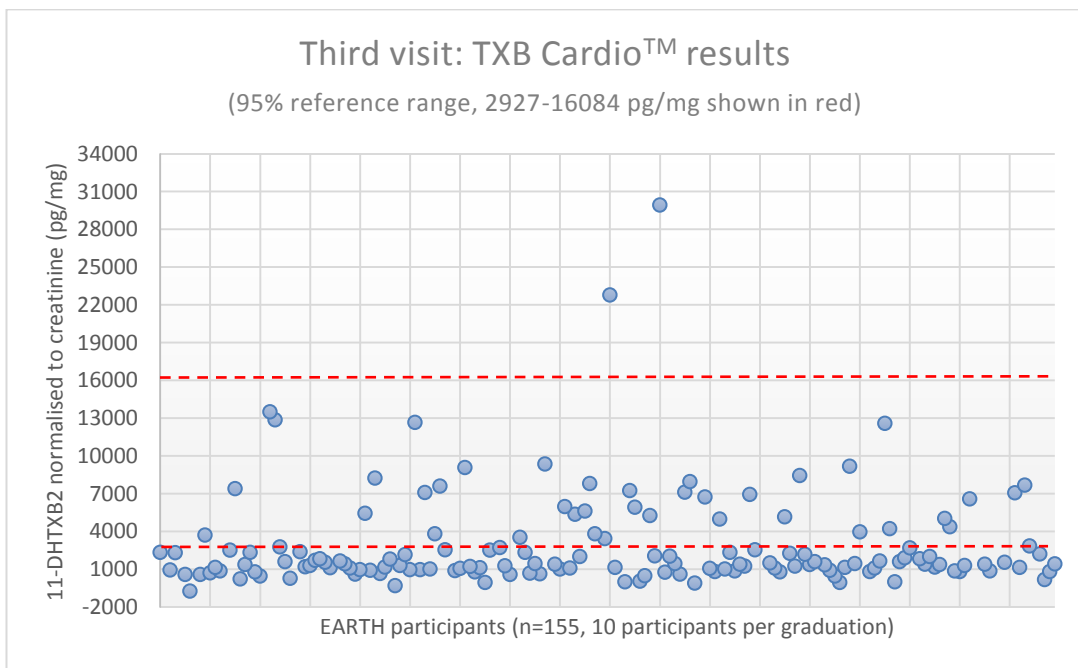
a) first visit

b) third visit

a)



b)



**Figure 8:** First visit TXB Cardio™ results (2927-16084, 95% reference range shown in red)

a) first visit

b) third visit

### Agreement between platelet function assays

Percentage agreements for participants with results for all three platelet function assays (n=171 at first visit, n=148 at third visit) are shown in Tables 8, 9 and 10. For all tests comparisons NPA (identification of participants with an expected platelet response to LDA treatment) was higher than PPA (identification of those with those whose platelets were non-responsive).

a)

		Multiplate™		PPA 33/(33+18) = 64.7% NPA 106/(106+14) = 88.3%
		+	-	
VerifyNow™	+	33	14	PPA 33/(33+14) = 70.2% NPA 106/(106+18) = 85.5%
	-	18	106	
		VerifyNow™		
		+	-	
Multiplate™	+	33	18	PPA 33/(33+18) = 64.7% NPA 106/(106+14) = 88.3%
	-	14	106	
<b>OPA (33+106)/(33+106+18+14) = 81.3%</b>				

b)

		Multiplate™		PPA 35/(35+13) = 73.0% NPA 91/(91+9) = 91.0%
		+	-	
VerifyNow™	+	35	9	PPA 35/(35+9) = 79.6% NPA 91/(91+13) = 87.5%
	-	13	91	
		VerifyNow™		
		+	-	
Multiplate™	+	35	13	PPA 35/(35+13) = 73.0% NPA 91/(91+9) = 91.0%
	-	9	91	
<b>OPA (35+91)/(35+91+13+9) = 85.1%</b>				

**Table 8:** Positive (PPA), negative (NPA) and overall agreement (OPA) for VerifyNow™ and Multiplate™ tests.

- a) First visit (5+<sup>0</sup>-20+<sup>6</sup> weeks).
- b) Third visit (33+<sup>0</sup>-35+<sup>6</sup>).

a)

		TXB Cardio™		PPA 32/(32+30) = 51.6% NPA 94/(94+15) = 86.2%
		+	-	
VerifyNow™	+	32	15	PPA 32/(32+15) = 68.1% NPA 94/(94+30) = 75.2%
	-	30	94	
		VerifyNow™		
		+	-	
TXB Cardio™	+	32	30	
	-	15	94	
<b>OPA</b> (32+94)/(32+94+30+15) = 73.7%				

b)

		TXB Cardio™		PPA 26/(26+14) = 65.0% NPA 90/(90+18) = 59.1%
		+	-	
VerifyNow™	+	26	18	PPA 26/(26+18) = 83.30% NPA 90/(90+14) = 86.5%
	-	14	90	
		VerifyNow™		
		+	-	
TXB Cardio™	+	26	14	
	-	18	90	
<b>OPA</b> (26+90)/(26+90+14+18) = 71.6%				

**Table 9:** Positive (PPA), negative (NPA) and overall agreement (OPA) for VerifyNow™ and TXB Cardio™ tests.

a) First visit (5+<sup>0</sup>-20+<sup>6</sup> weeks)

b) Third visit (33+<sup>0</sup>-35+<sup>6</sup>)



a)

		TXB Cardio™		PPA 36/(36+25) = 59.0% NPA 94/(94+16) = 85.5%
		+	-	
Multiplate™	+	36	16	
	-	25	94	
		Multiplate™		PPA 36/(36+16) = 75.0% NPA 94/(94+25) = 79.0%
		+	-	
TXB Cardio™	+	36	25	
	-	16	94	
<b>OPA (36+94)/(36+94+25+16) = 76.0%</b>				

b)

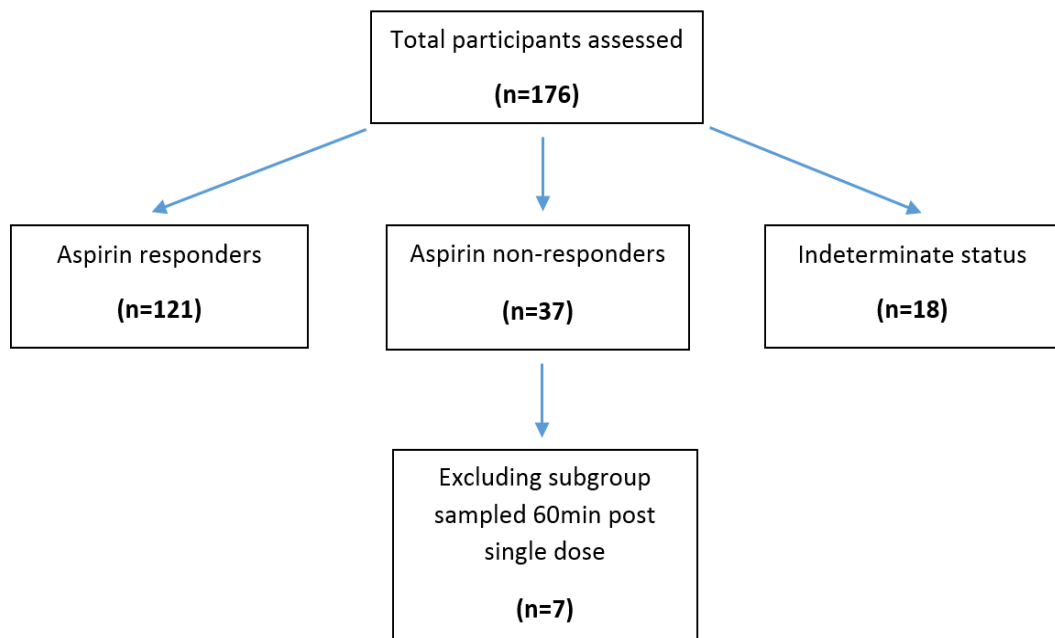
		TXB Cardio™		PPA 26/(26+14) = 65.0% NPA 90/(90+22) = 80.40%
		+	-	
Multiplate™	+	26	22	
	-	14	90	
		Multiplate™		PPA 26/(26+22) = 54.2% NPA 90/(90+14) = 86.5%
		+	-	
TXB Cardio™	+	26	14	
	-	22	90	
<b>OPA (26+90)/(26+90+14+22) = 78.4%</b>				

**Table 10:** Positive (PPA), negative (NPA) and overall agreement (OPA) for Multiplate™ and TXB Cardio™ tests.

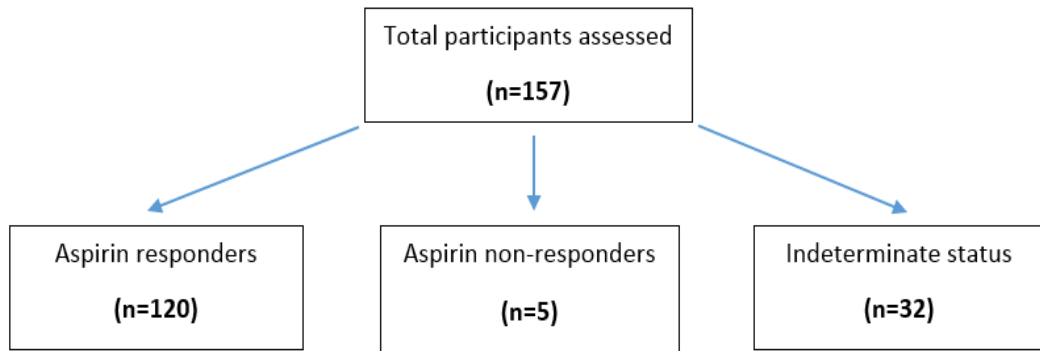
a) First visit (5+<sup>0</sup>-20+<sup>6</sup> weeks)

b) Third visit (33+<sup>0</sup>-35+<sup>6</sup>)

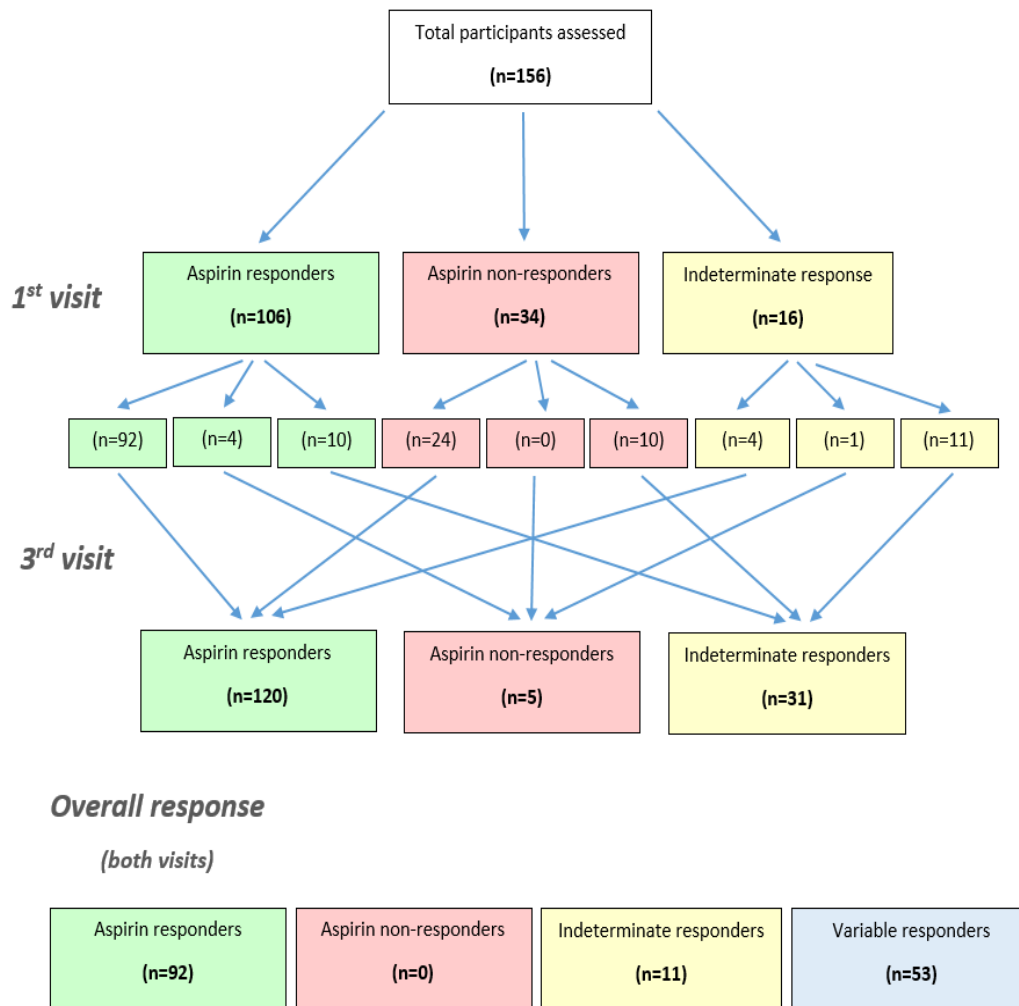
Assigned response to aspirin, incorporating platelet function data and detection of aspirin metabolites, is depicted per visit (Figures 9 and 10) and for 156 participants sampled across both visits (Figure 11). Overall, a higher proportion of participants were found to be aspirin responsive at the third visit, largely due to higher number of participants demonstrating the expected platelet response to aspirin at this visit (68.8% n=121 vs. 76.4% n=120). However, when examining longitudinal response to aspirin the most striking findings were that no participants were consistently non-responsive to aspirin at both the first and third visits. It was evident that 34.0% (n=53) demonstrated variable response to aspirin, changing aspirin response groups between the two visits. Closer examination of eleven participants with consistent indeterminate response to aspirin (platelets non-responsive and aspirin metabolites undetectable), revealed normal uterine artery Doppler's in all cases with one composite placentally-mediated adverse outcome in the group (Table 9).



**Figure 9:** Participants' response to aspirin at the first EARTH study visit.



**Figure 10:** Participants' response to aspirin at the third EARTH study visit.



**Figure 11:** Participants' Longitudinal response to aspirin during the EARTH study.

Case no.	Risk factors	Abnormal uterine artery Doppler? (Y/N)	Gestational age (at delivery)	Clinical outcomes
1	Previous pre-eclampsia, BMI >35.	N	33+ <sup>5</sup>	PPROM, IOL, NVD, livebirth, 36th centile.
2	Pre-existing type I diabetes.	N	34+ <sup>4</sup>	IUGR, pre-labour CS, livebirth, 1st centile, RDS, hyponatraemia, thrombocytopenia. Placenta-villous chorangiosis and focal ischaemia.
3	Previous pre-eclampsia, family history of pre-eclampsia.	N	35+ <sup>2</sup>	PPROM, IOL, NVD, livebirth, 7th centile.
4	Previous pre-eclampsia	N	38+ <sup>0</sup>	SGA, IOL, livebirth, 11th centile.
5	Chronic hypertension, family history of pre-eclampsia.	N	38+ <sup>0</sup>	IOL, NVD, livebirth, 45th centile.
6	Pre-existing type II diabetes.	N	38+ <sup>1</sup>	Deteriorating diabetic control, IOL, NVD, livebirth, 93rd centile.
7	Chronic hypertension, nulliparous, BMI>35.	N	38+ <sup>4</sup>	IOL, NVD, livebirth, 93rd centile.
8	Previous pre-eclampsia and IUGR.	N	38+ <sup>5</sup>	Spontaneous labour, NVD, livebirth, 13th centile.
9	Previous IUGR, inter-pregnancy interval >10 years, family history of pre-eclampsia.	N	38+ <sup>6</sup>	Suspected late-onset IUGR, IOL, NVD, livebirth, 10th centile.
10	Chronic hypertension	N	38+ <sup>6</sup>	Previous CS, pre-labour CS, livebirth, 52nd centile.
11	Previous pre-eclampsia	N	40+ <sup>2</sup>	Spontaneous labour, NVD, livebirth, 50th centile.

**Table 11:** EARTH Participants with consistent indeterminate response to aspirin across study visits.

\*PPROM=preterm prelabour rupture of membranes, IOL=induction of labour, NVD=normal vaginal delivery, IUGR=intrauterine fetal growth restriction, CS=caesarean section, RDS=respiratory distress syndrome, SGA=small for gestational age.

	<b>Aspirin responder (n=92)</b>	<b>Indeterminate response (n=11)</b>	<b>Variable response (n=53)</b>
<b>Maternal demographic characteristics</b>			
Mean maternal age (SD)	31.4 (5.3)	29.6 (5.3)	31.3 (5.3)
Mean body mass index (SD)	28.3 (6.6)	29.1 (5.9)	30.9 (7.4)
Nulliparous	27.2% (n=25)	9.1% (n=1)	13.2% (n=7)
Ethnicity			
Caucasian	82.6% (n=76)	72.7% (n=8)	83.0% (n=44)
Afro Caribbean	4.3% (n=4)	18.2% (n=2)	9.4% (n=5)
Asian	4.3% (n=4)	0.0% (n=0)	5.7% (n=3)
Mixed	4.3% (n=4)	9.1% (n=1)	1.9% (n=1)
Other	1.1% (n=1)	0.0% (n=0)	0.0% (n=0)
Chinese	3.3% (n=3)	0.0% (n=0)	0.0% (n=0)
Risk factors for pre-eclampsia			
High risk only	50.0% (n=46)	54.5% (n=6)	37.7% (n=20)
High and moderate	40.2% (n=37)	36.4% (n=4)	58.5% (n=31)
Moderate only	9.8% (n=9)	9.1% (n=1)	3.8% (n=2)
<b>Antenatal care</b>			
Median gestational age of commencing LDA (range)	10 (2-16)	10.5 (4-13)	10 (3-17)
Antihypertensive treatment	32.6% (n=30)	9.1% (n=1)	32.1% (n=17)
Admissions due to pre-eclampsia, gestational hypertension, IUGR	14.1% (n=13)	0.0% (n=0)	17.0% (n=9)
Median length of admission (range)	2.0 (1.0-6.0)	0.0 (0.0-0.0)	2.0 (1.0-13.0)
<b>Intrapartum care</b>			
Admissions to HDU/ITU	4.3% (n=4)	0.0% (n=0)	11.3% (n=6)
Median length of HDU/ITU admission (range)	1.0 (1.0-1.0)	0.0 (0.0-0.0)	1.0 (1.0-2.0)
Acute antihypertensive treatment	12.0% (n=11)	0.0% (n=0)	18.9% (n=10)
Magnesium sulphate treatment	3.3% (n=3)	0.0% (n=0)	9.4% (n=5)
Spontaneous labour	13.0% (n=12)	18.2% (n=2)	15.1% (n=8)
Induction of labour	51.1% (n=47)	81.8% (n=9)	50.9% (n=27)
<b>Delivery outcomes</b>			
Spontaneous preterm birth <37 weeks	3.3% (n=3)	0% (n=0)	9.4% (n=5)
Caesarean section	51.1% (n=47)	18.2% (n=2)	39.6% (n=21)

**Table 12:** Demographic characteristics, antenatal and intrapartum care for EARTH participants completing study follow-up (n=156).

\*IUGR=intrauterine fetal growth restriction, HDU=high dependency unit, ITU=intensive therapy unit.

	<b>Aspirin responder (n=92)</b>	<b>Indeterminate response (n=11)</b>	<b>Variable response (n=53)</b>
<b>MATERNAL CLINICAL OUTCOMES</b>			
Abnormal uterine artery Doppler	6.5% (n=6)	0.0% (n=0)	5.7% (n=3)
Pre-eclampsia	Total 16.3% (n=15)	Total 9% (n=1)	Total 15% (n=8)
≥37 weeks with AGA	8.7% (n=8)	9.1% (n=1)	7.5% (n=4)
≥37 weeks with SGA 5-9th cc	1.1% (n=1)	0.0% (n=0)	0.0% (n=0)
≥37 weeks with SGA <5th cc	1.1% (n=1)	0.0% (n=0)	1.9% (n=1)
<37 weeks with AGA	2.2% (n=2)	0.0% (n=0)	1.9% (n=1)
<37 weeks with SGA 5-9th cc	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
<37 weeks with SGA <5th cc	1.1% (n=1)	0.0% (n=0)	1.9% (n=1)
<37 weeks with IUGR	1.1% (n=1)	0.0% (n=0)	1.9% (n=1)
Gestational hypertension	5.4% (n=5)	0.0% (n=0)	7.5% (n=4)
Placental abruption	1.1% (n=1)	0.0% (n=0)	0.0% (n=0)
<b>FETAL AND NEONATAL OUTCOMES</b>			
IUFD	1.1% (n=1)	0.0% (n=0)	1.9% (n=1)
Isolated IUGR	1.1% (n=1)	9.1% (n=1)	3.8% (n=2)
Isolated SGA	Total 12.0% (n=11)	Total 9.1% (n=1)	Total 11% (n=6)
<5th customised centile	4.3% (n=4)	0.0% (n=0)	1.9% (n=1)
5-9th customised centile	7.6% (n=7)	9.1% (n=1)	9.4% (n=5)
Admissions to NICU	6.5% (n=6)	18.2% (n=2)	18.9% (n=10)
Median length of NICU admission (range)	1.5 (1.0-11.0)	30.0 (1.0-59.0)	3.0 (1.0-11.0)
RDS	5.4% (n=5)	9.1% (n=1)	5.7% (n=3)
Bronchopulmonary dysplasia	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Intraventricular haemorrhage	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Periventricular leukomalacia	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Seizures	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Hypoxic Ischaemic encephalopathy	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Necrotising enterocolitis	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
Neonatal death	0.0% (n=0)	0.0% (n=0)	0.0% (n=0)
<b>COMPOSITE PLACENTALLY MEDIATED ADVERSE OUTCOME</b>	17.4% (n=16)	18.2% (n=2)	20.8% (n=11)

**Table 13:** Maternal, fetal and neonatal outcome measures for EARTH participants completing follow-up (n=156).

\*AGA=appropriate for gestational age, SGA=small for gestational age, IUFD=intrauterine fetal death, IUGR=intrauterine fetal growth restriction, NICU=neonatal intensive care unit, RDS=respiratory distress syndrome, cc=customised centile.

<b>Response to aspirin</b>	<b>Pre-eclampsia X<sup>2</sup> (p value)</b>	<b>Composite outcome X<sup>2</sup> (p value)</b>
<b>Indeterminate</b>	0.296 (0.586)	0.004 (0.948)
<b>Inconsistent</b>	0.040 (0.841)	0.213 (0.645)

**Table 14:** Pearson's chi-square analysis of pre-eclampsia and composite placentally-mediated adverse outcomes.

## 5.6 Discussion

Both point-of-care devices (VerifyNow™ and Multiplate™) were used in our laboratories during EARTH were easy to maintain, reliable through quality control and easy to operate. In contrast to previously reported poor test agreement, and a subsequent broad range of reported incidence of aspirin non-responsiveness (Michelson, 2004, Snoep et al., 2007, Marshall et al., 1997, Eikelboom et al., 2002, Weber et al., 2002), we found reasonable test agreement. This is likely to be due to selection of the tests, all of which are considered 'COX-specific', and more reflective of the effect of aspirin on the platelet. The highest agreement was between Multiplate™ and VerifyNow™, which is perhaps expected as both operate on whole blood and use arachidonic acid as the agonist. However, this information was particularly reassuring in the case of VerifyNow™, which we had not previously used and for which we worked with the non-pregnant, manufacturer's reference range. It is possible that true agreement between Multiplate™ and VerifyNow™ may be further improved with development of a pregnancy-specific cut-off. A 95% reference range for VerifyNow™ could be established by cross-sectional sampling in aspirin naïve pregnant women at low-risk of pre-eclampsia, in the same manner as those we have established for Multiplate and TXB Cardio™.

Results from the EARTH subgroup undergoing observed dosing of their first dose of LDA demonstrate that whilst a discernible effect from a single dose is evident in over 95% of women, the scale of this effect varies considerably between individuals (Figures 5a and 5b). In the observed dosing subgroup, following a single dose of 75mg dispersible aspirin 63.3% (n= 31) and 56.6% (n= 30) of participants' platelet function results remained inside the aspirin naïve reference ranges for VerifyNow™ and Multiplate™, respectively. This effect reflects aspirin's pharmacodynamic action and sampling at a short interval on day one is likely to reflect only a proportion of the final platelet response. It would be informative to sequentially assess platelet function on successive days of LDA dosing to

ascertain time to achieve platelet function results outside the aspirin naïve reference ranges, and time to maximal platelet response in pregnant women. Additional factors affecting pharmacokinetics of LDA, specific to pregnancy, may also be influential when assessing the platelet effects of a single dose. Gastric transit time decreases significantly in pregnancy due to progestogenic effects on smooth muscle, which may be important for speed of gastric absorption of LDA and antiplatelet action in the portal circulation and time required to cause a measurable change in platelet function using these tests. Women included in this subgroup were not asked to alter their daily routine, including eating and drinking, prior to sampling. Subsequently, there may be variable absorption of LDA reflected in platelet function due to differing stomach contents. These factors would need to be controlled to assess the contribution of individual variation in platelet response to a single dose.

Accurate assignment of response to aspirin has two important facets 1) confirmation of platelet exposure to aspirin 2) assessment of platelet response to aspirin measured by platelet function. Due to the limitations of traditionally used qualitative and semi-quantitative aspirin adherence assessments (detailed in Chapter 3), we opted to incorporate two exact assessments of LDA metabolites to determine platelet exposure. The key finding when LDA adherence was incorporated in this way, in contrast to the studies described in Chapter 2, is that consistent aspirin non-responsiveness across study visits could not be demonstrated in any one individual. Importantly, individual aspirin response varied considerably between sampling time-points in the clinical study, with a third of participants changing aspirin response groups at each visit.

However, even when platelet response and metabolite detection data are combined, due to LDA's rapid clearance, it is not always be feasible to assign definitive aspirin response status. This results in an 'indeterminate' response group composed of individuals whose



platelets do not demonstrate the expected effects of LDA, and in whom aspirin metabolites cannot be detected. Accordingly, it is possible that this group may contain individuals who are in fact non-responsive to LDA. Ten participants deemed aspirin non-responsive at the first visit, subsequently had indeterminate response at the third visit and one with an indeterminate response at the first visit became an aspirin non-responder at their subsequent visit. The definitions for the indeterminate group mean it is also primed to contain a significant proportion of individuals with suboptimal adherence both in the short-term (as evidenced by lack of aspirin metabolites) and medium-term (as evidenced by lack of expected platelet response). This group could be further interrogated by guiding the timing of LDA dose administration, to ensure the dose-sampling intervals fall within the detectable windows for NMR and LC: MS detection of aspirin metabolites. Another useful approach would be to re-assess platelet function in these individuals following directly observed dosing.

All aspirin response groups represented in the cohort (aspirin responsive, indeterminate response and variable response) were equivalent with regard to demographic characteristics and the antenatal and intrapartum care provided. There was no significant association between aspirin response and clinical outcomes of pre-eclampsia or the specified composite of placentally-mediated adverse outcomes. This was true both when comparing aspirin responders to those with indeterminate response ( $p=0.856$  and  $0.948$ ) or to those with inconsistent response (indeterminate response plus variable response groups,  $p=0.841$  and  $0.645$ ). It is possible that these findings have been influenced by small numbers within the indeterminate group ( $n=11$ ). The likely heterogeneous composition of both the indeterminate and variable groups would need to be addressed and fully controlled before considering whether re-examination in a larger cohort would be a reasonable proposal.

## **Conclusions**

When sampled at individual time-points in pregnancy, women who appear non-responsive to aspirin can be identified. However, we did not identify any women who were consistently non-responsive when data were analysed longitudinally. It is evident from the EARTH cohort that most women initially appearing non-responsive to aspirin become responsive with continued dosing. Accordingly, there was no significant association between any aspirin response group and placentally-mediated adverse clinical outcomes. However, the high degree of variation in individual response to aspirin across sampling time-points is likely to reflect issues with platelet exposure to aspirin. This may be due to problems with aspirin adherence, but dosing factors may also be influential. Further understanding of the causes of this variation in response may be beneficial in optimising the use of LDA in high risk pregnancies.

## **CHAPTER 6: Pharmacogenomics of aspirin**

## **6.1 Rationale**

Genetic factors involved in the mechanism of action and disposition of aspirin have been proposed as potential causes of variability in individual response to aspirin. The evolution of approaches to assess influential genetic factors, from candidate genes to genome-wide assessments and high-throughput sequencing, now allow systematic assessment of genetic associations related to drug response to be undertaken (pharmacogenomics, Chapter 1). There have been key successes with the implementation of genotype-guided treatment in cardiovascular medicine with two similarly important medications, clopidogrel and warfarin. In addition there has been a change in obstetric prescribing triggered by pharmacogenomic findings of the variable metabolism of codeine phosphate (pharmacogenomics, Chapter 1). This chapter addresses the aim of conducting an unbiased genome-wide assessment of genetic factors that may influence response to low-dose aspirin (LDA) in pregnant women at high risk of pre-eclampsia. This included an initial examination of existing literature addressing genetic factors related to response to aspirin. With this foundation, we designed and conducted a genome-wide association study from the EARTH study cohort, to identify variants associated with response to LDA (Chapter 1, Objectives 5a and 5b).

## **6.2 Literature review**

In 2008 a systematic review and meta-analysis of candidate gene studies (CGS) investigating genetic factors in aspirin non-responsiveness was performed (Goodman et al., 2008). The authors stipulated that included studies must include a definition of aspirin non-responsiveness based on a standardised laboratory or point-of-care assessment. Twenty-one CGS describing variants in 11 genes were identified. Twelve CGS examining five genes reported genotype frequency data, and were therefore suitable for meta-analysis (Table 1) (Szczeklik et al., 2000, Macchi et al., 2003, Gonzalez-Conejero et al.,

2005, Pamukcu et al., 2005, Papp et al., 2005, Bernardo et al., 2006, Fontana et al., 2006, Kranzhofer and Ruef, 2006, Lev et al., 2007, Lepantalo et al., 2006, Undas et al., 2001, Jefferson et al., 2005). These studies included both genders of healthy Caucasians and Caucasians with known cardiovascular disease.

The most prominent findings related to the PIA1/A2 variant in the *GP1IIa* gene, on chromosome 17 (Goodman et al., 2008). This gene encodes the platelet membrane receptor responsible for binding fibrinogen and VWF (GeneCards, 2017b). In 673 pooled aspirin responders and 295 aspirin non-responders, this variant was significantly associated with aspirin non-responsiveness in healthy individuals (OR 2.36, 95% CI 1.24, 4.49; P = 0.009), with no significant association remaining when data from healthy individuals and those with cardiovascular disease were combined (Table 1) (Goodman et al., 2008). A range of methods were used to assess platelet response to aspirin and the method selected influenced the level of association with the PIA1/A2 variant (Goodman et al., 2008). For the ten studies meta-analysed, aspirin non-responsiveness denoted by bleeding time and thrombin generation was strongly associated with PIA1/A2 carriage (OR 11.76 95% CI 3.05-45.37 p=0.003), whereas aspirin non-responsiveness assigned by light transmission aggregometry (LTA) or platelet function analyser (PFA-100) were not (Goodman et al., 2008). No other significant associations were identified in the other candidate genes assessed (Table 1) (Goodman et al., 2008). However, the meta-analysis was limited by a small number of studies eligible for inclusion and significant heterogeneity between studies (Goodman et al., 2008).

Gene (SNP/variant)	Primary study designs	Association	Population	Ethnicities	References
<b>GP1IIa/ITGB3</b> (PIA1/A2)	CGS	OR 1.14 (95% CI 0.84, 1.54) p= 0.4	Healthy individuals, cardiovascular disease	Caucasians	Szczeklik 2000 Undas 2000 Macchi 2003 Gonzalez-Conejero 2005 Pamukcu 2005 Papp 2005 Bernardo 2006 Fontana 2006 Kranzhofer 2006 Lev 2007
<b>GP1a/ITAG2</b> (C807T)	CGS	OR 1.37 (95% CI 0.81- 2.31) p=0.24	Healthy individuals, cardiovascular disease	Caucasians	Macchi 2003 Gonzalez-Conejero 2005 Bernardo 2006 Fontana 2006
<b>COX1/PTGS1</b> (A842G and C50T in linkage disequilibrium)	CGS	OR 1.07 (95% CI 0.41- 2.77) p=0.89	Healthy individuals, cardiovascular disease	Caucasians	Gonzalez-Conejero 2005 Kranzhofer 2006 Lepantalo 2006
<b>P2Y12/P2RY12</b> (H1/H2)	CGS	OR 0.90 (95% CI 0.48- 1.68) p=0.73	Healthy individuals, cardiovascular disease	Caucasians	Bernardo 2006 Fontana 2006 Lev 2007
<b>P2Y1/P2RY1</b> (A1622G)	CGS	OR 0.80 (95% CI 0.48- 1.33) p=0.39	Cardiovascular disease	Caucasians	Jefferson 2005 Lev 2007

**Table 1:** Meta-analysis data of SNPs reported in candidate gene studies up to 2008 (Goodman et al., 2008).

\*CGS=candidate gene study

### Search strategy

We aimed to review published reports of all CGS published after the systematic review and meta-analysis of CGS, and all genome-wide association studies (GWAS) assessing genetic associations with aspirin non-responsiveness. A scoping search revealed no GWAS or studies with novel methodologies e.g. sequencing had been reported prior to 2008. Therefore, the search strategy used by Goodman et al (Goodman et al., 2008) was adapted to include all methodologies, and to run from 1<sup>st</sup> January 2008 to 28<sup>th</sup> May 2017.

Electronic databases, MEDLINE and EMBASE were searched. The search terms used were; aspirin, acetylsalicylic acid, aspirin non-responsive, aspirin resistance, platelet, antiplatelet

combined with genetics, gene, mutation, polymorphism, genotype, genome, GWAS, sequencing. For inclusion, platelet response to aspirin was required to be measured by either a standardised laboratory or point-of-care technique, or by clearly defined clinical parameters. The search was limited to humans and English language. Following the electronic search, the reference lists of identified studies were searched and results were cross-checked with studies listed in the Pharmacogenomics Knowledgebase (PharmGKB, 2017). SNP associations examined in the meta-analysis by Goodman et al were considered in relation to more recent studies identified.

## **Results**

The search yielded 160 hits. After reviewing article types and titles, 43 articles required abstract review. Thirty-two of those from abstract review then proceeded to full text review and 17 met the criteria stipulated for inclusion. The studies identified included participants from Europe, North Africa, the United States of America, South America, India and China with participants from six ethnic groups (Caucasians, Arabs, African Americans, South Americans, Indians and Chinese). Studies included a highly variable percentage of female participants (8.6-90%) and most cohorts consisted of participants with mean age older than 40 years (Tables 2 and 3). Overall sixteen studies described a definition of aspirin non-responsiveness based on biochemical parameters and one used a clinical definition (Sharma et al., 2012).

The final 17 articles consisted of reports on; 10 CGS (Table 2) (Sharma et al., 2012, Lim et al., 2007, Dropinski et al., 2007, Herrera-Galeano et al., 2008, Jin et al., 2009, Momary et al., 2009, Abderrazek et al., 2010, Faraday et al., 2011, Xu et al., 2012, Fan et al., 2013, Wang and Tan, 2014, Yi et al., 2017), 4 GWAS (Table 3) (Johnson et al., 2010, Lewis et al., 2013, Edelstein et al., 2012, Qayyum et al., 2015) and 3 studies reporting other methodologies (Mathias et al., 2010, Voora et al., 2013, Postula et al., 2016). Other methodologies identified by the search included; genome-wide linkage and association

and next-generation sequencing (Postula et al., 2016) and RNA microarray profiling (Voora et al., 2013).

### Candidate Gene Studies

Our search identified a further nine CGS reporting significant associations of genetic variants with aspirin non-responsiveness, published between 2008 and 2014 (Momary et al., 2009, Fan et al., 2013, Xu et al., 2012, Wang and Tan, 2014, Abderrazek et al., 2010, Jin et al., 2009, Faraday et al., 2011, Sharma et al., 2012, Herrera-Galeano et al., 2008). This included four studies in Chinese populations, one each in Indian and Arab populations and the remainder included mixed ethnicities (Table 2). The majority of studies (n=7, 78%) focussed on individuals with pre-existing coronary artery disease (CAD), cerebrovascular disease (CVD) or family history of premature CAD, though three studies also included healthy individuals.

Eight studies (88% of CGS identified) used LTA with arachidonic acid (AA) as the agonist, providing an accepted cyclooxygenase (COX)-1-specific assessment of platelet aggregation and one study used PFA-100 collagen/epinephrine cartridges, a non-COX-specific assessment, reflecting global platelet function. The remaining study defined aspirin non-responsiveness clinically, as recurrent stroke or cerebrovascular death whilst on aspirin treatment (Sharma et al., 2012).

Ten variants in 6 genes were investigated, including the *GP1IIa/ITGB3* gene previously examined (Table 2). The *GP1b $\alpha$ /GP1B* gene, in similarity to *GP1IIa/ITGB3* (both on chromosome 17), encodes the alpha chain component of the platelet surface glycoprotein receptor GPIb and binds Von Willenbrand Factor (VWF) (GeneCards, 2017a). This process is important for haemostasis, in particular, for initial platelet adhesion to the endothelium following vascular injury and participates in platelet signalling to potentiate further platelet activation (GeneCards, 2017a). The platelet endothelial aggregation receptor 1 gene (*PEAR1*, chromosome 1), encodes an endothelial growth factor, containing a



transmembrane receptor involved in platelet-platelet contact induced platelet activation (Nanda et al., 2005). The multidrug resistance 1 gene (*MDR1*, chromosome 7), encodes an adenosine triphosphate (ATP) binding cassette involved in transport of a variety of drugs across the cell membrane known to affect bioavailability and distribution of several drugs (Brinkmann and Eichelbaum, 2001). However, aspirin is not known to be a substrate for MDR-1.

The *COX1/PTGS1* gene (chromosome 9) has clear biological plausibility in platelet response to aspirin as it encodes the COX-1 enzyme responsible for the conversion of AA to TXA<sub>2</sub> within platelets and is the principal pharmacological target of LDA. The *COX2/PTGS2* gene (chromosome 1) has some plausibility as it encodes the inducible COX-2 enzyme also central to prostaglandin biosynthesis, though COX-2 inhibition tends only to occur with high dose aspirin therapy.

Yi et al sequenced known candidate genes for 14 genetic variants in patients with acute ischaemic stroke (Yi et al., 2017). They also assessed, gene-gene interactions using generalized multifactor dimensionality reduction. The variants selected occurred in *COX1/PTGS1*, *COX2/PTGS2*, *GP1b/GP1BA*, *GPIIIa/ITGB3*, *P2Y1/P2RY1* and *P2Y12/P2RY12*. Single locus analysis indicated one significant variant (rs1371097), whilst generalized multifactor dimensionality analysis implicated three sets of influential gene-gene interactions (between variants in the *COX2/PTGS2*, *P2Y1/P2RY1*, *GPIIIa/ITGB3* genes) (Yi et al., 2017). Interestingly these interactive genotypes were also associated with early neurological deterioration (Yi et al., 2017).

Reference	Genes (SNPs)	Association	Allele/SNP frequency	Population (cohort)	Age (years)/ Female (%)	Ethnicity	Phenotyping
Herrera-Galeano 2008	<i>PEAR1</i> (rs2768759)	Associated with native platelet aggregation and platelet aggregation after aspirin. (p values reported per agonist)	Allele frequency 70.2% versus 17.7%. White AA 80 vs AC 392 vs. CC 455. Black AA 378 vs. AC 164 vs. CC 17.	Two generations, premature CAD (GeneSTAR)	White: 45.5/43 Black: 44.5/60.3	White, Black	LTA (ADP, collagen, epinephrine)
Jin 2009	<i>GP1ba/GP1BA</i> (VNTR)	More sensitive to aspirin ( <b>p= &lt; 0.05</b> )	AS (CC 59, non-CC 41) ASR (CC 2, non-CC 8). Whole cohort (BC 2 vs. BD 2 vs. CC 148 vs. CD 119 vs. DD 29)	Patients with stroke, healthy volunteers.	ASR: 57/90 AS: 60/45	Chinese	LTA (AA, ADP)
Momary 2009	<i>COX1/PTGS1</i> (rs3842787)	Associated with greater aspirin response (overall p-value <b>p=0.04</b> , African Americans <b>p=0.02</b> )	MAF 8% (all heterozygotes)	Primary and secondary stroke prevention	59/57	Caucasians, Hispanic, Asian and African Americans	LTA (AA, ADP, collagen, epinephrine, TRAP)
Abderrazek 2010	<i>GPIIIa/ITGB3</i> (PIA1)	HPR patients more homozygous for PIA1/A1 (65.4% vs. 47.7%, <b>p=0.015</b> )	HPR: PIA1/PIA1 53 vs. PIA1/PIA2 or PIA2/PIA2 28 non-HPR PIA1/PIA151 vs. PIA1/PIA2 or PIA2/PIA2 56.	Stable CAD	A1/A1: 59.3/8.6 A/A2 and A2/A2: 57.3/11.9	Arab	PFA-100 (collagen/epinephrine)
Faraday 2011	<i>PEAR1</i> (rs12041331)	Discovery: ( <b>p=2.22 x 10-8</b> ) European replication ( <b>p=1.64 x 10-5</b> ) African replication ( <b>2.31 x 10-27</b> )	MAF 9.1% (Europeans) 37.1% (Africans).	Families with a premature CAD (GeneSTAR).	44/60	European ancestry, African ancestry	LTA (ADP, collagen, epinephrine)

<b>Sharma 2012</b>	<b>MDR1</b> (rs1045642)	AR risk higher in patients with TT vs. CC genotype (TT vs. CC, $\chi^2=6.268$ ; <b>p=0.012</b> , OR 1.85 95% CI; 1.142–3.017) (Adjusted OR 2.465; 95% CI; 1.895–4.625 <b>p= &lt;0.001</b> ).	MDR-1 genotypes: cases 201 (TT) 256 (CT) 103 (CC), controls 137 (TT) 263 (CT) 160 (CC). Allele frequency: cases 658 (T) 462 (C), controls 537 (T) 583 (C)	Ischaemic stroke patients, healthy volunteers	Patients: 49.3/29.8 Controls: 49/30.9	Indian	Recurrent strokes and death on aspirin treatment.
<b>Xu 2012</b>	<b>COX2/PTGS2</b> (rs20417)	Mutation higher in AR vs AS ( <b>p=&lt;0.05</b> )	Allele frequency: Wild type 694 (96.39%), mutant 26 (3.61%)	Patients attending for routine health screening	Age range reported, 62-94/15.4	Chinese	LTA (ADP, collagen, epi), TEG 11-DHTXB2 PFA-100 (collagen/ADP), P selectin
<b>Fan 2013</b>	<b>COX1/PTGS1</b> (rs1330344)	Increased the risk of aspirin resistance by LTA AA + TEG AA (OR = 1.82, 95% CI 1.13–2.92, <b>p = 0.01</b> )	Genotypes: cases 41 (G) 35 (A), controls 307 (G) 477 (A)	Elderly patients with CVD	74-75.4/28.9-42.2 (across agonists)	Chinese	LTA (AA) TEG (AA).
<b>Wang 2014</b>	<b>GPIIIa/ITGB3</b> (PIA1)	Associated with aspirin resistance, ( <b>p=&lt;0.002</b> )	Genotypes: AR 224 (A1/A1) 12 (A1/A2), AS group: 214 (A1/A1)	Elderly patients	AS: 83.5/24.3 AR: 84/ 23.7	Chinese	LTA (AA, ADP)
<b>Yi 2017</b>	<b>P2RY1</b> ( rs1371097)	rs1371097 associated with AR & ASR ( <b>p=0.01</b> ) Gene-gene interactions for COX2/PTGS2, P2Y1/P2RY1, GPIIIa/ITGB3. I	Genotypes: AR & ASR (TT+CT) 114, (CC) 106. AS (TT+CT) 257 (CC) 373.	Ischaemic stroke patients	AR & ASR: 70.8/55.4 AS: 70/45.2	Chinese	LTA (AA, ADP)

**Table 2:** Significant associations of single nucleotide polymorphisms reported in candidate gene studies after 2008.

\*CAD=coronary artery disease, LTA=light transmission aggregometry, ADP=adenosine diphosphate, AA=arachidonic acid, AS=aspirin sensitive, ASR=aspirin semi-responder, AR=aspirin resistant, TRAP=thrombin receptor activating peptide, MAF= minor allele frequency, HPR=high platelet reactivity, TEG=Thromboelastography, 11-DHTXB<sub>2</sub>=11-dehydrothromboxane B<sub>2</sub>, CVD=cardiovascular disease.

### Genome-wide association studies

We identified four relevant GWAS published between 2010 and 2015 (Table 3). All GWAS included participants of European and African ancestry. GWAS were similar to CGS studies identified, in that they focussed on populations with stable CAD, family history of premature CAD or apparently healthy individuals. All GWAS used laboratory or point-of-care measures of platelet response to aspirin and 75% (n=3) incorporated LTA into their assessments. However, only one used LTA with AA as the agonist for LTA and all GWAS relied on non-COX specific agonists including ADP, collagen and epinephrine (Table 3).

The earliest GWAS by Johnson et al, assessed genetic variants related to increased platelet aggregation responses, assessed with a panel of non-COX specific agonists (Table 3) (Johnson et al., 2010). The study focussed on individuals symptom-free of CAD, with the discovery cohort comprised 3991 individuals of European ancestry and the replication cohort 840 individuals of African ancestry. Six genome-wide significant ( $5 \times 10^{-8}$ ) variants associated with increased platelet aggregation response were identified and replicated between the ethnic groups. The significance of the *PEAR1* gene was again highlighted by this GWAS. However, a different SNP to that previously reported in CGS (Table 2) was identified (rs12566888). This SNP was found to be associated with diminished platelet aggregation in response to ADP and epinephrine.

The rs1671152 variant identified in association with collagen lag-time is in the glycoprotein 6 gene (*GP6* chromosome 19). *GP6* is biologically plausible, for involvement in collagen-induced platelet aggregation as it encodes the platelet glycoprotein receptor responsible for collagen response (Johnson et al., 2010). The adrenoreceptor alpha 2A (*ADRA2A*) gene (chromosome 10) encodes the G-protein receptor subtype alpha 2A, principally involved in neurotransmitter regulation but also implicated in metabolism of a

range of drugs (OMIM, 2017). However, this receptor has not been described in relation to aspirin metabolism. The murine retrovirus integration site 1 homolog (*MRVI1*, chromosome 11) gene encodes a protein that is a component of a signalling complex, shown in mice to inhibit platelet aggregation. The rs7940646 variant was found to be associated with ADP and epinephrine induced platelet aggregation. The biological plausibility for variants in jumonji domain containing 1C (*JMJD1C*, chromosome 10) and sonic hedgehog (*SHH*, chromosome 7), not previously identified, is less clear. The *JMJD1C* gene encodes a co-activator for transcription factors (GeneCards, 2017d) and *SHH* encodes a protein important for embryonic patterning (GeneCards, 2017e).

A smaller GWAS with 241 healthy participants identified two novel genome wide-significant variants in African Americans only, both associated with PFA-100 collagen/epinephrine closure times (Table 3) (Edelstein et al., 2012). The Supervillin gene (*SVIL*, chromosome 10), encodes a protein with a role in assembling myosin and disassembling focal adhesions (GeneCards, 2017f). However, confirmatory experiments by the authors suggested a role for SVIL as an inhibitor of platelet adhesion (Edelstein et al., 2012). SVIL was shown to be expressed in platelets and murine platelets without SVIL contributed more to thrombus formation under high shear stress (Edelstein et al., 2012).

Two recent GWAS have verified the significance of the *PEAR1* variant rs12041331, initially identified in CGS. This variant has been shown to be significantly associated with decreased platelet aggregation to a panel of agonists, including COX-1 selective AA (Lewis et al., 2013, Qayyum et al., 2015). These studies included 3957 healthy individuals and stable CAD patients of European and African ancestry, from four independent cohorts (Lewis et al., 2013, Qayyum et al., 2015). Qayyum et al also identified two variants in the bone morphogenetic protein type 1a (*BMPR1A*, gene on chromosome 10), with genome-wide significance for association with decreased ADP-induced platelet aggregation (Table

3) (Qayyum et al., 2015). However, the biological plausibility of BMPR1A in influencing platelet aggregation is unclear.

Reference	Genes (SNPs)	Association	Allele/SNP frequency	Population (cohort)	Age/% female	Ethnicity	Phenotyping
Johnson 2010	<b>PEAR1</b> (rs12566888) <b>GP6</b> (rs1671152), <b>ADRA2A</b> (rs431194) <b>JMJD1C</b> (rs10761741), <b>MRV1</b> (rs7940646), <b>SHH</b> (rs2363910).	<b>p = 3.4 × 10<sup>-12</sup></b> <b>p = 4.6 × 10<sup>-13</sup></b> <b>p = 3.3 × 10<sup>-11</sup></b> <b>p = 1.6 × 10<sup>-8</sup></b> <b>p = 2.0 × 10<sup>-8</sup></b> <b>p = 4.5 × 10<sup>-8</sup></b>	Meta-analysis: MAF 35.8%, 30.9%, 38.6%, 19.8%, 39.3%, 26.6%.	Individuals symptom-free of CAD (FHS, Gene STAR)	No data (Supplementary file not accessible)	European ancestry, African ancestry	LTA (ADP, collagen, epinephrine)
Edelstein 2012	<b>SVIL</b> (rs7070678, rs10826650)	<b>p= 3.58 x 10<sup>-8</sup></b> <b>p= 4.43 x 10<sup>-8</sup></b>	MAF 32.7%, 34.0%	Healthy non-diabetics	AA: 35/69 EA: 35/50	European Americans, African Americans	PFA-100 (collagen/ADP)
Lewis 2013	<b>PEAR1</b> (rs12041331)	<b>p=7.66×10<sup>-9</sup></b>	White -/A 26 vs. G/G 116 Black -/A 45 vs. G/G 40	Healthy individuals (PAP) Hypertension, stable CAD (INVEST-GENES)	43.9-68.4 (across cohorts)/48.5	White, Black, South American, other	LTA (collagen)
Qayyum 2015	<b>PEAR1</b> (rs12041331) <b>BMPR1A</b> (rs11202221) <b>Intergenic variant</b> 18q22.3 locus (rs6566765)	<b>p=8.34 × 10<sup>-12</sup></b> (replication AA p= 4.6 × 10 <sup>-3</sup> ;EA p=4.47 × 10 <sup>-3</sup> ) <b>p = 4.8 × 10<sup>-8</sup></b> (replication AA p=7.71 × 10 <sup>-5</sup> , EA p=0.69) <b>p=3.59 × 10<sup>-8</sup></b> (replication AA p=0.34, EA p=1.3 × 10 <sup>-3</sup> )	MAF 35.8%, 7.3%, 39.7%	African American families with history of early onset CAD (GeneSTAR study) Healthy individuals (PGAP study)	35-45 (across cohorts) /55-72 (across cohorts)	African Americans, European Americans	LTA (AA, ADP, collagen, epinephrine)

**Table 3:** Significant associations of single nucleotide polymorphisms reported in genome-wide association studies.

\*MAF=minor allele frequency, CAD=coronary artery disease, LTA=light transmission aggregometry, AA=African ancestry, EA=European ancestry, PFA-100=platelet function analyser, ADP=adenosine diphosphate, AA=arachidonic acid.

### Studies with other methodologies

Recently several groups have used combined approaches to identify and verify the plausibility of genetic associations with platelet response to aspirin and to explore heritability and interactions between genetic factors.

Genome-wide linkage and association has been used successfully to determine heritable regions of genomic interest, apparently influencing native and post-aspirin platelet function (Mathias et al., 2010). A study by Mathias et al included 2077 healthy individuals with a family history of European and African ancestry (Mathias et al., 2010). Comprehensive phenotyping of platelet aggregation with a panel of COX-specific and non-specific agonists, resulting in 37 platelet-function phenotypes was undertaken (Mathias et al., 2010). A combined approach with multi-point sib-pair based linkage analysis and single-SNP association tests revealed a ~10 cM region on chromosome 5q11.2 linked to ADP-induced platelet aggregation (Mathias et al., 2010). A further 11 regions with suggestive evidence for linkage were identified between the ethnic groups (Mathias et al., 2010). After assessing platelet function phenotypes, it was apparent that linkage was highest for post-LDA measures of platelet aggregation, reflecting the likely heritability of platelet response to aspirin (Mathias et al., 2010). This combined approach allowed weighting of SNPs identified by GWAS with linkage evidence, increasing the number of SNPs reaching the significance threshold from 9 to 30 (Mathias et al., 2010).

Postula et al recently used targeted next-generation sequencing using Illumina HiSeq2000 to investigate the association of rare variants related to platelet response to aspirin, measured with PFA-100 collagen/epinephrine cartridges, VerifyNow™ Aspirin test and serum thromboxane B<sub>2</sub> (TXB<sub>2</sub>) in 384 European type II diabetics (Postula et al., 2016). Rare missense variants in 8 genes were found to be unequally distributed amongst 'increased' and 'normal' platelet function groups assigned according to PFA-100 (Postula et al., 2016).



The variants were identified in previously suspected plausible candidate genes including; *COX1/PTGS1*, *COX2/PTGS2*, thromboxane A synthase 1 gene (*TXBAS1*), prostacyclin synthase gene (*PTGIS*), *ADRA2A*, adrenoreceptor alpha 2B gene (*ADRA2B*), thromboxane A2 receptor gene (*TXBA2R*), and platelet ADP receptor gene (*P2Y1/ P2RY1*) (Postula et al., 2016). This was the first reported investigation of the potential influence of low frequency genetic variants (minor allele frequency <5%) on platelet response to aspirin, with the power to examine for associations with greater resolution than standard GWAS arrays.

Voora et al used RNA microprofiling to identify biomarkers linked to aspirin responsiveness in a small cohort of 128 healthy individuals and cardiology outpatients (Voora et al., 2013). Platelet function was assessed pragmatically with the platelet function score (PFS) and VerifyNow Aspirin test. Bayesian sparse factor analysis was used to identify sets of co-expressed transcripts, with proteomics to confirm transcripts in platelet proteins related to platelet function (Voora et al., 2013). A set of 60 co-expressed genes, 17 of which encoded proteins expressed in the platelet proteome and 6 of which independently related to PFS, were denoted the 'aspirin response signature' (ARS) (Voora et al., 2013). Interestingly, reclassification of cardiovascular risk status in the cohort was shown to be improved by including ARS (Voora et al., 2013). This approach highlighted the *GPIIb/ITGA2B* gene (chromosome 17), which independently influenced reclassification of risk status (Voora et al., 2013). As with other GP genes *GPIIb/ITGA2B* is a plausible biological candidate, encoding a platelet membrane receptor which mediates platelet aggregation and platelet-platelet interactions (GeneCards, 2017c).

### Limitations

Despite major developments in available technology and study designs, the majority of reports of variants significantly related to aspirin response continue to arise from relatively small-scale CGS. However, in contrast to the scoping search prior to 2008, there are now a

limited number of GWAS and newer methodologies including high-throughput sequencing. These newer methodologies, particularly those combining approaches, offer new perspectives on the heritability of platelet response, functional translation of significant variants and potential influence of low frequency or rare variants.

A frequently encountered problem with the CGS we identified was differential identification of variants. Attempts were made to match the variant to known SNPs using dbSNP (identifying the base and /or amino acid change stated and blasting the primer sequence to the gene). These methods proved unsuccessful, identifying non-exact matches with the risk of labelling the variants incorrectly. Reporting in this way limits the utility of the information and potential for independent assessments by other authors. We decided to search for and examine the significance of all SNPs in previously reported candidate genes represented in the EARTH data-set. In addition to problems with the identification of variants, differential reporting of genotype frequency data and ethnic diversity limits the proportion of data that would be amenable to meta-analysis.

Despite many of the variants reported being in plausible candidate genes relating to platelet function and interactions, we found limited and conflicting assessments of the clinical significance of the variants to date (Lewis et al., 2013, Voora et al., 2011, Le Hello et al., 2007), with no consideration of the cost-effectiveness assessment of testing. In assessing variants which may relate to platelet response to aspirin in pregnancy, we must consider clinical significance.

### **6.3 Methods**

#### **Genome-wide genotyping**

DNA samples from two hundred participants were prepared for genome-wide genotyping, including 22 aspirin-treated women from the pilot study and 178 from the EARTH cohort. DNA was quantified using the Picogreen fluorometric method, normalised according to the

receiving laboratory's standards with at least 0.5µg of DNA at a concentration of 10ng/µl. Samples were shipped to the Oxford Genomics Centre at Oxford University for genome-wide genotyping using the UK Biobank Axiom Array (Affymetrix)(UKBB, 2017).

#### **Data quality control: Per sample**

One-hundred-and-ninety-nine individuals passed the 'Affymetrix best practice workflow', including 22 from the pilot study and 177 from the EARTH study, with genotype data available for all 830,115 SNPs. A sample from one individual did not pass the 'Affymetrix best practice workflow' and was analysed using the 'SS1 rescue workflow', with data available for 778,843 SNPs. Due to the concerns over the quality of this isolated sample and the discrepancy of data available, we decided to exclude its data from downstream analysis. Additionally, the duplicated participant excluded from aspirin metabolite and platelet function analysis (Chapters 4 and 5, respectively) was identified and data arising from the second (latest) DNA sample donated by this participant was excluded from downstream analysis.

The genome-wide genotype data for the remaining 198 participants (22 from the pilot study and 176 from the EARTH study) underwent quality control (QC) procedures described below, both at individual sample levels and at the SNP level. Quality control was conducted using PLINK software version 1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>), which is freely available to download (Purcell et al., 2007). The command lines used to perform the QC process can be found in Appendix 5a.

#### Gender assessment

Reported female genders (pedigree sex) for all participants were inputted into the binary .fam file and assessed against the genotypic sex obtained using X chromosome homozygosity estimates. Males are expected to have an X chromosome heterozygosity estimate of >0.8, whereas this should be <0.2 for females. In the context of our studies,

with all female participants, discordant gender information is most likely to represent sample contamination. Gender discrepancy was observed in four participants (Table 4) and these were marked for removal on completion of the sample QC process.

Individual ID	Pedigree gender	Genotypic gender	X heterozygosity estimate
APP319	FEMALE	NOT ASSIGNED	0.2068
APP253	FEMALE	NOT ASSIGNED	0.2536
APP270	FEMALE	NOT ASSIGNED	0.4013
APP297	FEMALE	NOT ASSIGNED	0.2669

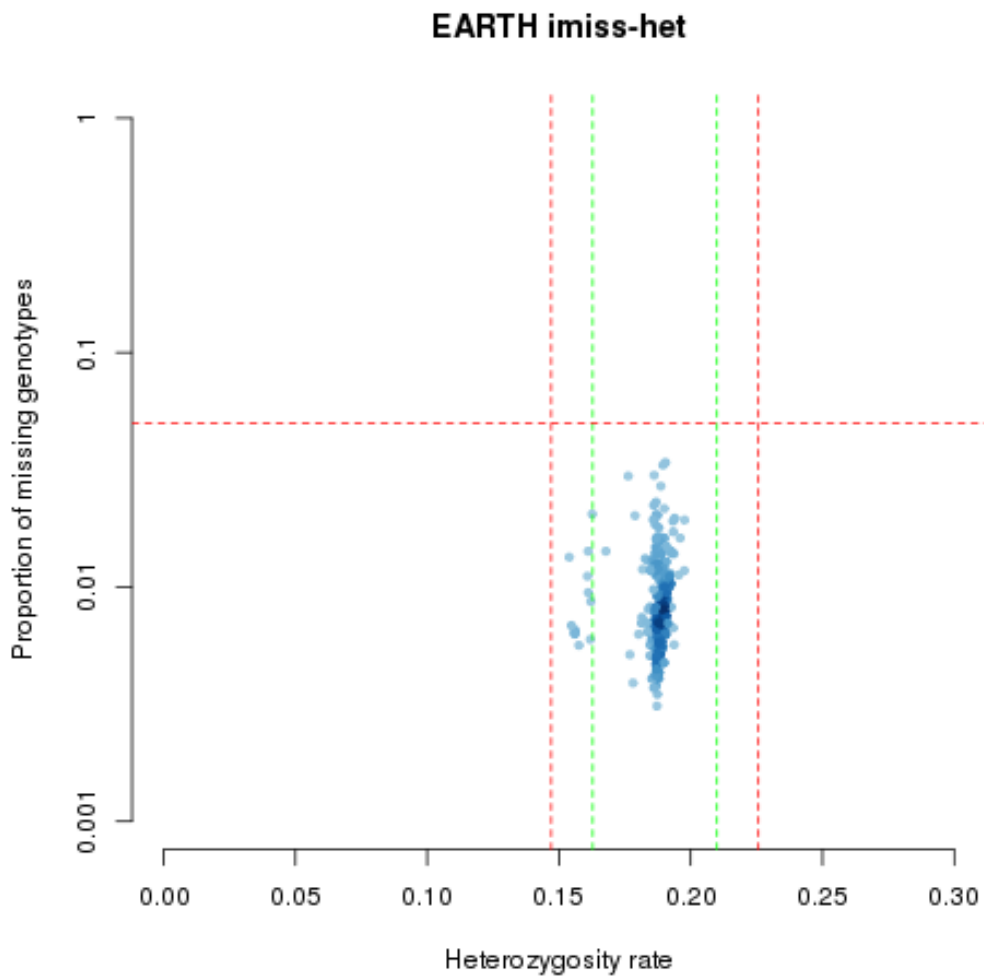
**Table 4:** Participants with discordant pedigree and genotypic gender information.

#### Missing data: Sample genotyping call rate

Samples with low DNA concentration and/or quality are more predisposed to have below average individual genotyping call rates. A threshold of > 95% individual call rate was pre-specified for inclusion in the analysis. No participants had  $\leq$  95% genotyping call rates (Figure 1).

#### Heterozygosity assessment

An excessive individual heterozygosity rate can signify sample contamination with DNA from another individual, whilst excessively reduced heterozygosity can indicate inbreeding, both of which undermine data quality. Non-autosomal chromosomes were removed from the data-set during this step to improve the accuracy of the heterozygosity assessments. The heterozygosity rate for each individual was calculated, no individuals had >3 standard deviations (SD) from the mean heterozygosity rate, 10 individuals had  $\leq$ 3 SD. As the heterozygosity rates for all outliers fell within  $5 \pm$  SD of the mean none required removal.



**Figure 1:** Individual genotyping call rate and heterozygosity rate.

\*Horizontal red dashed line indicates the 95% genotyping call rate applied, vertical green dashed lines indicate  $3 \pm SD$  from the mean heterozygosity rate, vertical dashed red lines indicate  $5 \pm SD$ .

#### Cryptic relatedness assessment

The presence of duplicated or closely related individuals in cohorts can introduce bias by the potential for over-representation of genotypes. A single duplicated individual had already been removed prior to this assessment.

Data free of non-autosomal SNPs were used to calculate identity by state (IBS) estimates for each pair of individuals, according to the average number of alleles in common at independent SNPs. Data regions of 50kb were pruned and correlation of SNPs assessed. To ensure that no pair of SNPs were correlated, one of each pair found to be above the applied

threshold ( $r^2 > 0.2$ , Linkage disequilibrium  $> 0.2$ ) was removed. IBS data were used to calculate identity by descent (IBD), an estimate of the degree of recent shared ancestry for each pair of individuals. Expected theoretical IBD thresholds (denoted by PI\_HAT) for ancestral relationships are shown in Table 5. A threshold mid-way between second and third degree relatives, PI\_HAT  $> 0.1875$ , was used to identify cryptic relatedness between samples. When the two individuals from each pair were compared, the individual with the lower genotyping call rate (higher F\_MISS value) was selected for removal. Ten unique IDs were recorded for removal at completion of the individual QC process (Table 6).

PI_HAT	Relationship
$\geq 0.98$	Duplicate or monozygotic twins
$\geq 0.5$	First degree relatives
$\geq 0.25$	Second degree relatives
$\geq 0.125$	Third degree relatives

**Table 5:** IBD thresholds for different ancestral relationships.

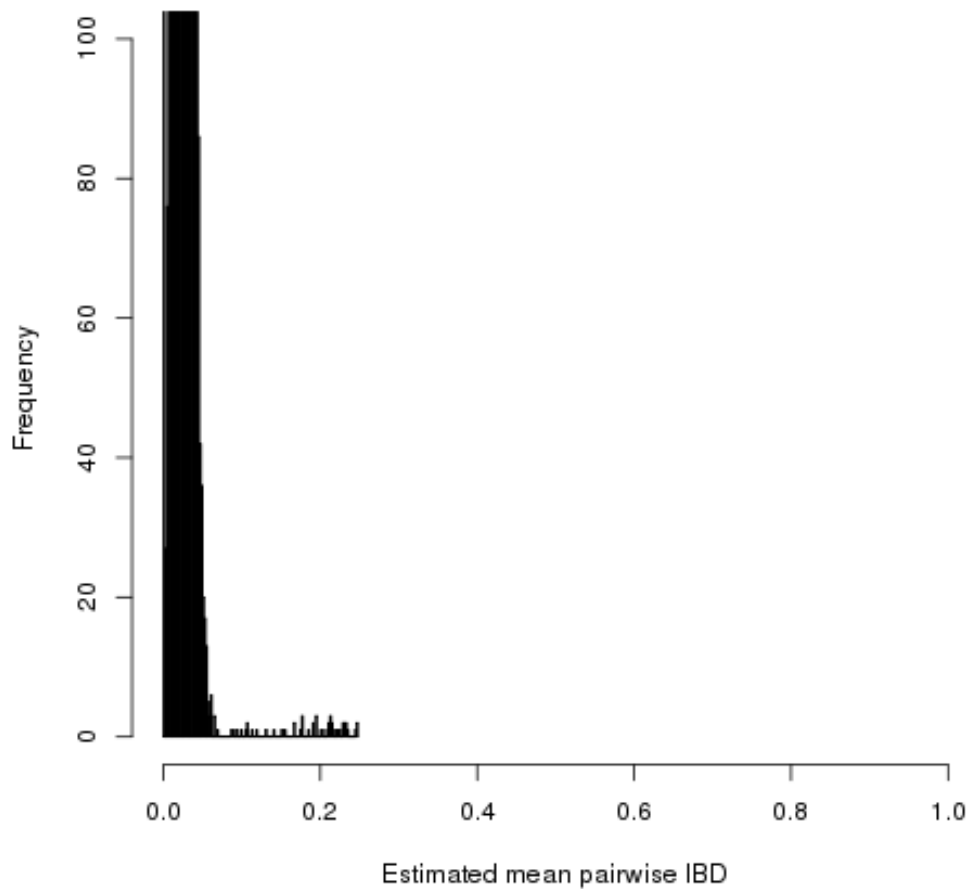
\*PI\_HAT indicates the identity by descent threshold.

Individual ID 1	F_MISS	Individual ID 2	F_MISS	PI_HAT
APP211	0.006368	APP254	0.02407	0.2165
		APP356	0.0142	0.2125
		APP361	0.066299	0.2289
		APP366	0.009463	0.2128
		APP373	0.005641	0.23
APP219	0.008672	APP270	0.005969	0.2322
APP253	0.006846	APP297	0.0134	0.2479
APP254	0.02407	APP356	0.0142	0.1903
		APP361	0.066299	0.2099
		APP366	0.009463	0.2001
		APP373	0.005641	0.212
APP302	0.006528	APP211	0.006368	0.2309
		APP254	0.02407	0.2135
		APP356	0.0142	0.2156
		APP361	0.066299	0.234
		APP366	0.009463	0.2234
		APP373	0.005641	0.2344
		APP12_069	0.0142	0.1957
APP319	0.01111	APP253	0.006846	0.2452
		APP297	0.0134	0.2475
APP356	0.0142	APP361	0.066299	0.2149
		APP366	0.009463	0.2026
		APP373	0.005641	0.2113
APP361	0.066299	APP366	0.009463	0.2206
		APP373	0.005641	0.2319
		APP12_069	0.0142	0.1958
APP366	0.009463	APP373	0.005641	0.2242
		APP12_069	0.0142	0.1911
APP373	0.005641	APP12_069	0.0142	0.1954

**Table 6:** Pairwise comparison of individuals with evidence of recent shared ancestry (PI\_HAT > 0.1875).

\* PI\_HAT indicates the identity by descent threshold. Shaded participants IDs were recorded for removal at completion of the individual QC process (n=10).

## Aspirin: IBD Estimation

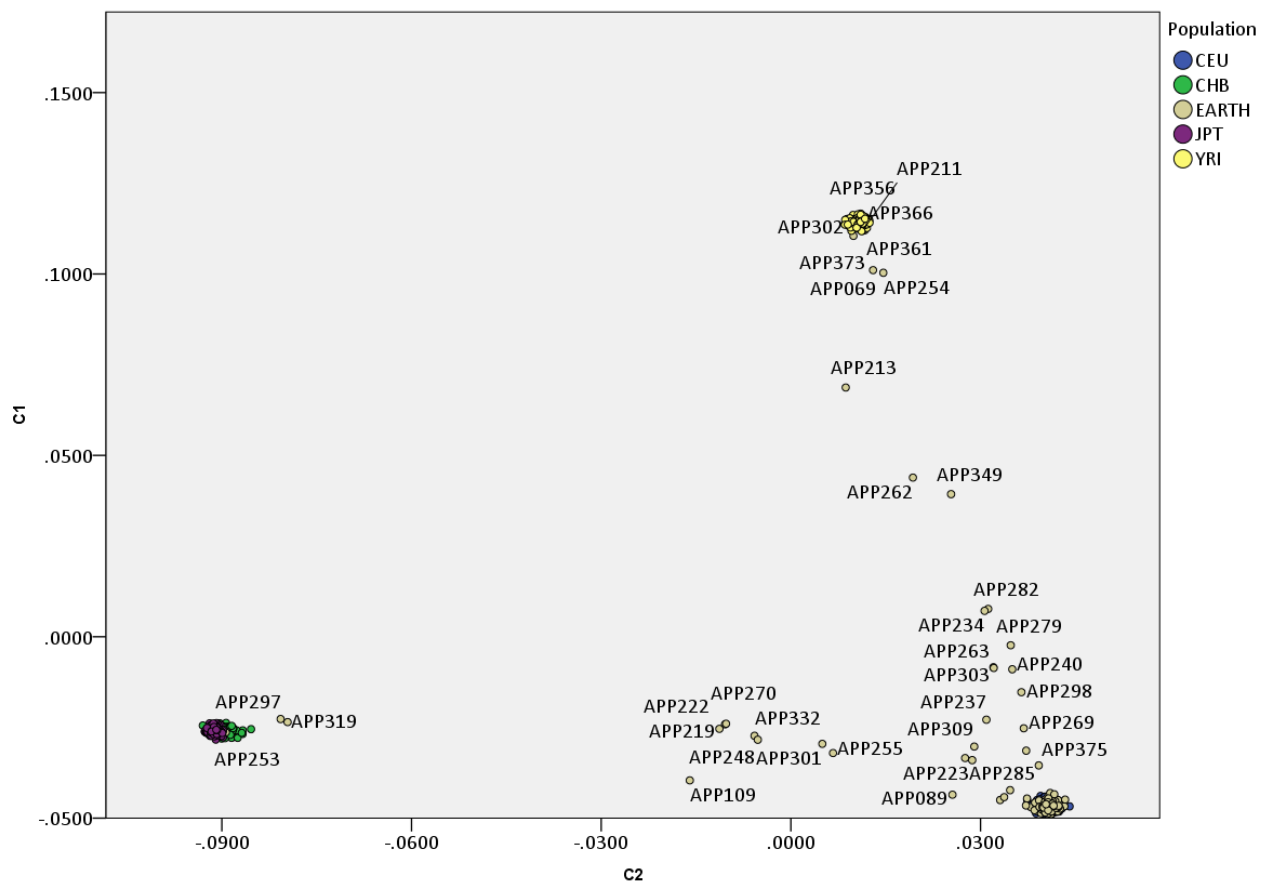


**Figure 2:** Cryptic relatedness of individuals in the pilot and EARTH study cohorts.

### Ethnicity assessment

Allele/genotype frequencies vary across different populations. A principal component analysis was used to assess ethnic origin and population structure from genotype data. Hapmap3 Caucasians (CEU), Han Chinese (CHB), Japanese (JPT) and Yoruba (YRI) were included as reference populations. Thirty-six ethnic outliers were identified according to their projection onto these four genetic variation principle components. These participants were recorded for exclusion at completion of the individual QC process (Figure 3). The remainder of pilot and EARTH study participants (n= 162) clustered within the Caucasian population were retained.





**Figure 3:** PCA plot of genetic ethnicities of pilot and EARTH study participants (C1= principal component 1, C2=principal component 2).  
 \*Ethnic outliers marked for removal are indicated by their participant numbers.

Thirty-six unique individual outliers generated at the gender, cryptic relatedness and ethnicity assessments were removed from the data-set before progressing to the SNP QC process (Table 4, Table 6, Figure 3).

Aspirin response phenotypes for 43 cases (variable aspirin responders), 73 controls (normal aspirin responders) and 46 excluded from downstream analysis (including indeterminate aspirin responders and pilot study participants) were inputted into the .fam file prior to SNP QC (n=162). Further explanation of aspirin response status and data used for phenotyping is provided both in Chapter 5 and the statistical analysis section of this chapter, respectively.

#### **Data quality control: Per single nucleotide polymorphism**

SNPs deviating from Hardy Weinberg Equilibrium ( $p < 1 \times 10^{-6}$ ) in controls only were removed (n=491). Monomorphic SNPs (n=100248) and SNPs with call rate <95% (n=0) were also removed prior to downstream analysis. After frequency and genotype pruning, the final data-set consisted of 695347 SNPs, and 116 individuals for downstream analysis (43 cases and 73 controls).

#### **Rationale for phenotyping decisions**

EARTH participants with platelet response data and aspirin metabolite detection data at both time-points ( $5^{+0}-20^{+6}$  weeks and  $33^{+0}-35^{+6}$  weeks) were analysed, from a possible total of 156 participants (Chapter 5). Participants with consistent indeterminate response to aspirin were excluded from the analysis due to the inability to verify adherence with LDA and hence accurately determine their response to LDA. Pilot study participants were excluded from this analysis. This decision was necessary due to the lack of VerifyNow data from the pilot study, to allow phenotyping between the pilot and EARTH cohorts to be appropriately matched.

## **Statistical analysis**

SPSS v.24 was used to examine clinical variables in the EARTH cohort data with potential effects on aspirin response. The variables assessed included; BMI, risk factors for pre-eclampsia (presence of high risk factors vs. moderate risk factors only) and whether LDA had been commenced prior to 16 week's gestation. The continuous variables, age and BMI, were analysed using analysis of variance (ANOVA) and the binary variables, degree of risk factors for pre-eclampsia and gestation at commencement of LDA were analysed with Pearson's chi-square test. Clinical variables with p values <0.05 were included as covariates in the subsequent logistic regression analysis of genome-wide genotype data.

Plink v.107 was used to add co-variate data to the binary file sets and to generate .assoc.logistic files necessary for further analysis (Purcell et al., 2007). The command lines used can be found in Appendix 5b.

Manhattan plots were generated using the genetics package of R v.3.1.1 (R, 2017). Two Manhattan plots were generated, one including BMI as a covariate and one without this covariate, to allow comparison of the significance of the resulting SNPs.

The threshold for genome-wide significance was  $5 \times 10^{-8}$  (WTCCC, 2007) and  $1 \times 10^{-5}$  was the suggested significant threshold (WTCCC, 2007). The presence of SNP 'towers' indicate regions of potential genomic interest. LocusZoom, a freely available web-based plotting tool (Pruim et al., 2010), was used to perform regional visualisation of these identified regions of interest.

## **6.4 Results: Genome-wide analysis of the EARTH cohort**

### **Participant characteristics**

Demographic, aspirin response and clinical outcome data for the complete EARTH cohort are described in Chapter 5 (Tables 6, 7, 11, 12, 13 and Figures 2, 3 and 4). Demographic

data for the 116 participants for which genome-wide genotyping data were analysed are shown in Table 7. Clinical outcome data for these participants are shown in Table 8.

	<b>Aspirin responder (n=73)</b>	<b>Variable responder (n=43)</b>
<b>Maternal demographic characteristics</b>		
Mean maternal age (SD)	31.1 (5.1)	30.8 (5.2)
Mean BMI (SD)	28.6 (7.3)	31.4 (7.7)
Risk factors for pre-eclampsia		
High risk only	50.1% (n=37)	32.6% (n=14)
High and moderate	38.4% (n=28)	62.8% (n=27)
Moderate only	11.0% (n=8)	4.7% (n=2)
<b>Antenatal care</b>		
Median gestational age of commencing LDA (range)	10.0 (2-16)	10.0 (3-17)
Antihypertensive treatment	34.2% (n=25)	27.9% (n=12)
Admissions due to pre-eclampsia, gestational hypertension, IUGR	13.7% (n=10)	16.3% (n=7)
Median length of admission (range)	2 (1-5)	2 (1-13)
<b>Intrapartum care</b>		
Admissions to HDU/ITU	4.1% (n=3)	7.0% (n=3)
Median length of HDU/ITU admission (range)	1.0 (1-1)	1.0 (1-2)
Acute antihypertensive treatment	13.7% (n=10)	16.3% (n=7)
Magnesium sulphate treatment	2.7% (n=2)	4.7% (n=2)
Spontaneous labour	11% (n=8)	14.0% (n=6)
Induction of labour	54.8% (n=40)	53.5% (n=23)
<b>Delivery outcomes</b>		
Spontaneous preterm birth <37 weeks	2.7% (n=2)	11.6% (n=5)
Caesarean section	50.7% (n=37)	39.5% (n=17)

**Table 7:** Demographic antenatal and intrapartum care data for EARTH participants for whom genome-wide genotyping data were analysed (n=116).

\*BMI=body mass index, LDA=low-dose aspirin, HDU=high dependency unit, IUGR=intrauterine fetal growth restriction, ITU=intensive therapy unit.

	<b>Aspirin Responder (n=73)</b>	<b>Variable Responder (n=43)</b>
<b>MATERNAL CLINICAL OUTCOMES</b>		
Abnormal uterine artery Doppler	5.5% (n=5)	9.3% (n=4)
Pre-eclampsia	Total 15.1% (n=11)	Total 11.6% (n=5)
≥37 weeks with AGA	6.4% (n=7)	9.3% (n=4)
≥37 weeks with SGA 5-9th customised centile	0.0% (n=0)	0.0% (n=0)
≥37 weeks with SGA <5th customised centile	1.4% (n=1)	0.0% (n=0)
<37 weeks with AGA	1.4% (n=1)	0.0% (n=0)
<37 weeks with SGA 5-9th customised centile	0.0% (n=0)	0.0% (n=0)
<37 weeks with SGA <5TH customised centile	1.4% (n=1)	2.3% (n=1)
<37 weeks with IUGR	1.4% (n=1)	0.0% (n=0)
Gestational hypertension	5.5% (n=4)	9.3% (n=4)
Placental abruption	1.4% (n=1)	0.0% (n=0)
<b>FETAL AND NEONATAL OUTCOMES</b>		
IUFD	0.0% (n=0)	2.3% (n=1)
Isolated IUGR	0.0% (n=0)	4.7% (n=2)
Isolated SGA	Total 13.7% (n=10)	Total 9.3% (n=4)
<5th customised centile	4.1% (n=3)	2.3% (n=1)
5-9th customised centile	9.6% (n=7)	7.0% (n=3)
Admissions to NICU	8.2% (n=6)	16.3% (n=7)
Median length of NICU admission (range)	1.5 (1-11)	3.0 (1-8)
RDS	5.5% (n=4)	4.7% (n=2)
Bronchopulmonary dysplasia	0.0% (n=0)	0.0% (n=0)
Intraventricular haemorrhage	0.0% (n=0)	0.0% (n=0)
Periventricular leukomalacia	0.0% (n=0)	0.0% (n=0)
Seizures	0.0% (n=0)	0.0% (n=0)
Hypoxic Ischaemic encephalopathy	0.0% (n=0)	0.0% (n=0)
Necrotising enterocolitis	0.0% (n=0)	0.0% (n=0)
Neonatal death	0.0% (n=0)	0.0% (n=0)
<b>COMPOSITE PLACENTALLY MEDIATED ADVERSE OUTCOME</b>	<b>16.4% (n=12)</b>	<b>18.6% (n=8)</b>

**Table 8:** Clinical outcome data for EARTH participants for whom genome-wide genotyping data were analysed (n=116).

\*AGA=appropriate for gestational age, SGA=small for gestational age, IUGR=intrauterine fetal growth restriction, NICU=neonatal intensive care unit.

There were no significant associations between age, degree of risk factors for pre-eclampsia (high versus moderate only) or whether LDA was commenced prior to or from 16 weeks and variable response to aspirin ( $p=0.729$ ,  $p=0.618$ ,  $p=0.257$ , respectively).

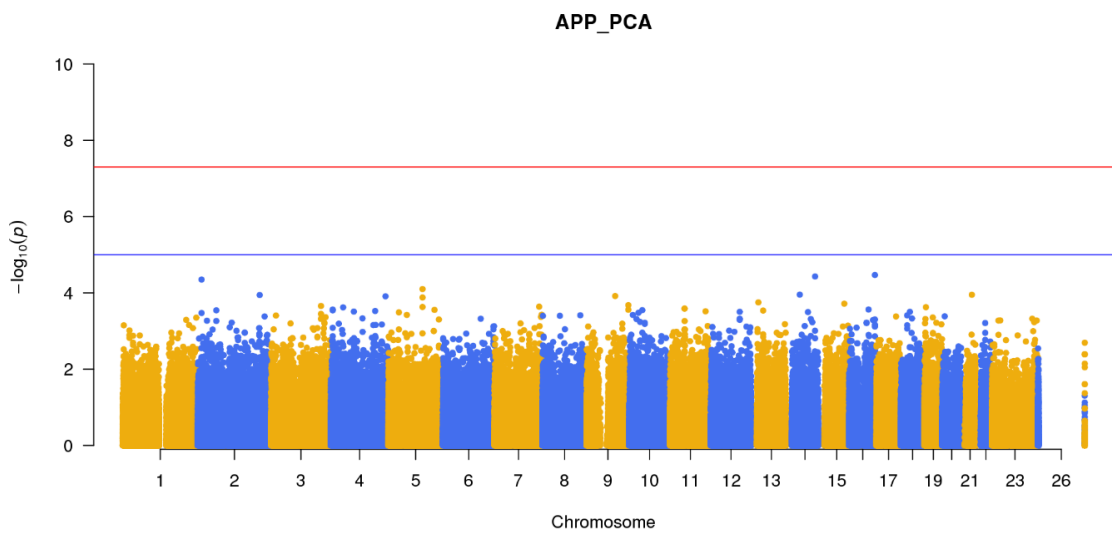
There was an association between BMI and variable response to aspirin which just

surpassed the threshold for significance ( $p=0.048$ ). BMI was subsequently added as a covariate for logistic regression of genome-wide genotype data.

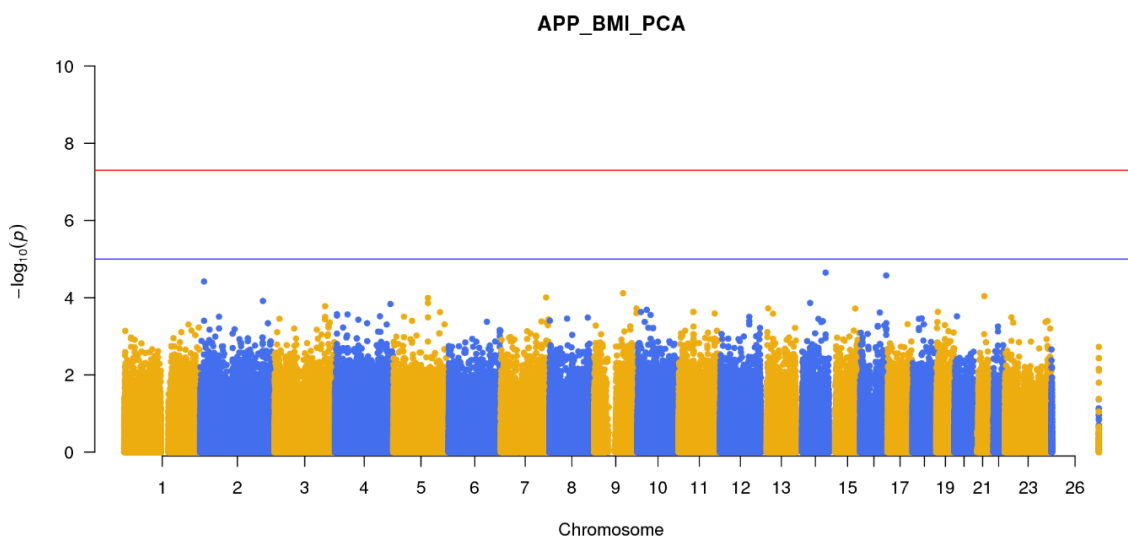
#### **Genome-wide association analysis**

When aspirin responders were compared to variable responders, no genome-wide significant association or suggested association was observed in the EARTH data-set (Figure 4a). Addition of BMI as a clinical covariate had no impact on the significance of genome-wide results (Figure 4b).

a)



b)



**Figures 4a and 4b:** Genome-wide Manhattan plot of (a) aspirin responders versus variable responders; (b) aspirin responders versus variable responders with BMI included as a clinical covariate.

\*Individual  $-\log_{10}$  p-values are plotted against their genomic position. The horizontal red line indicates the genome-wide significance threshold ( $5 \times 10^{-8}$ ). The horizontal blue line indicated the threshold for suggested significance ( $1 \times 10^{-5}$ ), an indicator of promising single nucleotide polymorphisms.

### **Single nucleotide polymorphisms with the lowest p-values**

Despite no SNPs surpassing suggestive and significant thresholds, the top three SNPs in the data-set, on chromosomes 16, 14 and 2, were seen to approach the suggestive threshold (Figures 4a and b, Table 9). Additionally, towers of SNPs on chromosomes 16, 14, 2, 5, 9 and 7 were identified (Figures 4a and b). Further examination of these regions was subsequently undertaken.



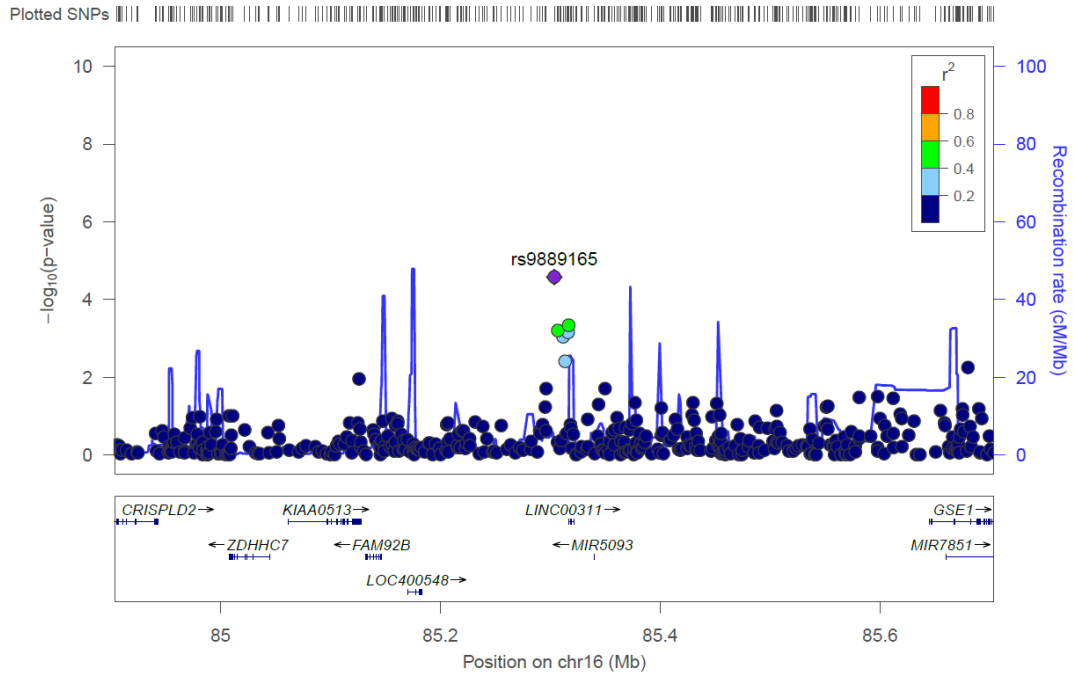
Chromosome	Closest gene	SNP ID	Position	Alleles	MAF (aspirin responders)	MAF (variable responders)	p-value
16	<i>LINC00311</i>	rs9889165	85303656	A/G	0.3356	0.6512	3.41 x 10 <sup>-5</sup>
14	<i>BDKRB2</i>	rs4905463	96681345	G/C	0.4028	0.1395	3.72 x 10 <sup>-5</sup>
2	<i>MIR3681HG</i>	rs1918735	12546256	A/G	0.3082	0.593	4.47 x 10 <sup>-5</sup>

**Table 9:** Top three SNPs identified from Manhattan plots (Figures 4a and b), with the lowest genome-wide p-values, physical positions are based on build 37 (NCBI) of the Human Genome.

\*MAF=minor allele frequency.

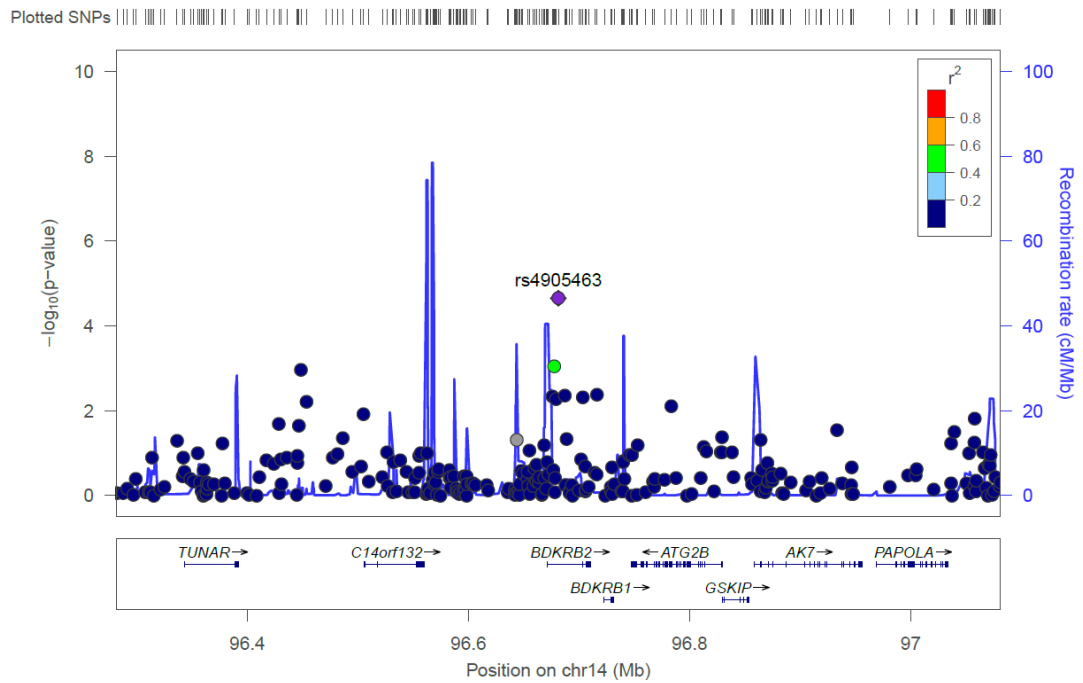
a)

### APP\_BMI\_PCA-Chr16



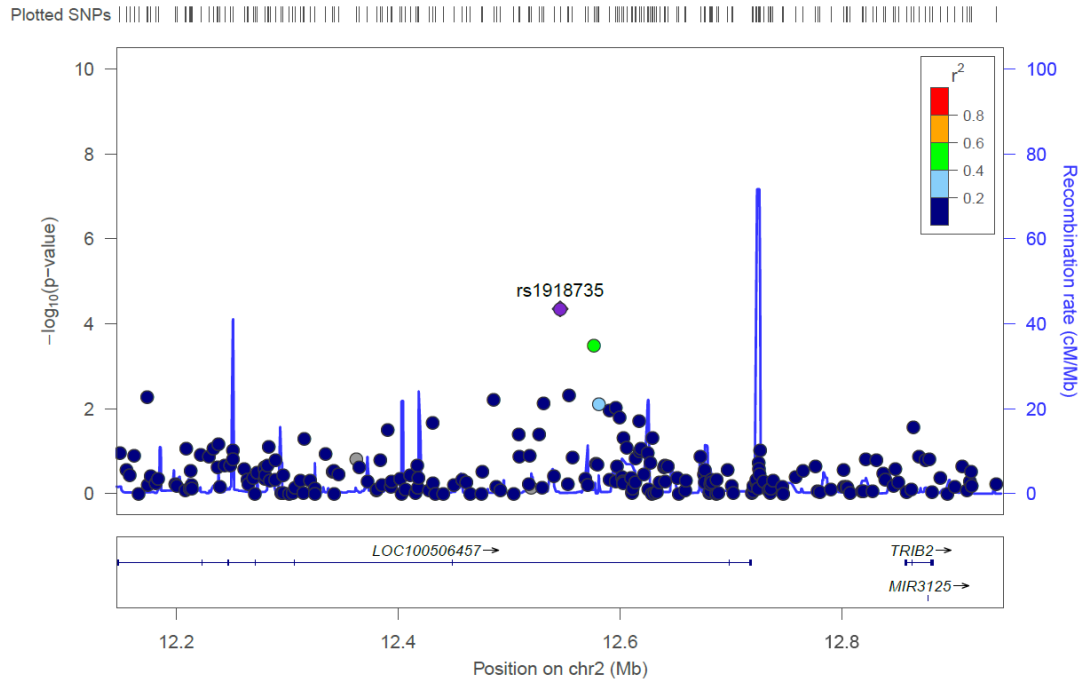
b)

### APP\_BMI\_PCA-Chr14



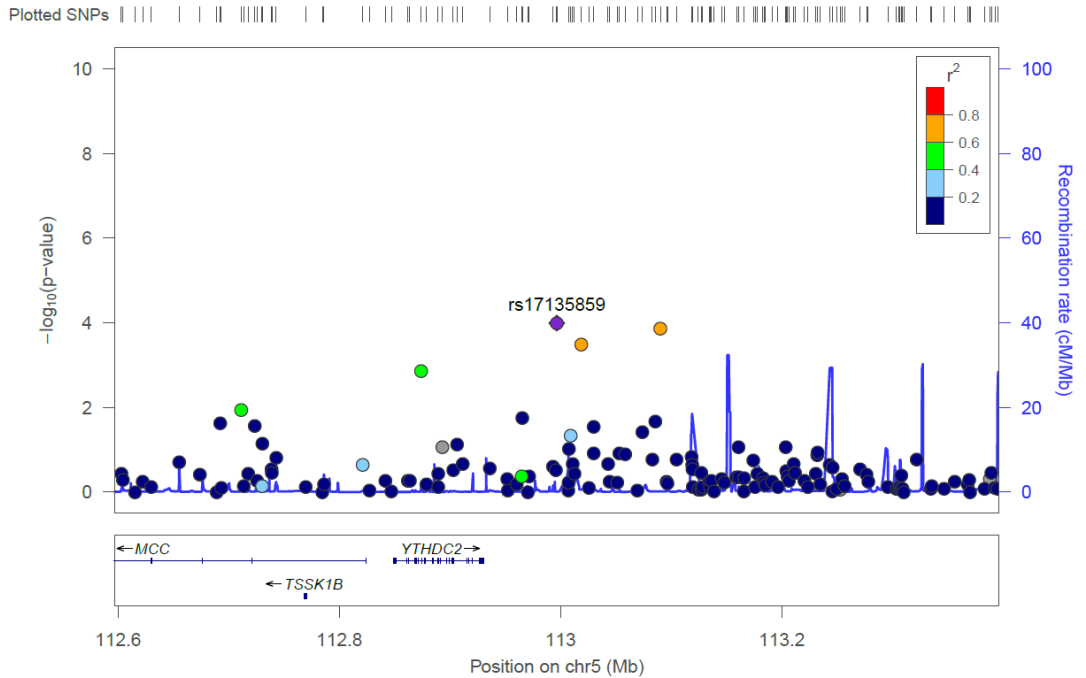
c)

### APP\_BMI\_PCA\_Chr2

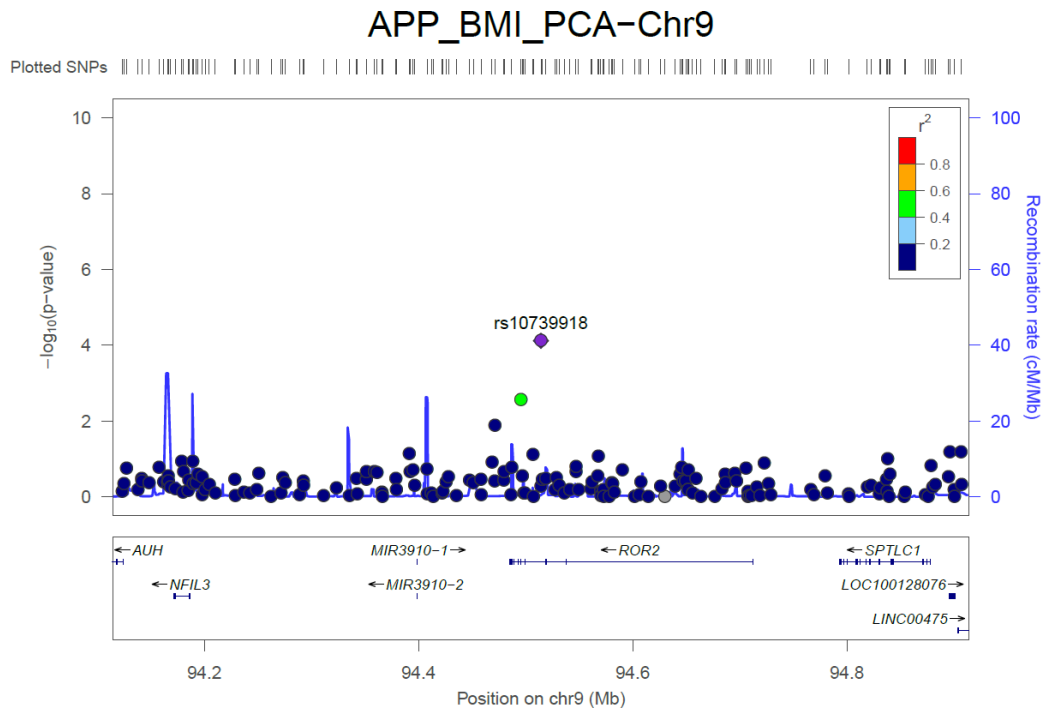


d)

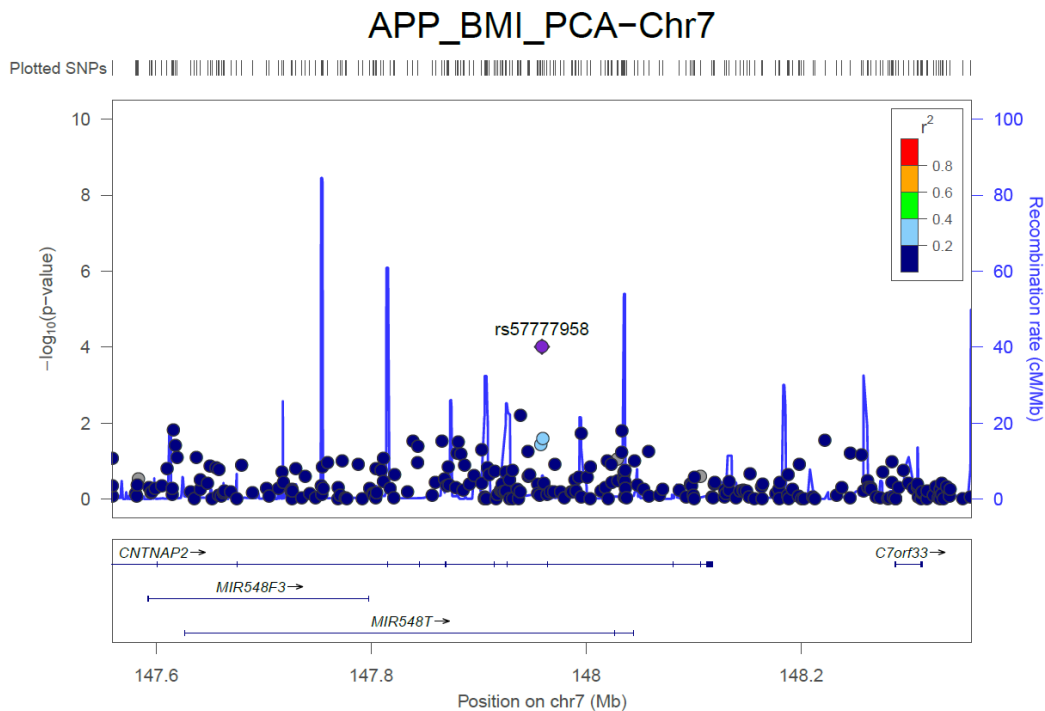
### APP\_BMI\_PCA\_chr5



e)



f)



**Figures 5a-f:** Regional association plots of chromosomes 16, 14, 2, 5, 9, 7 chosen for the appearance of SNP towers appearing in Manhattan plots, in order of increasing p-values.

\*73 Aspirin responders and 43 variable responders were compared. Target single nucleotide polymorphisms (SNPs) are indicated with purple diamonds. Coloured circles represent SNP p-values. The colour scale (indicated in the right legend) reflects the extent of linkage disequilibrium (LD,  $r^2$ ). SNPs with missing LD information are shown in grey.

Gene	SNP ID	Phenotyping method (literature)	GWAS p-value/s (literature)	Present in EARTH dataset?	GWAS p-value (EARTH)
<b>ADRA2A</b>	rs431194	COX-independent	3.3 x 10 <sup>-11</sup>	No	N/A
<b>BMPRIA</b>	rs11202221	COX-specific and COX independent	4.8 x 10 <sup>-8</sup>	No	N/A
<b>COX1/PTGS1</b>	rs3842787	COX-specific and COX independent	N/A	Yes	0.9753
	rs1330344	COX-specific	N/A	Yes	0.4063
<b>COX2/PTGS2</b>	rs20417	COX-specific and COX independent	N/A	Yes	0.06651
<b>GP6</b>	rs1671152	COX-independent	4.6 x 10 <sup>-13</sup>	No	N/A
<b>JMJDIC</b>	rs10761741	COX-independent	1.6 x 10 <sup>-8</sup>	No	N/A
<b>MDR1</b>	rs1045642	Clinical only	N/A	Yes	0.342
<b>MRVII</b>	rs7940646	COX-independent	2.0 x 10 <sup>-8</sup>	Yes	0.8243
<b>PEAR1</b>	rs2768759	COX-independent	N/A	Yes	0.07078
	rs12041331	COX-independent	7.66 x 10 <sup>-9</sup> 8.34 x 10 <sup>-12</sup>	No	N/A
	rs12566888	COX-independent	3.4 x 10 <sup>-12</sup>	Yes	0.2852
<b>P2RY1</b>	rs1371097	COX-specific and COX independent	N/A	No	N/A
<b>SHH</b>	rs2363910	COX-independent	4.5 x 10 <sup>-8</sup>	No	N/A
<b>SVIL</b>	rs7070678	COX-independent	3.58 x 10 <sup>-8</sup>	No	N/A
	rs10826650	COX-independent	4.43 x 10 <sup>-8</sup>	No	N/A
<b>(intergenic)</b>	rs56566765	COX-specific and COX independent	3.59 x 10 <sup>-8</sup>	No	N/A

**Table 10:** Single nucleotide polymorphisms reported by candidate gene studies and genome-wide association studies to be significantly associated with platelet response to aspirin.

\*COX=cyclooxygenase.

Gene	Chromosome	Position	No. of SNPs in EARTH cohort	Lowest GWAS p-value
<b>COX1/PTGS1</b>	9	125132809-125157982	11	0.06433
<b>GPIa/ITGA2</b>	5	52285156-52390609	25	0.1653
<b>GPIIIa/ITGB3</b>	17	45331208-45390077	17	0.1084
<b>GP1b<math>\alpha</math>/GP1BA</b>	17	4835312-4838325	3	0.3237
<b>P2Y1/P2RY1</b>	3	152552736-152555845	1	0.9802
<b>P2Y12/P2RY12</b>	3	151054631-151102600	10	0.1544

**Table 11:** Single nucleotide polymorphisms in the EARTH data-set from candidate genes previously reported to be significantly associated with platelet response to aspirin.

\*Physical positions are based on build 37 (NCBI) of the Human Genome.

## 6.5 Discussion

Despite no genome-wide significant or suggestive SNPs was found from this analysis, a detailed assessment was made of SNPs with the lowest p-values, with corresponding appearances of 'towers' within genotyped data. In order of ascending p-value, we identified signals for SNPs and towers on chromosomes 16, 14, 2, 5, 9 and 7. All but rs57777958, on chromosome 7, were identified as intronic variants.

The regional plot generated for chromosome 16 (Figure 5a) indicates good evidence of a tower of SNPs. The top SNP rs9889165, lies close to a long intergenic non-protein coding RNA 311 (*LINC00311*) and has no previously reported clinical significance. There was also good evidence of a tower for chromosome 14 (Figure 5b). The top SNP, rs4905463, is located closest to Bradykinin receptor B2 (*BDKRB2*), a protein coding gene. *BDKRB2* encodes a bradykinin receptor, involved in vascular and smooth muscle regulation (NCBI, 2017a). This receptor has recently been implicated in hypertension (Luo et al., 2016, Gu et al., 2016) and pre-eclampsia (Valdes et al., 2016).

Higher resolution assessments of chromosome 2 and 5 indicated limited evidence for SNP towers (Figures 5c and 5d). Additionally, the genes closest to the top SNPs, MIR3681 host gene (*MIR3681HG*, also ref to as *LOC100506457*) and *LOC107986441* have no functional significance (NCBI, 2017c) (NCBI, 2017f, NCBI, 2017d).

Regional assessments for chromosomes 9 and 7 both showed limited evidence of SNP towers (Figures 5e and 5f). Both top SNPs, rs10739918 and rs57777958 relate to genes involved with fetal development. Receptor tyrosine kinase like orphan receptor 2 (*ROR2*), on chromosome 9, encodes a transmembrane cell-surface receptor, required for early development of chondrocytes and the growth plate and mutations are associated with skeletal dysplasias (NCBI, 2017e). Similarly, contactin associated protein like 2 (*CNTNAP2*) encodes a neurexin protein involved in nervous system development and specifically

interactions between neurons and glia (NCBI, 2017b). This gene is one of the largest human genes, spanning 1.5% of chromosome 7, and implicated in neurodevelopmental disorders (NCBI, 2017b).

After examining SNPs previously reported to be significantly associated with response to aspirin occurring in the EARTH genotyped data-set, it was apparent that none approached genome-wide significance (Table 10). 59% (n=10) were not present in the genotyped data-set. Additionally, all SNPs from previously proposed candidate genes present in our data-set had comparably high p-values (Table 11). Such genomic regions with previously identified significant SNPs can be optimised by imputing the EARTH data. However, when considering the findings of other authors in relation to the genetics of platelet response to aspirin in pregnant women there are two key issues; population comparability and phenotyping. None of the cohorts identified in our searches are comparable in terms of mean age or disease status. The majority of studies, CGS, GWAS and other methodologies have focussed on cardiovascular and cerebrovascular populations, with inherently older participants and high numbers of males. As aspirin non-responsiveness is a relatively new area of exploration in obstetric research, there have been no prior studies examining genetic factors by any methodology. Sixty-five percent of studies (n=11) used LTA, the traditional gold standard assessment, for phenotyping. However, only 59% (n=10) of studies overall assessed platelet aggregation in response to a COX-1-specific agonist, whereas all studies included non-COX-specific assessments. Subsequently, most significant findings reported to date, relate to non-COX-specific platelet aggregation pathways, not directly influenced by aspirin. If the research question centres on response to aspirin, phenotyping should be COX-1-specific, reflecting aspirin's pharmacological action.

To address both these issues, we have identified a validation cohort. This cohort consists of aspirin-treated pregnant women and we also have access to aspirin-naïve healthy pregnant controls to include in a case-control GWAS. Both groups have undergone COX-specific platelet function testing and have aspirin metabolite data to allow matched phenotyping. With this combined population we will perform imputation to improve our SNP coverage and comparability to published data. During the analysis of EARTH data we strictly adhered to recommended per individual QC thresholds and there is scope to individualise these thresholds to optimise future analyses. This is especially relevant for the gender assessments, with data just outside recommended limits as we can be certain of entirely female cohorts. Similarly for cryptic relatedness, within small regional populations, from which both the EARTH and validation cohorts arise there is a reasonable chance of second and third degree relations. A more favourable balance may be reached to retain individuals for analysis whilst not introducing an unacceptable level of bias into the data.

## **6.6 Conclusions**

There is an established body of evidence from CGS, GWAS and studies of other methodologies indicating several variants in genes with functional plausibility related to platelet aggregation. However, the comparability of these data to response to the EARTH cohort is limited due to the advanced age of participants, preponderance for males, lack of any other obstetric cohorts and reliance on non-COX specific phenotyping. In this unbiased genome-wide assessment of variants in relation to longitudinal platelet response to aspirin we found no evidence of either genome-wide significant or suggestive associations. There were no signals to indicate genes with biological plausibility in relation to platelet response to aspirin in our data-set and no correlation with specified clinical outcomes. Several top SNPs and towers relate to genes with no functional significance or genes related to fetal development, to be expected in pregnant participants. We have



already identified a suitable validation cohort and plan for imputation of genotyped data to improve genomic coverage and comparability.

**CHAPTER 7: Placental growth factor and tests  
and the soluble FMS-like tyrosine kinase  
1/Placental growth factor ratio and their  
relationship to aspirin use**

## **7.1 Rationale**

The aim of the work in this chapter was to assess if aspirin non-responsiveness relates to recognised markers of declining placental function, addressing Objective 4b (Chapter 1, Figure 10). This was achieved by parallel testing of third trimester maternal serum and plasma samples from the EARTH cohort with three commercially available placental growth factor (PlGF) and FMS-like soluble tyrosine kinase-1 (sFlt-1)/PlGF ratio tests. We first assessed agreement between the tests and their predictive accuracy for pre-eclampsia in the EARTH cohort. Potential associations between abnormal test results and abnormal maternal and fetal Doppler ultrasound indices and individual response to aspirin were then explored.

## **7.2 Background to the tests**

PlGF is a glycosylated protein from the vascular endothelial growth factor family. In pregnancy, PlGF is synthesised by the placental trophoblast and involved with placental angiogenesis and vascular adaptation (Levine et al., 2004). Subsequently, PlGF tracks functional placental development, with circulating levels peaking between 26 and 30 week's gestation and declining during the remainder of the 3<sup>rd</sup> trimester (Knudsen et al., 2012). In the context of placental disease, circulating PlGF may rise insufficiently and its decline may be premature or accelerated (Levine et al., 2004).

sFlt-1 is a circulating anti-angiogenic factor and splice variant of the vascular endothelial growth factor (VEGF) receptor synthesised primarily by trophoblast and sequesters PlGF (Khalil et al., 2008). With normal placentation, circulating sFlt-1 is stable during the first and second trimesters, increasing during the third trimester from 33 weeks (Levine et al., 2004). In placental disease with reduced PlGF, there may be higher circulating sFlt-1 and escalating sFlt-1/PlGF ratios (Zeisler et al., 2016). These findings, often detectable several weeks before clinical manifestations, have prompted interrogation of PlGF and sFlt-1 for screening and diagnosis of pre-eclampsia (Rana et al., 2007, Romero et al., 2008).

PIGF and sFlt-1/PIGF ratios out-perform standard biochemical assessment methods for the prediction of adverse outcomes in suspected pre-eclampsia (Rana et al., 2012, Sibude et al., 2012). PIGF tests have superior area under the receiver operating curve (ROC) compared to systolic blood pressure (BP), diastolic BP and proteinuria assessments (0.87 vs. 0.67, 0.66, 0.76, respectively) (Chappell et al., 2013). A prospective multicentre study concluded that when pre-eclampsia is suspected prior to 35 weeks, plasma PIGF below the 5<sup>th</sup> centile has high sensitivity (0.96 95% CI 0.89-0.99) and high negative predictive power (0.98 95% CI 0.93-0.99) for pre-eclampsia requiring delivery within 14 days (Chappell et al., 2013). PIGF measurements have the potential to identify the majority of pre-eclampsia cases, minimise false negatives and may allow women with low-risk results to return to usual care (Cnossen et al., 2009).

In 2016 the UK National Institute for Health and Care Excellence (NICE) conducted an appraisal of four commercially available diagnostic tests for pre-eclampsia (Alere Triage PIGF test, Roche Elecsys immunoassay sFlt-1/PIGF ratio, Perkin Elmer DELFIA Xpress PIGF 1-2-3 test, and BRAHMS sFlt-1 Kryptor/BRAHMS PIGF plus Kryptor PE ratio)(NICE, 2016). Integration of Alere PIGF and Roche sFlt-1/PIGF ratio tests with clinical assessment and follow-up was recommended, to rule out pre-eclampsia in symptomatic women between 20+<sup>0</sup> and 34+<sup>6</sup> weeks.

We aimed to evaluate agreement of PIGF tests (Alere and Perkin Elmer) and the sFlt-1/PIGF ratio and compare their predictive accuracy for third trimester pre-eclampsia in high-risk women. We also aimed to examine for associations between these markers, uteroplacental Doppler and women's platelet response to aspirin.

### **7.3 Methods**

#### Study Design

Our cohort comprised women from the Estimating Aspirin ResisTance in High risk women (EARTH) study, a prospective cohort study designed to assess whether response to aspirin is associated with adverse pregnancy outcomes. The study was funded by Wellbeing of Women following external peer review. Roche diagnostics provided sFlt-1 kits free of charge. Both Wellbeing of Women and Roche did not influence the conduct of the study, analyses or content of the manuscripts. The study was also supported by the Liverpool Centre for Women's Health Research patient and public involvement group and approved by the Liverpool Research Ethics Committee (REC reference no. 13/NW/0764, Appendix 3a).

#### Study procedures

Women were recruited between 5<sup>+0</sup>-20<sup>+6</sup> week's gestation from antenatal clinics at Liverpool Women's Hospital between January 2014 and September 2015. All women were prescribed low-dose aspirin because they had two or more moderate risk-factors or a single high risk factor for pre-eclampsia, according to NICE criteria (NICE, 2010a).

Participants had uterine artery Doppler examinations at 20<sup>+0</sup>-23<sup>+6</sup> week's gestation. Between 33<sup>+0</sup> and 35<sup>+6</sup> weeks maternal plasma and serum samples were obtained for measurement of PlGF and sFlt-1. At the same time-point platelet function was assessed with cyclooxygenase (COX)-specific assays using maternal whole blood samples for Multiplate™ Impedance Aggregometry ASPI test (Roche Diagnostics, Switzerland) VerifyNow™ Aspirin test (Accumetrics, San Diego, CA). Maternal urine samples were collected at the same time-point to measure urinary 11-dehydrothromboxane B<sub>2</sub> (11-DHTXB<sub>2</sub>) via the TXB Cardio™ test (Randox, UK).

Maternal samples were simultaneously tested for aspirin metabolites. Plasma salicylic acid (SA) was measured using liquid chromatography mass spectrometry (LC: MS) and urinary salicylic acid (SUA) was measured using nuclear magnetic resonance (NMR).

#### Outcome variables

Following delivery, maternal and neonatal electronic and paper case records were reviewed to ascertain clinical outcomes. Pre-eclampsia was defined according to NICE criteria as new onset hypertension, systolic BP  $\geq$  140 mmHg and/or diastolic BP  $\geq$  90mmHg (Korotkoff V) on at least two occasions four hours apart, with proteinuria (spot urine protein  $\geq$  2+, protein: creatinine ratio  $\geq$ 30 mg/mmol) (ACOG, 2002). Severity was classified as mild (140-149/90-99mmHg), moderate (150-159/100-109) or severe ( $>$  160/110 mmHg)(NICE, 2010a). IUGR was defined as birthweight  $<$  5<sup>th</sup> centile, calculated using the GROW bulk centile calculator (GROW, 2016a) in combination with at least one abnormal fetal Doppler index (umbilical artery Doppler PI  $>$ 95<sup>th</sup> centile, reduced, absent, or reversed end diastolic flow, middle cerebral artery PI  $<$ 5<sup>th</sup> centile) or post-mortem classification following intrauterine fetal death (IUFD).

Multiplate<sup>TM</sup> and TXB Cardio<sup>TM</sup> results were assessed against 95% pregnancy-specific reference ranges calculated from a local cohort of 87 aspirin naïve pregnant women at low risk of pre-eclampsia with normal pregnancy outcomes (Chapter 1, page 42, Table 6). As a pregnancy-specific reference range for VerifyNow<sup>TM</sup>, is not currently available, results were assessed against the manufacturer's reference range with  $<$  550 aspirin reaction units (ARU) indicating appropriate aspirin response.

Appropriate platelet response was defined as at least two out of three platelet function test results being outside of the pre-specified reference ranges. Appropriate aspirin adherence was defined as either evidence of appropriate platelet response or detection of aspirin metabolites either by LC: MS or NMR. Participants with no evidence of

appropriate platelet response but detectable aspirin metabolites were denoted as aspirin non-responders.

Alere recommends classification of PIGF results as highly abnormal (<12pg/ml), abnormal (12-99 pg/ml) and normal ( $\geq 100$  pg/ml) (Chappell et al., 2013). For our analysis abnormal was defined as PIGF <100pg/ml and normal as PIGF  $\geq 100$  pg/ml. Roche advise an sFlt-1/PIGF ratio cut-off of < 38 to rule out pre-eclampsia over the next week and a rule in cut-off for the next 4 weeks of  $\geq 38$ . We defined normal as sFlt-1/PLGF ratio <38 and sFlt-1/PIGF ratio  $\geq 38$  as abnormal (Zeisler et al., 2016). Perkin Elmer advises determination of gestational-day-specific 5th and 10th centile cut-offs for each participant. This was done individually in Excel 2010, using formulae provided by Perkin Elmer. We grouped results as follows:  $\geq 10$ th centile (normal), <10th centile (abnormal).

#### Test procedures

The platelet function assays conducted are considered COX-specific, reflecting the irreversible inhibition of COX-1 by aspirin. Whole blood samples for platelet function were analysed within three hours of collection. Both the Multiplate™ and VerifyNow™ analysers are semi-automated and intended for point-of-care use. Multiplate™ detects the change in electrical impedance when platelets aggregate on the electrode sensors, measured using an arachidonic acid agonist (ASPI test) to assess COX inhibition due to aspirin. VerifyNow™ also uses arachidonic acid as an agonist (Aspirin test) and measures a change in optical signal as activated platelets bind and aggregate fibrinogen-coated beads. TXB Cardio™ is a measure of 11-DHTXB<sub>2</sub>, the stable urinary metabolite of thromboxane A<sub>2</sub> (TXA<sub>2</sub>), formation which is inhibited by aspirin's effects on COX. Samples were aliquoted and stored at -80 °C within three hours of collection and subsequently transferred on dry ice to Randox Laboratories, Ireland for analysis.

Samples for measurement of PIGF and sFlt-1 were aliquoted and stored at -80 °C and analysed in batches on completion of the clinical study. For the Alere Triage PIGF test, plasma aliquots were thawed and tested according to the manufacturer's instructions (Alere, Waltham, MA). The Alere Triage PIGF test is a point-of-care test, a fluorescence immunoassay that provides a result within 15 minutes. The test detects free plasma PIGF at a lower limit of 9 pg/ml and quantifies measurements in the range of 12-3000pg/ml.

The Roche Elecsys immunoassay sFlt-1/PIGF ratio operates on the Elecsys and the Cobas e automated analysers (Roche Diagnostics, Switzerland). Serum samples were transferred on dry ice to the Royal Liverpool University Hospital clinical chemistry laboratory and analysed according to the manufacturer's instructions. The test measures PIGF relative to sFlt-1, incorporating results from two sandwich electrochemiluminescence immunoassays, and provides results within 20 minutes. The lower limit of detection for sFlt-1 is 10 pg/ml (quantifiable from 15 pg/ml) with a measurable range of 10-85,000 pg/ml. The lower limit of detection for PIGF is 3 pg/ml (quantifiable from 10 pg/ml), with a measurable range of 3-10,000 pg/ml.

The Perkin Elmer DELFIA Xpress PIGF 1-2-3 test is compatible with the 6000 DELFIA Xpress random access analyser (Perkin Elmer, Finland). Serum samples were shipped on dry ice to Perkin Elmer laboratories for analysis according to their protocol. The Perkin Elmer DELFIA Xpress PIGF 1-2-3 test provides test results within 30 minutes, with a lower limit of detection of 1.9 pg/ml (quantifiable from 3.3 pg/ml) and measurable range of 1.9-4000 pg/ml.

#### Statistical analysis

Results from the two PIGF tests and the sFlt-1/PIGF ratio were dichotomised into 'normal' and 'abnormal' groups, previously described. Positive percent agreement (PPA) was calculated by dividing total agreed positives between tests by the agreed positives plus



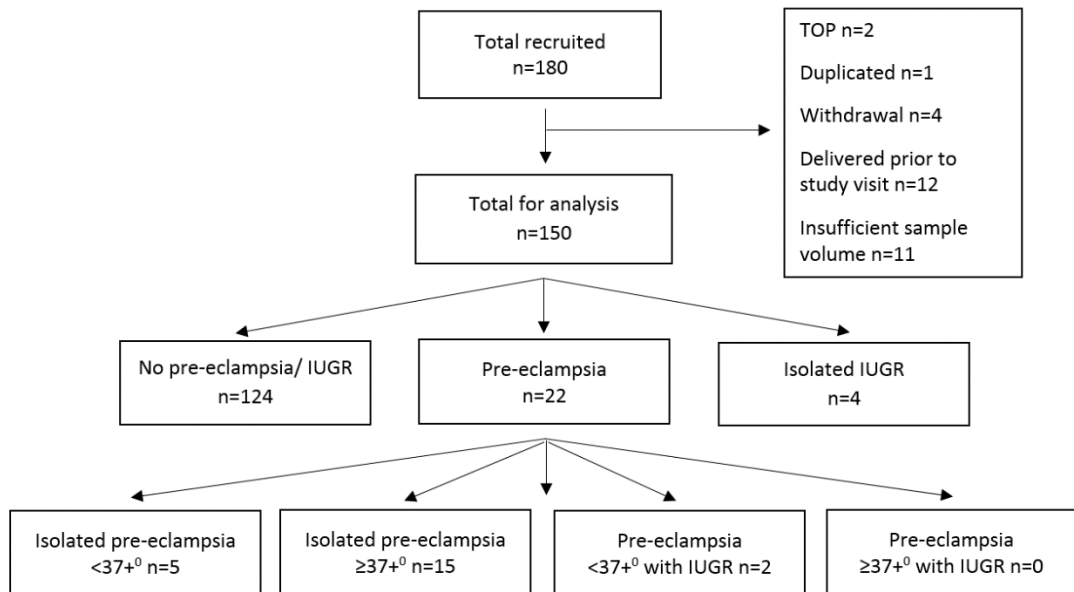
the negatives for the test under comparison only. Negative percent agreement (NPA) was calculated by dividing agreed negatives between tests by agreed negatives plus positives for the test under comparison only (Tables 3a-c). Overall percent agreement (OPA) was calculated by dividing agreed positives plus agreed negatives by the total tests performed for the following comparisons 1) Alere and Perkin Elmer 2) Perkin Elmer and Roche 3) Roche and Alere (Tables 3a-c).

Raw PIGF measurements and inverted sFlt-1/PIGF ratios were inputted to construct ROC curves and calculate area under the curve (AUC) to compare the predictive power of the tests. ROC curves including PIGF tests (Alere Perkin Elmer) and sFlt-1/PIGF ratio (Roche) were produced for each outcome; 1) all pre-eclampsia 2) pre-eclampsia requiring delivery prior to 37 week's gestation.

We examined for associations between 'increased risk' PIGF and/or sFlt-1/PIGF ratio results and; 1) mean uterine artery Doppler pulsatility index (PI)  $\geq 1.45$  between 20<sup>+0</sup> and 23<sup>+6</sup> weeks 2) response to aspirin by applying the Pearson's chi-square test for independence across the variables. The Bonferroni correction was applied to adjust p-values for the effect of multiple analyses, the corrected p-value of 0.025 (from 0.05) was applied when assessing the significance of results. Data were analysed using SPSS v.24 for Windows (IBM, Chicago, IL).

#### 7.4 Results: Test agreement

A flow chart of participants and clinical end-points are shown in Figure 1 with further details for pregnancies with adverse outcomes in Table 2. Demographic characteristics of the 150 participants for whom there were adequate samples to conduct all PIGF and sFlt-1/PIGF ratio tests are shown in Table 1.



**Figure 1:** Flow diagram of participants.

\*IUGR=intrauterine growth restriction.

<b>MATERNAL CHARACTERISTICS</b>	<b>Total analysed (n=150)</b>
Mean maternal age (SD)	31.3 (5.4)
Nulliparous	20.7% (n=31)
Risk factors for pre-eclampsia	
High only	45.3% (n=68)
High plus moderate	46.0% (n=69)
Moderate only	8.7% (n=13)
<b>Delivery outcomes</b>	
Spontaneous preterm birth <37 weeks	2.7% (n=4)
Caesarean section	45.3% (n=68)
<b>Maternal outcomes</b>	
Pre-eclampsia	Total 14.7% (n=22)
≥37 weeks with AGA	8.0% (n=12)
≥37 weeks with SGA <10 <sup>th</sup> customised centile	2.0% (n=3)
<37 weeks with AGA	2.0% (n=3)
<37 weeks with SGA <10 <sup>th</sup> customised centile	1.3% (n=2)
<37 weeks with IUGR	1.3% (n=2)
Gestational hypertension	6.0% (n=9)
Placental abruption	0.7% (n=1)
<b>Fetal and neonatal outcomes</b>	
IUFD	0.7% (n=1)
Isolated IUGR	2.7% (n=4)
Isolated SGA	Total 10.7% (n=16)
<5 <sup>th</sup> customised centile	7.3% (n=11)
<10 <sup>th</sup> customised centile	10.7% (n=16)
Admissions to NICU	Total 11.3% (n=17)
RDS	47.1% (n=8)
Jaundice	52.9% (n=9)
Hypoglycaemia	17.6% (n=3)
Median length of NICU admission (range)	3 (1-59)

**Table 1:** Demographic characteristics and clinical outcomes of high risk women with blood samples available for all tests.

\*SD=standard deviation, AGA=appropriate for gestational age, SGA=small for gestational age, IUFD=intrauterine fetal death, IUGR=intrauterine fetal growth restriction.

In total, 26 women had an adverse pregnancy outcome (17%), whilst 65 women (43%) had at least one abnormal PIGF or sFlt-1/PIGF ratio result. There were 59 abnormal results with Alere PIGF, 55 with Perkin Elmer PIGF and 22 with the Roche sFlt-1/PIGF ratio. All three tests were abnormal in 15% of women. There was good agreement between the two PIGF tests with an OPA of 89.3% (Table 3a). The agreement between the two PIGF tests and sFlt-1/PIGF ratio was considerably lower, 74.7% with Alere PIGF and 78.0% with Perkin Elmer PIGF (Tables 3b and 3c).

Case no.	Gestation (sampling)	Gestation (delivery)	PIGF pg/ml (Alere, Perkin Elmer)	sFlt-1/PIGF ratio (Roche)	Pre-eclampsia severity	Livebirth or IUFD	Customised BW centile
Isolated pre-eclampsia							
1	33+4	34+4	<12, 47	96.8	Moderate	Livebirth	0.8
2	34+3	35+3	90, 50.6	506.7	Moderate	Livebirth	2.4
3	33+5	35+5	<12, 64.3	138.3	Moderate	Livebirth	95.1
4	33+6	36+3	19.9, 81.6	50.1	Moderate	Livebirth	59.1
5	34+4	36+5	15.8, 118.7	95	Severe	Livebirth	36.5
6	34+2	37+0	62.6, 301.1	23.7	Mild	Livebirth	21.1
7	35+4	37+0	30.3, 101.8	47.1	Severe	Livebirth	2.1
8	33+1	37+0	63.3, 198.7	9.2	Moderate	Livebirth	50.7
9	35+4	37+1	15.1, 35.6	43.9	Moderate	Livebirth	2.7
10	33+0	37+1	1270, 1920.5	1.4	Mild	Livebirth	19.4
11	35+1	37+1	240, 326.8	4.1	Severe	Livebirth	68.5
12	35+0	37+2	170, 279.1	5.9	Moderate	Livebirth	98.5
13	33+0	37+6	1560, 1.7	1	Moderate	Livebirth	42.4
14	34+1	38+1	46, 129.7	17.7	Severe	Livebirth	8.5
15	34+0	38+1	12.5, 44.6	29.2	Moderate	Livebirth	70.1
16	34+0	38+3	137, 151.1	13.1	Moderate	Livebirth	54.7
17	33+3	38+5	34.3, 100.2	25	Moderate	Livebirth	13.4
18	34+6	39+0	<12, 70.2	149.2	Mild	Livebirth	65.7
19	35+4	39+4	319, 523.8	4.4	Moderate	Livebirth	38.2
20	34+1	39+4	146, 218	8.9	Severe	Livebirth	43.8
Pre-eclampsia with IUGR							
21	33+4	33+6	<12, 45.5	153.2	Severe	Livebirth	0.1
22	34+3	35+1	<12, 68.8	111.9	Severe	Livebirth	1.8
Isolated IUGR							
23	34+2	34+4	34.2, 180.1	23.5	NA	LB	0.9
24	33+4	36+4	141, 222	7.1	NA	LB	2.8
25	35+6	37+1	158, 182.2	7.9	NA	LB	1
26	33+0	38+5	13.5, 76.9	52.6	NA	IUFD	0.1

**Table 2:** PIGF, sFlt-1/Ratio results for all cases with adverse pregnancy outcome.

\*PIGF=placental growth factor, sFlt-1=FMS-like soluble tyrosine kinase-1, IUFD=intrauterine fetal death. BW=birthweight. Abnormal PLGF or sFlt-1/PLGF ratio results shown in bold.

a)

		PIGF (Perkin Elmer)		PPA 49/(49+6) = 89.1% NPA 85/(85+10) = 89.5%
		+	-	
PIGF (Alere)	+	49	10	
	-	6	85	
		PIGF (Alere)		PPA 49/(49+10) = 83.1% NPA 85/(85+6) = 93.4%
		+	-	
PIGF (Perkin Elmer)	+	49	6	
	-	10	85	
<b>OPA (49+85)/(49+85+6+10) = 89.3%</b>				

b)

		sFlt-1/PIGF ratio (Roche)		PPA 22/(22+0) = 100.0% NPA 95/(95+33) = 74.2%
		+	-	
PIGF (Perkin Elmer)	+	22	33	
	-	0	95	
		PIGF (Perkin Elmer)		PPA 22/(22+33) = 40.0% NPA 95/(95+0) = 100.0%
		+	-	
sFlt-1/PIGF ratio (Roche)	+	22	0	
	-	33	95	
<b>OPA (22+95)/(22+95+0+33) = 78.0%</b>				

c)

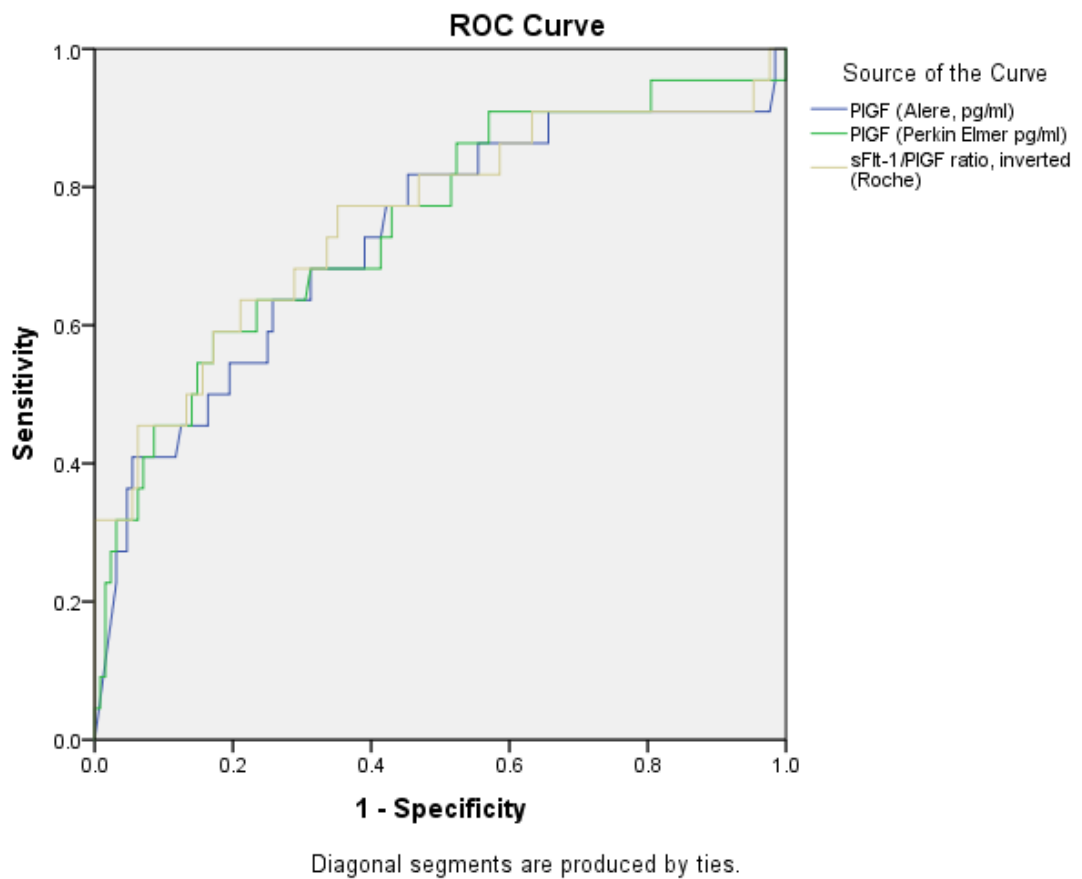
		sFlt-1/PIGF ratio (Roche)		PPA 22/(22+0) = 100.0% NPA 91/(91+37) = 71.1%
		+	-	
PIGF (Alere)	+	22	37	
	-	0	91	
		PIGF (Alere)		PPA 22/(22+37) = 37.3% NPA 91/(91+0) = 100.0%
		+	-	
sFlt-1/PIGF ratio (Roche)	+	22	0	
	-	37	91	
<b>OPA (22+91)/(22+91+0+37) = 74.7%</b>				

**Table 3a-c:** Calculation of positive (PPA), negative (NPA) and overall agreement (OPA) for placental growth factor (PIGF) tests and the FMS-like soluble tyrosine kinase-1 (sFlt-1)/PIGF ratio.

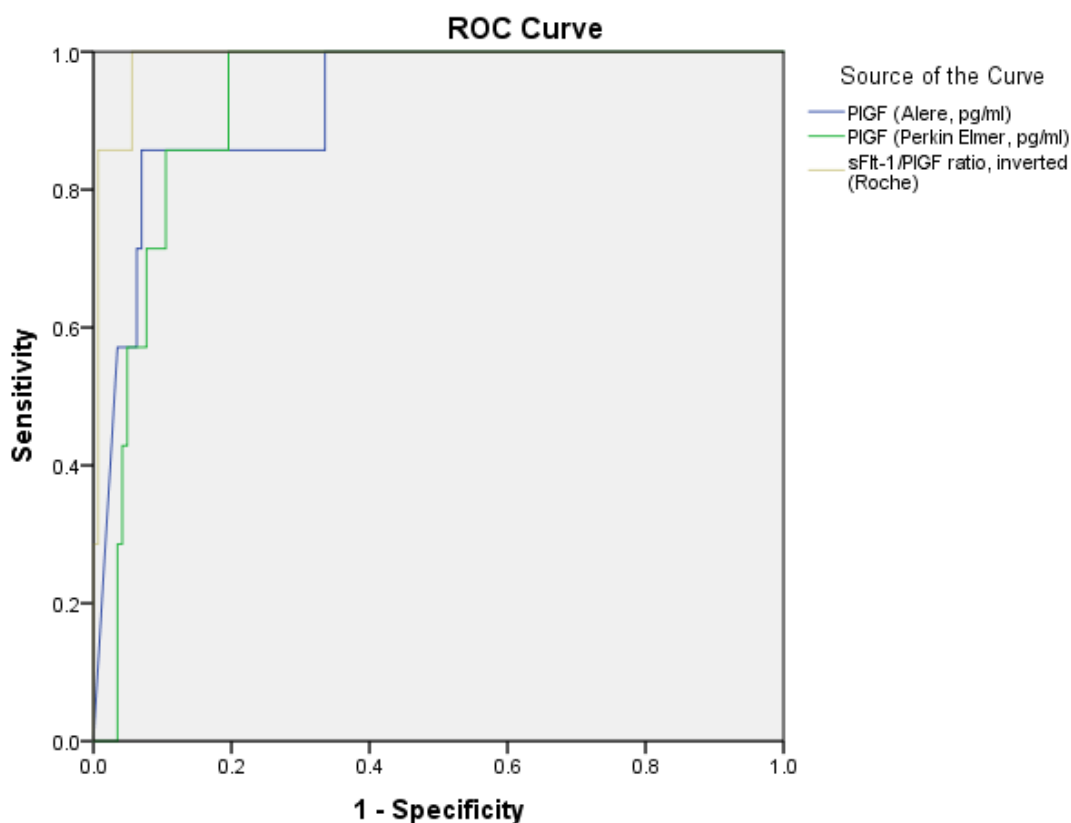
- a) Alere and Perkin Elmer PIGF tests.
- b) Perkin Elmer PIGF and sFlt-1/PIGF ratio.
- c) Alere PIGF and sFlt-1/PIGF ratio.

### 7.5 Results: Predictive accuracy for pre-eclampsia

All three tests have similar AUC for prediction of all pre-eclampsia ranging from 0.702 to 0.746 (Figure 2). In the subgroup that required delivery before 37 weeks (n=7) the two PIGF tests had identical AUCs (0.923) although the Perkin Elmer test had a somewhat narrower 95% CI (Perkin Elmer 0.869 to 0.977; Alere 0.838 to 1). The AUC for the sFlt-1/PIGF ratio was higher (0.988) with a narrow 95% CI of 0.97 to 1 (Figure 2).



**Figure 2:** Placental growth factor (PIGF) tests and FMS-like soluble tyrosine kinase-1 (sFlt-1)/PIGF ratio for prediction of 22 cases of pre-eclampsia of whom 7 needed delivery before 37 weeks.



Diagonal segments are produced by ties.

**Figure 3:** Placental growth factor (PIGF) tests and FMS-like soluble tyrosine kinase-1 (sFlt-1)/PIGF ratio for prediction of 7 cases of pre-eclampsia requiring delivery prior to 37 week's gestation.

**7.6 Results: Tests of association with maternal and fetal Doppler indices and response to aspirin**

149 of 150 participants had uterine artery Doppler assessments between 20<sup>+0</sup> and 23<sup>+6</sup> weeks. Twelve women (8%) had an abnormal PI of 1.45 or above. There were more abnormal PIGF or sFlt-1 ratio results when uterine artery Doppler PI was abnormal (75% versus 39%,  $\chi^2$  5.47 p=0.019).

149 of 150 participants provided sufficient sample volumes for platelet function testing and aspirin metabolite detection. We could not confirm aspirin adherence in 31 women (21%) and have excluded them from this part of the analysis. Five of 118 with confirmed adherence were deemed to be non-responsive and two of them had PIGF and/or sFlt-1/PIGF ratios indicating 'increased risk' of pre-eclampsia (40%). 48 of 113 (42.5%) aspirin

responsive participants had PIGF and/or sFlt-1/PIGF ratios indicating increased risk. There was no significant association between platelet response to aspirin and abnormal PIGF and/or sFlt-1/PIGF ratio results ( $\chi^2 0.12$   $p=0.913$ ).

## **7.7 Discussion**

### Main findings

Low circulating PIGF and elevated sFlt-1 reflect the pathophysiological process in pre-eclampsia, accordingly both plasma and serum PIGF and the sFlt-1/PIGF ratio have good diagnostic test accuracy in suspected pre-eclampsia (Chappell et al., 2013, Zeisler et al., 2016). Our findings suggest that these markers have utility for third trimester prediction of pre-eclampsia, particularly for cases prompting preterm delivery.

We found good agreement between the two PIGF tests. The agreement between PIGF tests and the sFlt-1/PLGF was limited, an effect driven by low PPA. This appears to be secondary to an overall lower number of abnormal results with the sFlt-1/PIGF ratio.

As expected, women with abnormal uterine artery Doppler were also more likely to have abnormal PIGF and/or sFlt-1/PLGF and vice versa. However, we failed to find such an association with individual response to aspirin.

### Strengths

Our prospective cohort provided an opportunity for side-by-side comparison of these tests in an unbiased, blinded manner. It was reassuring to find good overall agreement between the Alere and Perkin Elmer PIGF tests, despite tests using different media and having different parameters. Comparisons involving the sFlt-1/PIGF ratio, though perhaps offering a different perspective on the placental pathology, are more problematic. However, we also found the Roche sFlt-1/PIGF ratio had a higher AUC for prediction of preterm pre-eclampsia than other tests with slightly narrower 95% CIs. Further



assessment in sufficiently large cohorts would be required to draw firm conclusions about differences in performance between these tests.

To our knowledge, this is the first attempt to assess for associations between women's response to aspirin and markers of placental function known to be associated with placental disease. It is of central importance that any attempt to examine platelet response to aspirin takes account of aspirin adherence. Due to inherent biases of commonly used adherence assessments, such as questionnaire methods and pill-counting/weighing we opted to rely on detection of stable aspirin metabolites. Despite this, we could not demonstrate an association between response to aspirin and markers of placental function. Five participants in our cohort were denoted aspirin non-responsive and it is possible that a more minor association may be revealed in a larger cohort. It is probable that the protective effects of aspirin in high risk pregnancy are more complex than antiplatelet action alone. Our assessment did reveal 21% the cohort with both undetectable platelet response and metabolites which may reflect insufficient platelet exposure to aspirin due to suboptimal dosing and/or adherence issues. Pregnancy-specific aspirin pharmacokinetics and pharmacodynamics are poorly understood and current dosing is extrapolated from non-pregnant populations. Given the modest risk reduction attributable to the 75mg once daily dose currently endorsed in the UK (Askie et al., 2007, NICE, 2010a), there is an opportunity to improve knowledge in this area and optimise preventative treatment.

#### Limitations

We acknowledge that this study was not powered to assess the predictive accuracy of PIGF and sFlt-1/PIGF ratio tests for placental disease. An important limitation when comparing the individual predictive performance of the tests for each outcome is the relatively small number of cases represented (22 with pre-eclampsia, including 7 with

preterm pre-eclampsia). Subsequently, the upper and lower bounds of the 95% CIs for the tests overlap. From this analysis, we cannot state with certainty which test has the best clinical utility. It is reassuring that all three tests performed comparatively well for prediction of pre-eclampsia, which was consistently high for prediction of earlier onset disease. However, the narrower confidence intervals obtained with the sFlt-1/PIGF ratio, suggest there may be a measurable difference in test performance warranting further interrogation in an appropriately powered study.

### Interpretation

Most PIGF studies to date have focussed on prediction of pre-eclampsia, and associations with SGA and IUGR, in the absence of pre-eclampsia have not been assessed adequately. Studies have used a variety of customised and population-based birthweight centile cut-offs between the 3<sup>rd</sup> and 10<sup>th</sup> centiles, with, or without abnormal Doppler indices (Levine et al., 2004, Taylor et al., 2003, Romero et al., 2008). Some recent case-control studies have used more uniform definitions combining Hadlock estimated fetal weight <10<sup>th</sup> confirmed by birthweight <10<sup>th</sup> population centile with abnormal Doppler (Benton et al., 2012) (Herraiz et al., 2014, Gomez-Roig et al., 2015). These studies demonstrate 75-100% associations of IUGR by these definitions with abnormal PIGF or sFlt-1/PLGF ratio (Benton et al., 2012) (Herraiz et al., 2014) (Gomez-Roig et al., 2015). However, studies to date have been uniformly restricted by limited cases of isolated IUGR. Unfortunately, in our cohort there were only four cases of isolated IUGR and therefore we chose not to analyse predictive performance of the tests for this outcome. We were fully aware that defining IUGR by birthweight < 5<sup>th</sup> customised centile and abnormal uteroplacental Doppler would be very restrictive. However, we feel strongly that definitions of IUGR applied in clinical research must be stringent to be clinically meaningful.

## 7.8 Conclusions

There is good agreement between commercially available PIGF tests with more limited agreement between PIGF tests and sFlt-1/PIGF ratio. In women at high-risk of pre-eclampsia the PIGF tests assessed and the sFlt-1/PIGF ratio have good predictive accuracy for pre-eclampsia below 37 weeks both with and without IUGR. Within the limited cohort size and small outcome numbers in this study, all tests perform at adequate levels for these clinically important outcomes. Further side-by-side comparison in cohorts with higher numbers of well-defined outcomes may help define any subtle differences in test performance and place agreement between PIGF tests and the sFlt-1/PIGF ratio in context. There may also be some capacity for these tests to predict isolated 3<sup>rd</sup> trimester IUGR. However, assessment in appropriately powered studies with a strict definition of IUGR is vital.

## **CHAPTER 8: Discussion and conclusions**

## **8.1 Aims addressed**

The primary aims addressed by my thesis include investigating whether the aspirin non-responsiveness phenomenon exists in pregnant women at high risk of pre-eclampsia, assessing if platelet response to aspirin relates to adverse clinical outcomes and investigating if genetic factors may impact on individual response to aspirin. Overall, I aimed to identify any findings with a potential for stratification of women at risk of pre-eclampsia and/or with potential to optimise future preventative treatment.

When commencing this work we had identified early signals of aspirin non-responsiveness in a small number of observational studies in high risk obstetric populations. The issues in the existing cardiovascular literature afforded an opportunity to refine the clinical questions for obstetric populations, and design a focussed clinical study with which to address them.

## **8.2 Key findings**

Different terminology has been used to describe variable response to aspirin (Fitzgerald and Pirmohamed, 2011). I have opted to refer to aspirin non-responsiveness as opposed to resistance as the pharmacological evidence reported to date does not indicate a typical picture of drug resistance. Aspirin non-responsiveness is more reflective of lack of usual platelet effects of low-dose aspirin (LDA) and can also indicate lack of appropriate clinical response. I also elected to describe aspirin adherence, focussing on participant/patient involvement in decisions around medication taking, versus compliance with clinician-imposed therapy.

Examination of the existing body of evidence for aspirin non-responsiveness revealed key issues; miscellaneous platelet and clinical definitions and the use of numerous cyclooxygenase (COX)-specific and COX-independent 'diagnostic tests' (Chapter 2). These issues were compounded by limited evidence of adherence assessments both in studies

reporting on aspirin non-responsiveness, and randomised controlled trials (RCTs) assessing aspirin effectiveness. There was preponderance for subjective, qualitative and semi-quantitative methods where adherence had been assessed (Chapter 3). A wide range of incidences of aspirin responsiveness and conflicting evidence of clinical significance have been reported. These conflicting findings are to be expected as aspirin non-responsiveness, and associated terms, are currently umbrella terms covering variable root causes.

Initial work was undertaken to select platelet function tests targeting LDA's principal pharmacological action, inhibition of COX-1, and to establish relevant reference ranges accounting for the normal haematological changes of pregnancy. To our knowledge, pregnancy-specific reference ranges for platelet function with which to assess the pharmacodynamic actions of aspirin have not previously been reported or applied (Chapter 1). Additionally, non-invasive, exact methods to detect LDA metabolites were developed. We were able to accurately detect aspirin's stable plasma and urine metabolites and describe a reliable strategy to assess LDA adherence, which was successfully incorporated in the EARTH study. From our experience, collection, storage and analysis of appropriate samples for this purpose can be seamlessly integrated into clinical study protocols. Dose-to-detection intervals are limited only by the pharmacokinetics of LDA. Improved accuracy of detection is feasible by manipulating either the timing of doses or sampling. A strategy of using exact assessments, augmented with qualitative data would be suitable for use by other clinical researchers to improve the reliability of adherence information and the accuracy of future evaluations of the clinical effectiveness of LDA (Chapter 4).

We also conducted longitudinal assessments of LDA's effects on platelets. In contrast to other authors, we found reasonable agreement between platelet function tests, between

72% and 85% overall, across gestational age time-points (Chapter 5). This level of agreement applies to all tests targeting COX-related platelet function. Additionally, for the majority of women with confirmed exposure to LDA there was good evidence of the expected pharmacodynamic effects of LDA (Chapter 5). In contrast to other studies, both in obstetrics and more broadly, we found no evidence of sustained aspirin non-responsiveness in any individual (Chapter 5). I believe three factors have led to this conclusion: i) the greater selectivity of the platelet function tests used in EARTH for the effects of LDA, ii) analysis of the cohort accounting for aspirin adherence, iii) the benefits of longitudinal testing. For EARTH, if aspirin non-responsiveness had been defined on the basis of lack of an expected platelet response alone, the incidence of aspirin non-responsiveness would be 15% (n=23). This incidence is comparable to the findings of other authors. If response to aspirin had been considered separately at the first and third visits, after exclusion of the subgroup sampled following a single LDA, the prevalence of aspirin non-responsiveness would be 4% and 3%, respectively.

We have already considered the significance of consistent indeterminate response to aspirin in relation to the pharmacokinetics of LDA and metabolite detection. The unexpected outcome in the EARTH cohort was the high degree of variable response to aspirin. Thirty-four percent of the cohort switched between aspirin response groups throughout their pregnancies (Chapter 5). This variable response to aspirin throughout pregnancy, highlighted in the EARTH cohort, is important. The largest individual changes in aspirin response were observed in participants switching from non-responsive or indeterminate groups to the responsive group in the third trimester (18%, n=28). However, there was also appreciable movement in the opposite direction (9%, n=14). These variable responses suggest the main issues centre around aspirin adherence and/or suboptimal dosing.

In the EARTH cohort, individual response to aspirin was assessed against biomarkers and ultrasound markers for placental function and against clinical outcomes, chosen for their reflection of clinically important placental disease. The most striking findings, addressing the primary aim of this research, were the lack of any significant associations between aspirin response status and markers of placental function or the specified clinical outcomes; pre-eclampsia occurring at any gestation or composite placentally-mediated adverse outcome (Chapter 5). Additionally, an unbiased genome-wide assessment of genetic variants and response to aspirin revealed no genome-wide significant or suggestive associations. These findings support the notion that other intrinsic factors, such as physiological or disease characteristics affecting the required dose or extrinsic factors, principally adherence are more influential on aspirin response (Chapter 6).

### **8.3 Implications for research**

#### **Aspirin non-responsiveness**

The conclusion that when aspirin non-responsiveness is stringently defined, no cases were detected restricts the value of any further investigations of platelet response to low-dose aspirin. Whilst it remains possible that individuals who are genuinely non-responsive to aspirin may be found in larger cohorts, the lack of any cases in a cohort of 156 individuals assessed throughout pregnancy suggests that the numbers would be too small as a meaningful research cohort.

#### **Aspirin dosing and adherence**

Consideration of suboptimal adherence as the fundamental cause of a perceived lack of platelet response and misidentification of aspirin non-responsiveness has been ventured by other authors, and the findings within the EARTH cohort support this.

Acceptability of and adherence to any proposed treatment are over-arching issues that should be fully considered in future evaluations. Accordingly, assessment of aspirin



adherence must factor in evaluations of the effectiveness of LDA to reduce the risk of pre-eclampsia and other placentally-mediated complications. At present, a large body of evidence demonstrates inadequate aspirin adherence data for trial participants. This is of particular relevance considering the modest, 10%, risk reduction in pre-eclampsia attributed to current use of LDA following IPD meta-analysis (Askie et al., 2007). Inclusion of adherence assessments in trial protocols would facilitate stratification according to adherence status and allow re-evaluation of LDA to mediate risk in adherent individuals, both in individual trials and future meta-analyses.

There is a very limited pharmacokinetic evidence-base to support alterations in aspirin dosing during pregnancy. However, there is some evidence of decreased rate of absorption and increased distribution of the drug which align with pregnancy-induced physiological changes to the gastrointestinal and circulatory system, specific to the 75mg dispersible dose in current use in the UK (Chapter 1) (Rymark et al., 1994, NICE, 2010a). Subgroup analyses of trial data suggested a potential inverse association between actual aspirin dose and reduction in the risk of pre-eclampsia (NICE, 2010a, Duley et al., 2007). A recent meta-analysis, assessing doses ranging from 50 to 150mg, stratified by gestation of commencing LDA (prior to and from 16 weeks), renewed the argument to re-consider aspirin dosing (Roberge et al., 2017). The ASpirin for evidence-based PREeclampsia prevention (ASPREE) RCT has since demonstrated reduction in the risk of pre-eclampsia of 80% and 60%, prior to 32 and 37 weeks, respectively, with 150mg aspirin, compared to placebo (O'Gorman et al., 2016, CORDIS, 2017, Rolnik et al., 2017). The authors also endeavoured to assess aspirin adherence with both qualitative and semi-quantitative measures (verbal enquiries, participant diaries and pill counting) (O'Gorman et al., 2016, CORDIS, 2017).

Several characteristics of ASPRE may have influenced the commanding findings and warrant further scrutiny. There are key differences in the methods used to identify high risk women in the ASPRE cohort, as opposed to National Institute for Health and Care Excellence (NICE) risk factor-based screening, with evidence to suggest the superiority of the algorithm used in ASPRE (O'Gorman et al., 2016, O'Gorman et al., 2017). There was a high-level of aspirin adherence in ASPRE with 79.9% of participants reported to have taken 85% or more of their allocated aspirin doses, with no significant differences in adherence status between groups (Rolnik et al., 2017). These findings indicate a highly motivated, adherent population. Due to the previous signals of superior risk reduction in pre-eclampsia when aspirin is commenced prior to 16 weeks (Roberge et al., 2017), ASPRE participants commenced aspirin between 11 and 14 weeks (Rolnik et al., 2017). Similarly, due to a previous report that ingesting LDA eight hours or more after waking improves both ambulatory blood pressure and reduces the risk of hypertensive complications ASPRE specified evening dosing (Ayala et al 2013).

Subsequent studies should therefore aim to delineate the effects of gestation at which aspirin is commenced, dose/doses and their timing and the impact of aspirin adherence. There will be important opportunities in light of ASPRE to build-in detailed assessments of aspirin pharmacokinetics, pharmacodynamics and forgiveness at different doses in addition to strengthening data on short and long-term safety of aspirin at different doses. Whilst pharmacokinetic data are likely to be of use in determining the optimal safe and effective dose, knowledge of forgiveness may be utilised to outline both optimal and minimal adherence to ensure the intended pharmacodynamic and clinical effects. These pharmacokinetic and pharmacodynamic components may be embedded into clinical trial protocols but must be planned from the outset and written into funding applications. It is vital that such assessments are conducted in collaboration with pharmacology experts. More broadly, building clinical pharmacology into obstetric research in these ways is vital

to develop our currently limited pharmacological evidence-base in pregnancy and to progress our use of many drugs in pregnancy.

## **Genetic factors**

No plausible associations between any single nucleotide polymorphisms (SNPs) and response to aspirin support a clinically non-significant contribution of genetic factors in determining individual response to aspirin. These findings also support the assertions that, though genetic factors may still emerge, these are likely to be over-shadowed by other intrinsic and extrinsic contributions, which may include aspirin adherence and dosing. We plan to strengthen our genotyped data by two means. First, we plan to validate our data with a similar cohort of aspirin-treated pregnant women with matched phenotyping. We also plan to impute the complete data-set to improve SNP coverage within our cohorts to enable comparison of our findings to those of previous and future studies. An appropriate validation cohort has already been identified and we have collaborated for the past two years, to ensure matched phenotyping. We have now received maternal buffycoat samples from our validation cohort, from which to obtain maternal DNA for a genome-wide association study (GWAS).

Our initial intention was to conduct a case: control design GWAS examining SNPs associated with aspirin non-responsiveness in parallel with any future clinical study or trial assessing preventative treatments in aspirin non-responsive individuals. In light of the lack of aspirin non-responsiveness and lack of association between aspirin response status and adverse clinical outcomes, this would not be meaningful.

Pre-eclampsia is known to have significant heritability and a plethora of genetic factors have been ventured in relation to pre-eclampsia and placental disease. However, GWAS, as relatively new and constantly evolving technology has not been applied often to date, both in pre-eclampsia research and obstetrics as a whole. Two groups have undertaken GWAS of the association of variants with pre-eclampsia (Zhao et al., 2013, Zhao et al., 2012, Johnson et al., 2012). In 2012, the first GWAS examined deletions in Caucasian 177

cases and 116 controls (Zhao et al., 2012). Whilst no genome-wide significant SNPs were identified, enrichment for a deletion in the region of the Pregnancy-Specific Beta-1-Glycoprotein 11 (PGS11, chromosome 19) gene was observed in cases (Zhao et al., 2012). In a subsequent GWAS by the same group, utilising samples from the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study, groups of Afro-Caribbean, Hispanic and participants of European ancestry were assessed in a case-control study (133 cases versus 2917 controls) (Zhao et al., 2013). Again no genome-wide significant SNPs were detected, with no consistency between ethnic groups and lack of replication in an independent cohort (Zhao et al., 2013). A further case-control GWAS identified three genome-wide significant SNPs, in linkage disequilibrium close to the Inhibin beta subunit (INHBB, chromosome 2) gene (Johnson et al., 2012). However, they noted low sequence conservation in this region, indicative of low functional importance and failed to replicate their findings in a subsequent assessment (Johnson et al., 2012).

Whilst no replicated variants have yet been described, there is scope to examine these early signals in pre-eclampsia disease genomics. It is justified to conduct a large-scale case: control GWAS of pre-eclampsia disease genetics, utilising biobanked samples. The recent Screening for Pregnancy Endpoints (SCOPE) and Improved Pregnancy outcomes via early detection (IMPROVED) cohorts would be a valuable source of well-phenotyped individuals and rich meta-data for this purpose. With the availability of imputation, improved genomic coverage and the ability of modern arrays to include rare variants (MAF <1%), there is potential for meaningful findings. Additionally, variants may be further examined with high-throughput sequencing, or use of combined methodologies, embarked on by other groups to assess variant interactions, and functional significance. Projected sample sizes for case-control GWAS with differing MAF, power and alpha error parameters are outlined in Table 1. At low-moderate MAF levels detectable with a range of standard arrays used for GWAS (MAF of 0.03-0.05), with MAF in cases versus controls

of 2-3 times, appropriate numbers of cases and controls could be sought from existing cohorts for assessments with both 90% and 80% power and alpha error as low as 1% (Table 1). Similarly, if the UK Biobank Axiom array were to be used to examine rare variants (MAF 0.01), with MAF in cases versus controls of 4-5 times, sample sizes could be secured for assessments with both 90% and 80% power with alpha error to 1% (Table 1).

MAF in cases (0-1.0)	MAF in controls (0-1.0)	Power/ alpha error (%)/(%)	Sample size required (cases)	Sample size required (controls)
0.07	0.05	90/1	2718	8154
		80/5	1427	4281
0.05	0.01	90/1	311	933
		80/5	161	483
0.1	0.03	90/1	220	660
		80/5	114	342
0.1	0.01	90/1	105	315
		80/5	54	162
0.15	0.03	90/1	97	291
		80/5	51	153
0.15	0.01	90/1	60	180
		80/5	31	93

**Table 1:** Sample sizes required for case-control GWAS to assess pre-eclampsia disease genomics for different MAFs, power and alpha error, where ratio of cases to controls is 1:3. \*Power indicates the probability of detecting a real effect. Alpha error indicates the probability of incorrectly rejecting the null hypothesis

#### 8.4 Implications for clinical practice

The clinical significance of platelet response to aspirin is questionable. Based on this evidence, use of platelet response to aspirin as a marker of individual risk is not justified and there is currently no evidence to support the concept of testing for aspirin non-responsiveness in clinical obstetric practice.

With the existing safety record of LDA and recent findings regarding the effects of increased dose (Askie et al., 2007, ACOG, 2013, Rolnik et al., 2017, Duley et al., 2007), a first-line approach to re-assess and optimise the use of this drug in pregnancy is justified prior to further pursuit of alternative preventatives.

The discovery of high variability in the response to aspirin which may be caused through differences in adherence does have clinical relevance. Pregnant women at high risk of pre-eclampsia should receive an appropriate explanation of the evidence for aspirin in mediating their personal level of risk and be provided with the available information about the safety of aspirin for themselves and their fetuses, to support adherence. Discussions about adherence should be included in subsequent antenatal visits and specific concerns with aspirin-taking and adverse effects addressed as they arise. As evidence around alternative dosing and adherence emerges, it may also be possible to offer information about the impact of levels of adherence on risk reduction.

With the evolution of precision medicine the aim will be to use genomic testing for screening, monitoring of at risk individuals and diagnostics. Efforts are already underway to embed genomics into the NHS and to explore pre-emptive genotyping in clinical care (GE, 2017, Weitzel et al., 2016). There is significant scope to progress genomic information into the clinical environment for preventative, surveillance and therapeutic approaches in pregnancy. In the context of pre-eclampsia disease genomics, knowledge of risk variants may enable development of panels for screening. With the existing wealth of experience in combination models for pre-eclampsia screening and current interest in other omics and functional biomarkers a systems biology approach to this complex pathology may prove advantageous. There is already significant experience with pharmacogenomics in adverse drug reactions in non-pregnant populations which have had direct clinical applicability (Alfirevic and Pirmohamed, 2017). Despite the rise in genomic information on drug labels, only one finding, relating to codeine use, has had specific relevance for pregnant women (Chapter 1,) (FDA, 2007, EMA, 2013). There is currently an unexplored field of drug efficacy and safety in pregnancy, investment in which may aid understanding of pathological mechanisms and support safe prescribing.

## **8.5 Final conclusions**

When adherence is confirmed and platelet function assessed with tests reflecting LDA's impact on platelets throughout pregnancy, aspirin non-responsiveness was not identified. We could not demonstrate any link between platelet response to aspirin, markers of placental function, genetic factors or adverse clinical outcomes. We doubt that there is any merit in repeating studies to detect aspirin non-responsiveness in women at risk of pre-eclampsia. With no cases in 156 well-characterised individuals, true aspirin non-responsiveness at best is rare and not of significant magnitude to produce clinically important detriments or to be valuable for screening. Variable aspirin response strongly suggests that aspirin adherence may be a key determinant of LDA effectiveness. Aspirin adherence should be assessed in future trials to allow appropriate stratification of cohorts. Variable response may also indicate suboptimal dosing, which aligns with recent findings from the ASPRE study. There is now an excellent opportunity, in parallel with further assessments of alternative doses of aspirin, to deepen the pregnancy-specific pharmacokinetic and pharmacodynamic knowledge-base. Additionally, advances in technology available for genomics and the availability of biobanked maternal DNA from large well-phenotyped cohorts provides a strong foundation for robust pre-eclampsia disease genomics.



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## **Appendices**

### Cardiology RCTs reporting use of aspirin adherence assessments

Reference	Number of participants	Method	Definition of appropriate adherence	Number/proportion of non-adherent participants
Ikeda, Y et al 2014	14464	Self-reporting at follow-up contact	Not pre-specified	88.9% of patients reported that they were adherent in year 1; this decreased to 76.0% in year 5.
Mahaffey, K et al 2014	12515	Verbal enquiries at each follow-up visit	Not pre-specified	Not reported
Devereaux, PJ et al 2014	10010	Method not specified	Not pre-specified	80.4% of participants in the aspirin group and 82.4% in the placebo group took at least 80% of doses.
Weimar, C et al 2013	7212	Verbal enquiries at each follow-up visit	Not pre-specified	28.2% non-adherence
Lee, C et al 2014	5045	Verbal enquiries at each follow-up visit. If any antiplatelet medication had been discontinued, an attempt was made to determine the specific timing of discontinuation. If there was uncertainty about the timing, the referring cardiologist or general practitioner was contacted for additional information.	Not pre-specified	Adherence in the aspirin-alone group was 98.2 % (2361 of 2405) and 97.2 % (1975 of 2032) at 12 and 24 months, respectively. For the dual-therapy group, these adherence rates were 88.6% (2157 of 2435) and 79.4% (1625 of 2046), respectively.
Feres, F et al 2013	3119	Not specified	Not pre-specified	Adherence rate for aspirin at 90 days was 99.3%, for the short-term group vs 99.5% for the long-term group. At 1-year clinical follow-up, the rate of aspirin use was 98.9%, in the short-term group and 98.8% in the long-term group.

Dangas, G et al 2013	2997	Interviewed at follow-up visits	Classified as; 1) on DAPT during complete follow-up 2) off dual antiplatelet therapy (DAPT) during complete follow-up 3) not faithfully compliant/unclear 'irregular use'	Within 30 days 12/74 16.2%, confirmed discontinuation. Non-adherence to DAPT varies over time. It is strong within the first month, remains important until six-month follow-up but fades afterwards.
Castellano, JM et al 2014	2118	Morisky-Green medication adherence questionnaire (MAQ). Pill counts also performed.	MAQ score $\geq 16$ identifies good adherence. A pill count between 80% and 110% was considered good adherence. Patients were considered to be "adherent" if they achieved good adherence scores with both methods—pill count and MAQ.	Baseline adherence via MAQ->90% of patients had a score $>16$ , 45.5% score=20. Adherence with MAQ and pill counts was control-141 (41%) polypill-178 (50.8%) p value 0.019
Gwon, HC et al 2012	1443	Verbal enquiries at each follow-up visit	Not pre-specified	Adherence to the study protocol was 71.2% of the 6-month DAPT group and 93.2% of the 12-month DAPT group at 12 months.
Hermanides, RS et al 2012	1309	Observed dosing	Not pre-specified	Not reported
Suh, JW et al 2011	960	method not explicitly stated	Not pre-specified	Not reported
Borghi, C et al 2012.	771	Compliance checked at each study visit' verbal enquiry	Subjects taking $\geq 75\%$ of drug dose	Treatment adherence was high and comparable between groups (ASA zofenopril group 97%, ASA ramipril group 96%).
Youn, YJ et al 2014	630	Face-face or telephone enquiries at each follow-up	Not pre-specified	Not reported
Lee, S et al 2011	499	Questionnaires at follow-up visits	Not pre-specified	75 participants were excluded due to drug discontinuation. Study drug discontinuation for adverse events and other reasons was more common in the triple versus the dual group (18.8% vs. 12.2%, p

				= 0.02), and most study drug discontinuations occurred within 3 months after randomization.
Stone, GW et al 2011	243	Not specified	Not pre-specified	JOSTET group aspirin use at discharge was 98.3%, 8 months 93.5%, 9 months 93.5%. Bare metal stent group, aspirin use at discharge was 100%, 8 months 88.7%, 9 months 87.1%.
Campo, G et al 2014	224	verbal enquiries at each follow-up visit	Not pre-specified	Adherence >95% in the long DAPT regimen and >97% in the short DAPT regimen). At 6 months, the use of aspirin plus clopidogrel was 95% in the short DAPT regimen versus 98% in the long DAPT regimen (p = 0.7). A 1 year, it was 15% versus 97% (p < 0.01), whereas after 18 months it was 2% versus 96% (p < 0.01), respectively.
Park, JB et al 2013	127	Questionnaire (by telephone or face-face)	<80% planned doses (listed as protocol violation)	Test group, 98.2%; control group, 98.9%; P = 0.272) both groups included aspirin in intervention.
Kim, IS et al 2011	126	Interview, pill count, survey at 30-day follow-up visit	Complete compliance (100%)	All patients tolerated therapy well and did not discontinue the study regimens.
Basili, S et al 2014	91	Verbal questioning prior to enrolment	Participants confirmed taking 100md/day aspirin for ≥7 days pre-PCI	Not reported
Yang, TH et al 2013	40	Not specified	Not pre-specified	Not reported

## Obstetric RCTs reporting use of aspirin adherence assessments

Reference	Adherence assessment	Method	Definition of appropriate adherence	Number/proportion of non-adherent participants
Golding, JA et al 1998	Yes	Monitored for compliance at follow-up visits	Not pre-specified	1904 /3023 in the aspirin group deemed adherent.35 stopped aspirin due to adverse effects. (Analysed in compliant, non-compliant subgroups).
Rotchell, YE et al 1998	Yes	Asked the last known date of taking pills at postnatal visit	Not pre-specified	Not reported
Caritis, S et al 1998	Yes	Pill counts (adherence assessed prior to enrolment), and at follow-up visits with direct questioning.	Deemed adherent if had ingested great than/equal to 50% of pills from a 10 placebo pill pack (5 pills or more/7 days) prior to enrolment.	Median tablets taken, 100 (aspirin group), 99 (placebo group). 93% of all women took half or more of their pills, 9% took at least 80%.Incidence of preeclampsia did not differ significantly between the groups.
Harrington, K et al 2000	Yes	Aspirin packets checked at follow-up visits	Not pre-specified	The adherence rate for aspirin was 97.2%, with a mean of 3.1 tablets missed per patient during the duration of treatment.
Anonymous. ECPA 1996	Yes	Random sample of cohort interviewed (88 women). Dates of stopping aspirin recorded in post-delivery follow-up forms.	Not pre-specified	88% of a sample of 88 women confirmed they had taken >75% of scheduled tablets. Date of stopping aspirin recorded for 967 women, 90% stopped after 75% of time randomisation-delivery completed, 69% stopped after 95% of this time.
Yu, CKH et al 2003	Yes	Telephone calls and random pill counts at follow-up visits.	Not pre-specified	24 discontinued aspirin. Percentage tablets taken 95, 80-100 (median, 1QR).
Rogers, MS et al 1999	Yes	Remaining drugs assessed at follow-up visits	Not pre-specified	All patients completing follow-up (90%) deemed adherent, 10% delivered elsewhere, therefore no data.
Ayala, DE et al 2013	Yes	Pill Counts	Max. expected no. of 175 tablets ingested (medication for 25 weeks)	Average tablets ingested, 171 (aspirin groups) 167 (placebo group). No observed differences in groups prescribed different times of day.



Health and Life Sciences Committee on Research Ethics (Human participants, tissues and databases)

17 January 2017

Dear Dr Navaratnam,

I am pleased to inform you that your application for research ethics approval has been approved. Details and conditions of the approval can be found below:

Reference: 1138  
Project Title: Aspirin  
breakdown products in healthy women  
Principal Investigator/Supervisor: Dr Kate Navaratnam  
Co-Investigator(s): Dr Marie Phelan  
Lead Student Investigator: -  
Department: Women's and Children's Health  
Reviewers: Dr Catrin Eames, Dr Mal Horsburgh  
Approval Date: 17/01/2017  
Approval Expiry Date: Five years from the approval date listed above

The application was **APPROVED** subject to the following conditions:

**Conditions**

- All serious adverse events must be reported via the Research Integrity and Ethics Team ([ethics@liverpool.ac.uk](mailto:ethics@liverpool.ac.uk)) within 24 hours of their occurrence.
- If you wish to extend the duration of the study beyond the research ethics approval expiry date listed above, a new application should be submitted.
- If you wish to make an amendment to the research, please create and submit an amendment form using the research ethics system.
- If the named Principal Investigator or Supervisor leaves the employment of the University during the course of this approval, the approval will lapse. Therefore it will be necessary to create and submit an amendment form using the research ethics system.
- It is the responsibility of the Principal Investigator/Supervisor to inform all the investigators of the terms of the approval.

Kind regards,

Health and Life Sciences Committee on Research Ethics (Human participants, tissues and databases)

edreseth@liverpool.ac.uk

0151 795 4358



## **AMV Study**

### **Aspirin metabolites in healthy female volunteers**

Version 1.0 01/11/16

MAIN SPONSOR: University of Liverpool

FUNDERS: Wellbeing of Women

STUDY COORDINATION CENTRE: University of Liverpool

REC reference: TBC

#### **Study Team**

Chief Investigator: Dr Kate Navaratnam

Co-Investigator: Dr Marie Phelan



### **Study Coordination Centre**

For general queries, supply of Study documentation, and collection of data, please contact:  
Study Coordinator: Dr Kate Navaratnam

Address: First floor, Centre for Women's Health Research, Crown Street, Liverpool, L8 7SS.

Telephone: 0151 795 9567

Email: [Kate.Navaratnam@liv.ac.uk](mailto:Kate.Navaratnam@liv.ac.uk)

### **Clinical Queries**

Clinical queries should be directed to Dr Kate Navaratnam

### **Sponsor**

The University of Liverpool is the research Sponsor for this Study. For further information regarding the sponsorship conditions, please contact:

Alex Astor

Head of Research Support – Health and Life Sciences

University of Liverpool

Research Support Office

2nd Floor Block D Waterhouse Building

3 Brownlow Street

Liverpool L69 3GL

[sponsor@liv.ac.uk](mailto:sponsor@liv.ac.uk) <mailto:Astor@liv.ac.uk>

### **Funder**

Wellbeing of Women (RC), London, UK

## STUDY SUMMARY

This protocol describes the AMV Study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the Study. Problems relating to this Study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

## GLOSSARY OF ABBREVIATIONS

HRA	Health Research Authority
REC	Research Ethics Committee
AMV study	Aspirin Metabolites in healthy female Volunteers study
ASA	Acetyl salicylic acid, aspirin
SUA	Salicyluric acid
GA	Gentisic acid
SA	Salicylic acid
NSAID	Non-steroidal anti-inflammatory drug
NMR	Nuclear Magnetic Resonance
NICE	National Institute of Clinical Excellence
EARTH study	Estimating Aspirin ResisTance in High-risk women study
AE	Adverse event
SAE	Serious adverse event
TSP	Selectively deuterated trimethylsilyl propionate

**KEYWORDS:** Aspirin, adherence, metabolites, women, female, urine.

**TITLE:** Aspirin metabolites in healthy female volunteers (AMV Study).

**DESIGN:** Urine sample collection from healthy volunteers.

**AIMS:** To allow accurate nuclear magnetic resonance detection of aspirin's urinary metabolites and identify dose-urine sampling intervals for low-dose aspirin to. This will allow development of a reliable non-invasive aspirin adherence test.

**POPULATION ELIGIBILITY:** 30 healthy non-pregnant women aged 18-45 years, not currently taking aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) with no allergies or adverse reactions related to NSAID use.

**OUTCOME MEASURES:** Detection of aspirin's principal urinary metabolite, salicyluric acid (SUA) and minor urinary metabolites; gentisic acid (GA) and salicylic acid (SA). Construction of dose to urine sampling time-course curves for aspirin metabolite detection.

**DURATION:** 3 months (12 weeks)

## **1. INTRODUCTION**

### **1.1 BACKGROUND**

Pre-eclampsia is a serious multi-system disorder of pregnancy which adversely affects maternal endothelium and placental function. 2-8% of low-risk, healthy pregnant women will develop pre-eclampsia and 15% of women at high-risk making it a consistent top three cause of maternal morbidity and mortality worldwide<sup>1</sup>. Low-dose aspirin/acetylsalicylic acid (ASA, 75mg once daily) is advocated by the National Institute of Clinical Excellence (NICE) for women at high-risk of pre-eclampsia as it reduces the risk by 10%<sup>1,2</sup>.

Aspirin resistance, insufficient suppression of platelet activity could begin explain why a significant proportion of high-risk pregnant women develop pre-eclampsia despite treatment with low-dose aspirin<sup>3,4</sup>. However, the most cited cause of aspirin resistance identified in clinical studies to date is suboptimal aspirin adherence<sup>5,6</sup>. Suboptimal adherence tends to increase with polypharmacy and is widespread though difficult to accurately quantify. Very little is known regarding adherence to aspirin in pregnant women prescribed the drug to reduce the risk of pre-eclampsia. Randomised trials evaluating aspirin effectiveness both in non-pregnant and pregnant populations show over-reliance on qualitative assessments of adherence (enquiries, questionnaires) with inherent vulnerabilities in accuracy and semi-quantitative measures (medication pill counting and weighing) with the risk of tampering<sup>7,8</sup>. Quantitative assessments of adherents via detection of drug levels and/or drug metabolites may provide useful adherence information, but require further development.

### **1.2 RATIONALE FOR CURRENT STUDY**

We recently conducted a prospective cohort study Estimating Aspirin ResisTance in High Risk women (EARTH) study , during which we recruited 180 women at high-risk of pre-eclampsia prescribed aspirin, assessed longitudinal platelet activation and developed the method for detection of urinary aspirin metabolites.

Women in the EARTH study took aspirin 75mg once daily, at varying individual times according to their own schedule and preferences. Subsequently, urine samples provided for NMR detection of urinary metabolites cover broad dose-sample intervals of 1-24 hours.

The purpose of the aspirin metabolites in healthy female volunteers' (AMV) study is to identify reliable dose to urine sample collection intervals that will allow reliable detection of aspirin's urinary metabolites using NMR methods. This will aid with interpretation of EARTH Study data with conclusions regarding aspirin adherence in the cohort. The AMV study will allow more detailed understanding of this novel quantitative method to begin integrating it into future clinical research protocols as a measure of aspirin adherence.

## **2. STUDY OBJECTIVES**

- We aim to detect aspirin's principal urinary metabolite (SUA) and minor urinary metabolites (GA, SA) in volunteer urine using Nuclear Magnetic Resonance (NMR) Spectroscopy.
- We will identify dose-urine sampling intervals for low-dose aspirin to allow accurate nuclear magnetic resonance detection of aspirin's urinary metabolites.
- We aim to use this information as the basis to develop a reliable non-invasive aspirin adherence test.

### **3. STUDY DESIGN AND SAMPLE SIZE CALCULATION**

During this study we will collect urine samples only. We plan to recruit 30 healthy non-pregnant women aged 18-45 years will be recruited. (Please refer to section 6 for further information). It is necessary to recruit 30 women as 20 individuals are required to produce informative time-course curves and enable us to specify 95% confidence intervals for the earliest and latest time-points to detect aspirin's metabolites in urine samples. From previous experience analysing the EARTH study urine samples using these techniques, recruiting 30 women will account for women choosing to withdraw, not being able to provide all specified samples and potential technical problems that arise from samples. Technical issues may include sample contamination and baseline noise that mean spectra are less reliable for interpretation.

Duration: 3 months (12 weeks) from recruitment of the first participant.

#### **3.1 STUDY OUTCOME MEASURES**

- Identification of aspirin's principal urinary metabolite (SUA) and minor urinary metabolites (GA, SA) in volunteers' urinary NMR spectra.
- Construction of accurate aspirin dose to urine sampling intervals for detection of aspirin's urinary metabolites.

### **4. PARTICIPANT ENTRY**

We will circulate a poster via University staff and student email and display this in the Centre for Women's Health Research and NMR Centre to raise awareness of the AMV study. The posters will contain contact details for the researchers to be approached by women interested in taking part. The researchers will have a discussion with interested women about what taking part involves and will check their eligibility. Eligible women will receive a participant information leaflet and will have time to consider this and ask any questions that arise prior to deciding to take part.

#### **4.1 INCLUSION CRITERIA**

- Female
- Not pregnant
- Aged 18-45 years old
- Has not reached menopause (has a menstrual cycle)
- BMI less than 30
- No chronic medical conditions
- Not taking aspirin, NSAIDS, steroids, anticoagulants

- Has previously taken aspirin and other NSAIDS with no adverse reactions or allergies

#### **4.2 EXCLUSION CRITERIA**

- Less than 18 years old
- Greater than 45 years old
- Pregnant
- BMI of 30 or greater
- Pre-existing medical conditions including; asthma, diabetes, high blood pressure, autoimmune diseases, inflammatory bowel disease, gastritis, gastro-oesophageal reflux disease.
- Already taking aspirin, other antiplatelet agents (e.g. dipyridamole), NSAIDS, steroids (including prednisolone and steroid inhalers) or taking any anticoagulant medication.
- Any known adverse reactions or allergies to Aspirin or other NSAIDS.

#### **4.3 WITHDRAWAL CRITERIA**

Participants may opt not to continue with the study at any time during collection of samples or prior to anonymisation of samples on receipt in the NMR Centre. It will not be possible to withdraw samples from the study following anonymisation. This process will be discussed prior to enrolment and is detailed in the AMV study consent form.

The participant will be withdrawn from the study if they experience any AEs or SAEs (detailed in section 5.1). In the event of symptom occurring participants are advised to seek medical advice, and to use their judgement depending on severity to seek this advice appropriately, either from their GP or accident and emergency department. Participants are also requested to contact Dr Kate Navaratnam (mobile tel. no provided within participant information leaflet) as soon as feasible. This process will be discussed at enrolment and is detailed, along with contact information in the participant information leaflet.

### **5. ADVERSE EVENTS**

#### **5.1 DEFINITIONS**

**Adverse Event (AE):** any untoward medical occurrence in a patient or clinical study subject.

Suspected AEs to aspirin, including, but not limited to; rash, mucosal irritation, dyspepsia

Medical judgement will be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, will also be considered serious.

**Serious Adverse Event (SAE):** any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening** – refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Suspected SAEs due to aspirin, including, but not limited to; anaphylaxis, bronchospasm, bleeding from the gastrointestinal tract

## **5.2 REPORTING PROCEDURES**

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator, Dr Kate Navaratnam, in the first instance.

### **5.2.1 Non serious AEs**

All such events, whether expected or not, should be recorded.

### **5.2.2 Serious AEs**

An SAE form should be completed and faxed to the Chief Investigator within 24 hours. However, relapse and death due to <condition>, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the University of Liverpool REC where in the opinion of the Chief Investigator, the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator must also notify the Sponsor of all SAEs.

Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Please send SAE forms to: Dr Kate Navaratnam

Fax: 0151 795 9599 (For the Attention of Dr Kate Navaratnam)

Tel: 0151 795 9567 (Mon to Fri 09.00 – 17.00)

## 6. ASSESSMENT AND FOLLOW-UP

Eligibility will be assessed by a member of the research team and verbal information about the AMV study will be discussed. Prospective participants will be provided with a copy of the participant information leaflet, they will have time to read and discuss the information and ask questions before deciding if they wish to take part.

Eligible women that opt to take part will provide informed written consent using the AMV consent form.

Enrolled participants will be asked to provide urine samples over two non-consecutive days. The scheduling of the days is flexible and can be arranged with the investigators to ensure they are at the participant's convenience.

The participant will attend the Centre for Women's Health Research, on the Liverpool Women's Hospital Site at the start of each day. The 75mg aspirin dose will be directly supervised by Dr Kate Navaratnam, who is medically qualified and resuscitation trained to advanced level. Dosing will take place adjacent to a high-dependency area with recourse to immediate use of resuscitation facilities in the case of a rare adverse reaction. Participants will remain in the Centre for Women's Health Research for 30 minutes following the initial dose.

On arrival participants will be asked to provide a urine sample (labelled with the time and '0 hours') then immediately directly observed taking one 75mg dispersible aspirin tablet dissolved in 100ml water. Subsequent samples will be obtained according to hours elapsed from the time of the aspirin dose (as below). Participants will be asked to record the date and 24hr clock timing on each sample provided.

Day 1 urine samples (minimum of 2ml) at 0, 1, 3, 5, 7, 9, 11, 24hrs

Day 2 urine samples (minimum of 2ml) at 0, 2, 4, 6, 8, 10, 12, 24hrs

Samples do not require immediate refrigeration/freezing and can be returned to the investigators at the Centre for Women's Health Research when convenient for study participants. On arrival urine will be allocated a random laboratory number (1-50), the allocated number will then be crossed off the list. No log will be kept to link identifying information and laboratory number for participants and the samples will then be considered fully anonymised. Urine will be centrifuged to produce cell free samples. Anonymised, labelled samples will be transferred on foot in a shatter proof sealed container to the NMR Centre where they will be logged and securely stored at -80 degrees Celsius for NMR processing in batches.

### Sample processing and NMR methods

Samples of urine will be centrifuged to remove precipitant and stored at -80°C for analysis by NMR for presence of metabolites of acetyl salicylic acid (ASA). Metabolite levels and resonance positions will be determined from time-course data derived from healthy female. Samples will be prepared according to established protocols [Beckonert 2007] to a final sample composition of 50% urine, 10% <sup>2</sup>H<sub>2</sub>O, 500mM sodium phosphate buffer (pH 7.4), 100µM trimethylsilyl propionate (Sigma, UK) and 0.1% sodium azide. Spectra will be acquired on a 700MHz Bruker spectrometer equipped with a 5mm TCI cryoprobe using a 1H 1D NOE pulse sequence (98304 points, 2048 transients) at 27°C. Presence of ASA metabolites will be identified in a semi-automated manner using Chenomx pattern

recognition software (Chenomx, Canada) with positive identification subject to strict identification criteria.

## **7. STATISTICS AND DATA ANALYSIS**

### Metabolite Identification

Individual NMR spectra will be analysed using Chenomx pattern recognition software and expert review in the University of Liverpool NMR Centre. <sup>1</sup>H NMR of ASA and its metabolites give rise to distinctive signals in the aromatic region. These signals can be searched via use of the metabolite identification package, Chenomx (Alberta, Ca). The Chenomx package contains a library of over 350 human and drug metabolites and contains a peak fitting algorithm that enables automated appraisal of the NMR spectra to obtain a maximal fit between library peaks and experimental data.

### Data normalisation and integration

Following metabolite identification the peaks identified will then be integrated with respect to the reference signal, TSP. Using Topspin 3.2 (Bruker, Coventry, UK). This will produce signal intensities representative of the effective concentration of the sample. Urine, as a waste product does not have a fixed dilution. Subsequently, normalisation of these values will be conducted with respect to the total peak volume (in the absence of residual water contribution) observed in the samples. Time-course curves will be constructed to determine the detectable time range from dose-urine sampling for low-dose aspirin in healthy women.

## **8. REGULATORY ISSUES**

### **8.1 ETHICAL APPROVAL**

The Chief Investigator will obtain approval from the University of Liverpool Research Ethics Committee and Health Research Authority (HRA). The study will be submitted to the research site (University of Liverpool) for Confirmation of Capacity and Capability. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

### **8.2 CONSENT**

Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent forms will be obtained by research team members who are appropriately trained in Good Clinical Practice. The right of the participant to refuse to participate without giving reasons must be respected. All participants are free to withdraw at any time up until the samples can still be identified (prior to arrival in the Centre for Women's Health Research Laboratory) and anonymisation with a randomly allocated laboratory number. This will be stated on the consent form. It will also be discussed with the participant and specified on the AMV study consent form that the participant retains the right to withdraw without giving reasons and without prejudicing further treatment.



### **8.3 CONFIDENTIALITY**

The Chief Investigator will preserve the confidentiality of participants taking part in the study and will abide by the Data Protection Act. All personal data will be stored in the Centre for Women's Health Research office in a locked filing cabinet. The research office is a locked room with limited access to research staff only. All data will be irreversibly anonymised.

### **8.4 INDEMNITY**

The University of Liverpool holds Indemnity and insurance cover with Marsh UK LTD, which apply to this study.

### **8.5 SPONSOR**

The University of Liverpool will act as Sponsor for this study. It is recognised that as an employee of the University the Chief Investigator has been delegated specific duties, as detailed in the Sponsorship Approval letter.

### **8.6 FUNDING**

Wellbeing of Women are funding this study.

### **8.7 AUDITS**

The study may be subject to inspection and audit by the University of Liverpool under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

## **9. STUDY MANAGEMENT**

The day-to-day management of the study will be coordinated through Dr Kate Navaratnam.

## **10. END OF STUDY**

Participants will be recruited during a 3 month (12 week period); a maximum of 50 women will be recruited

## **11. ARCHIVING**

Data will be fully anonymised, and due to the scientific value this will be securely stored in accordance with the University of Liverpool's IT Policy indefinitely.

## **12. PUBLICATION POLICY**

Data will be fully anonymised and no identifiable personal information will be used for publications. Results will be presented at scientific conferences and prepared for publication in a peer-reviewed journal. We will not provide individual feedback of results to participants, but will direct participants to freely available conference proceedings and publications if they express an interest in this.

### 13. REFERENCES

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2. Askie LM, Duley L, Henderson-Smart DJ, Stewart LA. Fast track-articles: antiplatelet agents for prevention of pre-eclampsia: a meta-analysis of individual patient data. *Lancet* 2007; 369:1791–8.
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6. Navaratnam K, Alfirevic A, Alfirevic Z. Low dose aspirin and pregnancy: how important is aspirin resistance? *BJOG* 2016; 123: 1481-88.
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8. Vrijens B, De Geest S, Hughes DA, Przemyslaw K, Demonceau J, Ruppert T, et al. A new taxonomy for describing and defining adherence to medications. *Br J Clin Pharmacol*. 2012;73(5):691-705.

## Participant Information Leaflet

# Aspirin Metabolites in Healthy Female Volunteers (AMV) Study

You are being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. Please also feel free to discuss this with your friends or relatives if you wish. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.

Thank you for reading this.

### 1. What is the purpose of the study?

Aspirin is one of the most widely prescribed medicines worldwide. Aspirin is commonly used to reduce the risk of heart attacks and stroke. In pregnant women aspirin is important to reduce the risk of women getting high blood pressure and delivering small babies.

Despite lots of research studies using aspirin it is difficult to measure whether people are taking their aspirin tablets as prescribed. If doses are missed or aspirin is stopped this may lead to increased risks for the person. This can also mean researchers underestimate of aspirin's potential to reduce risks for patients.

The AMV study will look for aspirin's natural breakdown products in urine of volunteers who have taken one dose of aspirin. We aim to use this information to create a new test to decide if a person has taken their aspirin.

### 2. Why have I been chosen to take part?

You have been chosen as you are a member of staff or student (or a friend or relative of a member of staff or student). The study requires healthy women aged 18 to 45 years old.

### 3. Do I have to take part?

Participation is voluntary, the researcher will discuss the study with you and provide you with a participant information leaflet. You will have an opportunity to read this and the researcher will answer any questions you have.

You are free to withdraw at any time whilst your urine samples can still be identified. Unfortunately, after samples reach the Nuclear Magnetic Resonance (NMR) Centre they are anonymised and cannot be traced back to you, this means we are unable to withdraw your samples from that point. If you wish to withdraw from the study you do not need to give an explanation and there will be no disadvantage to you.

#### **4. What will happen if I take part?**

If you decide to take part in the AMV study the researcher will check that it is safe for you to take part and that you meet the entry criteria for the study.

The researcher will ask you to read and sign a consent form the researcher will also sign this form. You will be provided with one copy of your consent form and one copy will be kept by the research team.

You will be asked to attend the Centre for Women's Health Research to start the study on each day. You will be asked to donate urine samples on two separate days (not one after the other), the researcher will agree the dates and start times for you to take part to make sure this is convenient for you.

At the start of each day you will meet the researcher, they will ask you to provide a urine sample. Immediately after this the researcher will ask you to take one aspirin tablet (75mg dispersible aspirin dissolved in 100ml of water), they will watch you take this and record the date/time. This date/time will be '0 hours'. Following the aspirin dose you will be asked to wait in the Centre for Women's Health Research to make sure you have not had any serious allergic reaction to aspirin, this is very rare. The Centre for Women's Health Research is attached to an NHS Hospital with full facilities and researcher is medically qualified to help you in the rare case of an allergy you did not previously know about.

Following the 30 minute period the researcher will check that you feel well. If so, you do not need to stay in the research centre and are free to go about your normal daily activities. The researcher will give you small containers for urine samples. They will ask you to write the date/time that you collect each urine sample on the side of the container.

**On Day 1** you will be asked for urine samples at 0, 1, 3, 5, 7, 9, 11, 24 hours from the time of taking aspirin.

**On Day 2** you will be asked for urine samples at 0, 2, 4, 6, 8, 10, 12, 24 hours from the time of taking aspirin.

The aspirin breakdown products are stable, as a result samples do not need refrigeration or freezing. You will be asked to return your samples to the Centre for Women's Health Research. The researcher will discuss this process with you and agree a convenient time for you. When your samples arrive in the laboratory they will be given a random laboratory number, from this point they cannot be traced back to you. The samples will be taken by the research team to the NMR Centre.

In the NMR Centre urine samples will be stored in a locked -80 degree Celsius freezer with access restricted to research study staff. The results will be fully anonymous and cannot be traced back to you. Results will be stored on a University computer in a password protected file and backed up on the University Server each night to ensure they cannot be lost. Due to the usefulness of this data will be kept by the University forever.

## **5. Expenses and / or payments**

Unfortunately no payments or reimbursements will be available for this study. Taking part is voluntary and the timing of the two days you will need to provide urine samples on can be arranged for days that are convenient for you. The researcher will discuss this with you.

## **6. Are there any risks in taking part?**

There is a very small risk of an allergic reaction to aspirin or an intolerance that you did not already know about. For this reason, we will only ask you to take part if you have taken aspirin in the past with no problems.

Some people with asthma find that aspirin and other drugs in the same family (non-steroidal anti-inflammatory drugs) trigger asthma attacks for them. For this reason, women with asthma and women with any previous adverse allergic reactions or intolerances to non-steroidal anti-inflammatory drugs will not be able to take part.

The researcher will check that you do not have any previous problems with aspirin or non-steroidal anti-inflammatory drugs and do not have asthma before you take part.

As part of the AMV study you will be asked to provide urine samples on two separate days. Before agreeing to take part you should consider if this will be convenient for you.

**7. Are there any benefits in taking part?**

Any useful information from the AMV study is unlikely to directly benefit you directly, and will not benefit your career development. Taking part in the AMV study may benefit future research in this area and benefit patients in the future.

**8. What if I am unhappy or if there is a problem?**

If you are unhappy, or if there is a problem, please feel free to let us know by contacting Dr Kate Navaratnam 0151 795 9567 and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer at [ethics@liv.ac.uk](mailto:ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

**9. Will my participation be kept confidential?**

If you meet the entry criteria and wish to take part we will record your name on the consent form. No further data will be collected. Your personal information will be stored securely in a locked filing cabinet in the Centre for Women's Health Research with access restricted to the research study staff.

On arrival in the laboratory your urine samples will be given a random laboratory number. As a result, samples and the results of the study cannot be traced back to you.

**10. What will happen to the results of the study?**

Participants will not be identifiable in any presentations or publications and all results will be anonymous.

Results of the AMV Study will be presented at scientific conferences and published in peer reviewed journals. If you are interested to read the full results please contact Dr Kate Navaratnam or Dr Marie Phelan who will be happy to provide you with details of presentations or publications which will you can read.

**11. What will happen if I want to stop taking part?**

You are free to withdraw, without explanation at any time when we are still able to identify your urine samples. Unfortunately after samples reach

the laboratory they are given a random number and cannot be traced back to you, this means we are unable to withdraw your samples from that point.

## **12. Who can I contact if I have further questions?**

Please contact:

Dr Kate Navaratnam  
First Floor, Centre for Women's Health Research, University of Liverpool,  
Crown Street, L8 7SS  
0151 795 9567  
07889970973  
[Kate.Navaratnam@liv.ac.uk](mailto:Kate.Navaratnam@liv.ac.uk)

Dr Marie Phelan  
Nuclear Magnetic Resonance Centre, University of Liverpool, Crown Street,  
L69 7ZB  
0151 795 4398  
[Marie.Phelan@liv.ac.uk](mailto:Marie.Phelan@liv.ac.uk)

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**Research Ethics Committee**

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**PARTICIPANT CONSENT FORM**

**Title of Research:** Aspirin Metabolites in Healthy Female Volunteers (AMV) Study

**Researchers:** Dr Kate Navaratnam, Dr Marie Phelan

1. I confirm that I have read and have understood the information sheet dated [Version 1.0 01/11/16] for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my participation is voluntary and that I am free to withdraw at any time when my urine samples can still be identified, without giving any reason and without my rights being affected.
3. I understand my anonymised urine samples will be gifted to the University of Liverpool for use in future research without further consent.
4. I agree to take part in the above study.

_____ Participant Name	_____ Date	_____ Signature
_____ Researcher	_____ Date	_____ Signature

**Principal Investigator:**  
Dr Kate Navaratnam  
Centre for Women's Health Research  
University of Liverpool  
0151 795 9567  
Kate.Navaratnam@liv.ac.uk

**Co-Investigator:**  
Dr Marie Phelan  
NMR Centre  
University of Liverpool  
0151 795 4398  
Marie.Phelan@liv.ac.uk

**Version 1.0 01/11/16**

Version 1.0  
01/11/16



**NRES Committee North West - Liverpool Central**

HRA NRES Centre - Manchester  
3rd Floor  
Barlow House  
4 Minshull Street  
Manchester  
M1 3DZ

Telephone: 0161 625 7818  
Facsimile: 0161 625 7299

17 December 2013

Dr Ana Alfirevic  
University of Liverpool  
The Wolfson Centre for Personalised Medicine  
Department of Molecular and Clinical Pharmacology  
Block A Waterhouse Buildings, 1-5 Brownlow Street, Liverpool  
L69 3GL

Dear Dr Alfirevic

**Study title:** Estimating Aspirin ResiTance in High-risk women  
(EARTH)  
**REC reference:** 13/NW/0764  
**Protocol number:** 1.0  
**IRAS project ID:** 135034

Thank you for your letter of 06 December 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Mrs Carol Ebenezer, [nrescommittee.northwest-liverpoolcentral@nhs.net](mailto:nrescommittee.northwest-liverpoolcentral@nhs.net).

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Ethical review of research sites**

**NHS sites**

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

01/11/15

## Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

*Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.*

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

*Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.*

*For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.*

*Sponsors are not required to notify the Committee of approvals from host organisations*

## Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett ([catherineblewett@nhs.net](mailto:catherineblewett@nhs.net)), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

## Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Advertisement	1	04 September 2013
Investigator CV	Alfirevic	15 March 2013
Investigator CV	Navaratnam	14 October 2013
Letter from Sponsor		16 October 2013
Participant Consent Form	2	12 November 2013
Participant Information Sheet	2	12 November 2013
Protocol	1.2	27 November 2013

REC application	3.5	14 October 2013
Referees or other scientific critique report		13 September 2013
Response to Request for Further Information		06 December 2013

### Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

### After ethical review

#### Reporting requirements

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

#### Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/NW/0764	Please quote this number on all correspondence
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We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at <http://www.hra.nhs.uk/hra-training/>

With the Committee's best wishes for the success of this project.

Yours sincerely



**Mrs Julie Brake**  
Chair

Email: [nrescommittee.northwest-liverpoolcentral@nhs.net](mailto:nrescommittee.northwest-liverpoolcentral@nhs.net)



17<sup>th</sup> December 2013

Direct dial: 0151 702 4346  
Email: [gillian.vernon@lwh.nhs.uk](mailto:gillian.vernon@lwh.nhs.uk)

Dear Dr Ana Alfirevic

**LWH0989 Estimating Aspirin Resistance in High Risk Women (EARTH)**

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by Trust R&D. This approval relates to the documentation listed below:

- Ethics approval letter dated 17<sup>th</sup> December 2013; REC Reference 13/NW/0764
- Protocol Version 1.2 27<sup>th</sup> December 2013

The research is registered on the Trust's R&D database under the reference LWH0989, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) are University of Liverpool and Liverpool Women's NHS Foundation Trust.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles of Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf. Our Trust R&D Department must be kept informed of regulatory amendments, updates and approvals – this is your responsibility as site investigator.

It is also your responsibility to assure the confidentiality and protection of patient identifiable information. To gain a thorough understanding of your information governance responsibilities, the Trust R&D Department recommends that you refer to the NHS IG Toolkit, accessing the online training materials where necessary ([www.connectingforhealth.nhs.uk/igtrainingtool](http://www.connectingforhealth.nhs.uk/igtrainingtool)).

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely

**Gillian Vernon**  
Research & Development Manager

## **Estimating Aspirin ResisTance in High-risk women (EARTH)**

**Alfirevic A (Chief Investigator), Navaratnam K (PI), Pirmohamed M, Martlew V, Alfirevic Z.**

### **BACKGROUND**

Pre-eclampsia is multisystem disorder of pregnancy that presents with high blood pressure and proteinuria after 20 week's gestation [1]. It complicates 2-8% of pregnancies and remains a leading cause of maternal and perinatal morbidity and mortality worldwide[2]. Globally, an estimated 40,000 maternal deaths occur as a result of pre-eclampsia [3]. In the UK, pre-eclampsia accounts for 18% of direct maternal deaths [4], and is the most common pregnancy-related cause of admission to intensive care units [5].

The pathogenesis of pre-eclampsia is not fully understood, but inadequate re-modelling of the uterine spiral arteries during placentation, resulting in reduced placental blood supply has been implicated [6]. The resultant reperfusion injury leads to placental ischaemia and villous immaturity. The increased loss of placental material into the maternal circulation is proposed to cause maternal endothelial cell dysfunction, with resultant widespread vasoconstriction, microthrombi formation and reduction in circulating blood volume. This maternal vascular response is thought to be, at least in part, secondary to derangements in the coagulation cascade that include platelet activation, increased production of Thromboxane A<sub>2</sub>, a platelet-derived vasoconstrictor and platelet aggregator [7] and reduced production of the vasodilator and platelet inhibitor prostacyclin from endothelial cells [8]. This observation led to the hypothesis that antiplatelet agents such as aspirin, which inhibit Thromboxane A<sub>2</sub>, may prevent pre-eclampsia and its complications.

A systematic review of individual patient data evaluating antiplatelet agents for prevention of pre-eclampsia confirmed a reduction in the risk of pre-eclampsia (10%) and preterm birth <34 weeks (10%) with aspirin therapy [9] (Table 4 Appendix). However, it did not demonstrate a clear difference in the effectiveness of aspirin for any particular subgroup of women because of the small numbers in these groups. There is a suggestion that a higher aspirin dose (80 to 150 mg) may be more beneficial, but data are limited. There is no known increase in the risk of adverse effects on pregnancy with low or higher dose aspirin [10]. NICE has recently recommended the use of low dose aspirin in pregnant women at increased risk of pre-eclampsia (NICE guideline 'Hypertension in Pregnancy' (NICE clinical guideline 107, August 2010). Various risk factors for pre-eclampsia have been identified, based on obstetric and medical history [11] however, it is unclear which women will benefit most.

Aspirin (acetylsalicylic acid) is one of the oldest and most commonly prescribed medications for various indications; around the globe, approximately 40,000 tons of aspirin are produced every year. Aspirin is readily absorbed in the upper gastrointestinal tract, with peak plasma levels within

40 minutes and platelet inhibition within one hour of ingestion [12]. Aspirin irreversibly inhibits the platelet COX-1 enzyme by acetylation of the serine residue (Ser529). This alteration in structure prevents the binding of the arachidonic acid to the catalytic site (Tyr385) thereby inhibiting the synthesis of prostaglandin H-synthase PGH<sub>2</sub>. Subsequently, generation of Thromboxane (TxA<sub>2</sub>) and TxA<sub>2</sub>-induced platelet aggregation is inhibited for the lifespan of the platelet (10 days).

### **Aspirin resistance**

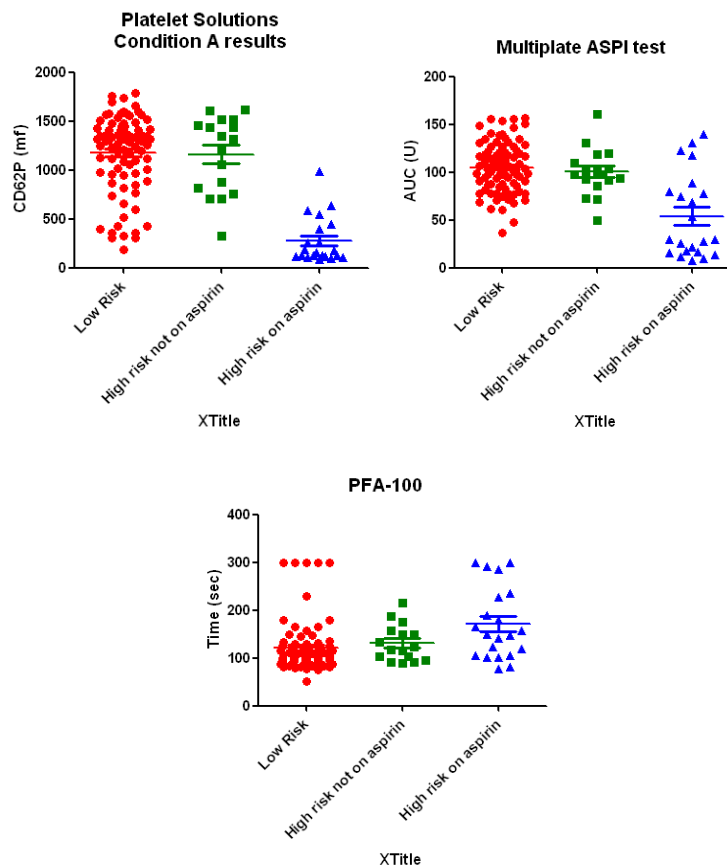
Reduced response to aspirin is well established in cardiovascular medicine. It has been defined biochemically as a failure to inhibit platelet function, or clinically as an increased risk of cardiovascular events despite aspirin therapy at recommended doses, and can be observed in up to 40% of patients [13, 14]. Aspirin resistance has been demonstrated in healthy individuals [15], as well as those with cardiac disease [16, 17]. Various reasons have been proposed for the reduced response to aspirin, including non-compliance, drug interactions, enhanced platelet turnover and genetics [18]. A recent prospective study in patients on two antiplatelet agents after coronary stenting, demonstrated that aspirin resistance could be overcome by increasing the dose of aspirin [19].

Whether aspirin resistance is also seen in pregnant women at risk of pre-eclampsia is not clear, but it could provide an explanation for why some women develop pre-eclampsia despite taking aspirin. If confirmed, the knowledge could potentially be applied to models of pre-eclampsia prediction or modification of preventative therapy. One study has assessed a strategy of platelet function testing and individualising dose of aspirin in women at risk of pre-eclampsia (higher dose for inadequate response to aspirin) using a platelet function analyser (PFA-100)[20]. This study found that the incidence of pre-eclampsia was lower in women who were tested for aspirin resistance whilst on aspirin, compared to those who were not tested. Among the group who were tested, the incidence of pre-eclampsia was higher in those who needed an increase in their aspirin dose compared to those who did not (26% vs 9%). The explanation given for this observation was that modification in dose may have been made too late in gestation to make a difference to outcome. These findings need to be interpreted with caution as data are from a retrospective observational study with incomplete balancing of risks. Some risk factors for pre-eclampsia were not distributed equally among the two groups, and information was missing on other interventions that may have impacted on subsequent development of pre-eclampsia. The reference target used to diagnose low biochemical response to aspirin was based on that developed from a mixed adult population rather than pregnant women. Another study [21] has recently reported that women with biochemical aspirin resistance had worse clinical outcomes, including pre-eclampsia, fetal distress and small for gestational age infants. They defined aspirin resistance as u11-dTXB<sub>2</sub> concentration (a urinary metabolite of thromboxane A<sub>2</sub>) in the highest quartile, and also by using the resistance index, which was calculated as the difference between u11-dTXB<sub>2</sub> concentration of each woman treated

with aspirin and the median value at the same gestational age measured in the control group of pregnant women not on aspirin. Laboratory tests were performed at 18 to 22 weeks of gestation, 28 to 32 weeks of gestation and 16 to 32 weeks after delivery in 75 women (43 on aspirin for high-risk pregnancies, over half with antiphospholipid syndrome or SLE) and 35 not on aspirin (24 healthy pregnancies and 8 with SLE).

### Pilot study results

The normal reference ranges for women at low risk of pre-eclampsia, and women at high risk with or without aspirin are not influenced by gestational age. Results for each cohort will be amalgamated, and for EARTH women 11+0-20+6 will be included.



**Figure 1.** Platelet activity in pregnancy in low-risk women and high-risk women using Platelet solutions, Multiplate™ and PFA100 point-of-care systems (*unpublished pilot data*).

## **Choice of platelet activation/function tests for EARTH**

### Excluded

PFA 100 and Platelet Solutions were ruled out due to unreliable and variable results.

### Included

Multiplate™ ASPI test (reliable, point of care)

VerifyNow™ Aspirin point of care system, used in cardiovascular and stroke medicine.

Urinary 11-Dehydroxythromboxane B<sub>2</sub>,

### Additional study procedures

P-selectin, E-selectin, Thrombomodulin, ICAM-3 markers of vascular injury associated with placental disease (measured from maternal plasma and serum).

Storage of maternal DNA.

Preparation and storage of placental samples.

## **STUDY AIM**

To investigate aspirin responsiveness as a potential target for personalised strategies for the prevention of pre-eclampsia. A secondary aim is to store and extract DNA for a candidate gene approach to investigate genetic pathways in the mechanism of action and disposition of aspirin.

## **STUDY OBJECTIVES**

1. To define the proportion of women at high-risk for pre-eclampsia who are biochemically non-responsive to aspirin.
2. Assess if biochemical non-responsiveness is related to clinical outcomes and aspirin resistance.
3. Identify genetic factors in the aspirin pathway that correlate to platelet function and pre-eclampsia.

## **METHODS**

This is a prospective cohort study which will be performed at the Liverpool Women's Hospital NHS Foundation Trust. We will aim to recruit 100 high-risk women compliant with aspirin therapy in the first half of pregnancy (5<sup>0</sup>-20<sup>+6</sup> weeks). It is estimated that 180 total participants will be required to ensure 100 women compliant with aspirin in the cohort. Participation will involve blood samples for platelet activation and function tests, urine collection, and blood collection for assessment of aspirin and DNA extraction. Participants will have a uterine artery Doppler examination (standard investigation for women at high-risk of pre-eclampsia) at 20<sup>0</sup>-23<sup>+6</sup> week's gestation. Follow-up at 33<sup>0</sup>-35<sup>+6</sup> will involve assessment of placental function, with blood and urine samples for PIGF testing, and platelet activation tests and assessment of aspirin compliance. An ultrasound scan will



be performed with uterine artery Doppler and fetal Doppler, assessment of fetal growth and liquor volume. Case notes for all women will be reviewed following delivery.

### **Setting**

High-risk women will be recruited from the antenatal clinics and ultrasound department at Liverpool Women's Hospital. We will aim to recruit either at the routine booking visit (5+0-13+6), the visit for serum Downs' Syndrome screening (15+0-16+6), or at the fetal anomaly scan (18+0-20+6).

### **Inclusion criteria**

High risk pregnant women with a viable single intrauterine pregnancy 5+0-20+6 week's gestation (EDD calculated from CRL at 1st trimester dating scan), prescribed aspirin 75mg daily.

High risk is defined in accordance with NICE guidance, a single high risk factor, or at least two moderate risk factors.[22]

High risk factors for pre-eclampsia [22]

Previous hypertensive disease in pregnancy

Chronic hypertension

Pre-existing type 1 or 2 diabetes

Chronic renal disease

Autoimmune disease e.g. Systemic Lupus Erythematosus, Antiphospholipid Syndrome

Moderate Risk factors for pre-eclampsia [22]

First pregnancy

Maternal age >40 years

BMI >35

Pregnancy interval >10 years

Family history of pre-eclampsia (first-degree relatives)

### **Exclusion criteria**

Women will be excluded if they are taking medication which could interfere with platelet function, such as anticoagulants (e.g. LMWH) or other antiplatelet agents (e.g. Clopidogrel).

### Primary outcome measure

- Biochemical non-responsiveness to aspirin. For EARTH, biochemical non-responsiveness is defined as response (outside the test-specific high risk on aspirin reference range) in platelet activation tests (Multiplate™, VerifyNow™ 11-Dehydrothromboxane B2).

### Secondary outcomes

- **Preeclampsia** defined as proteinuric hypertension (with onset after 20 weeks). Systolic BP≥140 mmHg and/or diastolic BP ≥90mmHg (Korotkoff V) on at least 2 occasions 4h apart, with proteinuria (spot urine ≥2+, protein: creatinine ratio ≥ 30 mg/mmol, or ≥ 300 mg/24h. With or without severe maternal complications.
- **Placental abruption**, evident at the time of delivery, on examining the placenta or from placental histology.
- **IUGR**. The estimated fetal weight at 33+0-35+6, and birthweight are assessed against customised centiles, adjusted for gestation, maternal weight, height, parity, ethnicity. IUGR is defined as less than 5<sup>th</sup> customised centile.
- **Abnormal fetal Doppler indices**. Umbilical artery Doppler (PI>95<sup>th</sup> centile, reduced, absent, or reversed end diastolic flow), evidence of brain-sparing in the middle cerebral artery, absent a-wave in the ductus venosus, or pulsatile umbilical vein.
- **Abnormal uterine artery Doppler**, mean PI >1.45MoM.
- Admission to neonatal unit and major **neonatal morbidity**, or **perinatal mortality**.

### Study procedures

Consent, interviews and venous blood samples will be taken by the EARTH research staff. Platelet function tests and PLGF-1 tests will be performed in the Centre for Women's Health Research laboratories. Results of Multiplate™ tests and VerifyNow™ will not be disclosed to the investigators or participants, they will be printed and securely stored by a trained laboratory technician for analysis by the research team on completion of the study. Blood samples for PIGF-1 will initially be frozen, and processed at completion of the study (following delivery of the final participant). Vascular injury panels (P-selectin, E-selectin, Thrombomodulin and ICAM-3) and assessment of serum salicylic acid concentration will be performed at the Wolfson Centre for Personalised Medicine. Placental samples will be collected by EARTH research staff, prepared and stored at -80 degrees Celsius in the Centre for Women's Health Research Laboratories.

Ultrasound scans will be coordinated with visits offered for standard antenatal care (20+<sup>0</sup>-23+<sup>6</sup>, and at 33+<sup>0</sup>-35+<sup>6</sup>) Ultrasound scans will be performed by the EARTH research staff (fully trained in fetal biometry and maternal-fetal Doppler assessments). The scan results will be presented to the Fetal Medicine team for review and clinical decision making. The EARTH research staff will not be

involved in the clinical decision making based on any scan results obtained during these visits. Non-invasive cardiac output, blood pressure and peripheral vascular resistance will be assessed by EARTH research staff using NICOM and arteriography equipment at 20+0-23+6 and 33+0-35+6. Any clinically relevant results will be referred to the clinical care team for further assessment.

**5<sup>0</sup>-20<sup>6</sup> visit**

Interview (coordinated with booking visit, serum screening, or anomaly scan)	To assess eligibility (gestational age calculated from early pregnancy ultrasound, prescribed aspirin).  Past medical, obstetric and family history.  Current medications.  Aspirin diary filed in patient's hand-held records for future use.
Consent	Written and verbal.
Blood and urine sampling	Venous blood sample (15ml). The participant will be observed taking 75mg dispersible aspirin. 15ml venous blood sample repeated 1 hour following aspirin dose  3ml Hirudin (Multiplate™), 2ml Citrate (VerifyNow™), 3ml EDTA (DNA storage). 4ml whole blood (serum to assess salicylate concentration, vascular injury panel). 3ml Citrate (vascular injury panel)  1-20ml urine sample (11-Dehydrothromboxane B <sub>2</sub> ).

**20<sup>0</sup>-23<sup>6</sup> visit**

Obstetric ultrasound scan	Mean uterine artery Doppler pulsatility index (PI)
NICOM/Arteriography	Non-invasive measurement of cardiac output, blood pressures and peripheral vascular resistance.

**33<sup>0</sup>-35<sup>6</sup> visit**

Aspirin Diary	Participants reminded by telephone 14 days prior to visit to commence aspirin diary. Dates, times, missed doses and other medications during time period 7days prior to visit recorded and collected by EARTH research team.
Blood and urine sampling	Single venous blood sample (12ml total) 3ml EDTA (PIGF) 3ml Hirudin (Multiplate™), 2ml Citrate (VerifyNow™), 2ml whole blood (serum to assess salicylate concentration, vascular injury panel), 2ml Citrate (vascular injury panel)  1-20ml urine sample (11-Dehydrothromboxane B <sub>2</sub> ).
Obstetric ultrasound scan	Mean uterine artery Doppler PI  Fetal Doppler (Umbilical artery PI, Middle Cerebral Artery PI, venous Doppler)  Fetal growth measurements (Bi-parietal diameter, head circumference, abdominal circumference, femur length), Liquor volume (Maximum vertical pool depth).
NICOM/Arteriography	Non-invasive measurement of cardiac output, blood pressures and peripheral vascular resistance.

**After Delivery**

Placental Tissue	Assessment of placental vasculature, biomarkers for preeclampsia and placental disease, proteomics and genomics.
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**Laboratory techniques**

**Multiplate™** impedance aggregometry [23] will be used to measure platelet activation and function. We will use 300µl of hirudin anticoagulated whole blood with ASPI test which is semi-automated and perform a dual measurement as internal control.

**VerifyNow™** will be used to measure platelet activation, using 3ml whole blood with 3.2% sodium citrate. The test incorporates the agonist arachidonic acid to activate platelets, and measures platelet function based upon the ability of activated platelets to bind fibrinogen. Fibrinogen-coated microparticles aggregate in whole blood in proportion to the number of unblocked platelet GP IIb/IIIa receptors. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads, measured as a change in optical signal.

**Urine 11-Dehydrothromboxane B<sub>2</sub>** will be determined from urine stored at -80°C using ELISA. <1500pg/mg indicates complete COX-1 inhibition, and appropriate response to aspirin.

**DNA storage, extraction, and analysis:** 9ml of whole blood withdrawn in EDTA coated tubes will be stored at -20°C for DNA extraction. Samples will be stored in a DNA archive with integrated robotics linked to the laboratory information management system with sample tracking using 2D bar-coding. Variants in candidate genes will be investigated in relation to aspirin non-responsiveness in pregnancy. Genome-wide approaches will be utilised if appropriate number of samples becomes available through collaborations.

**MSD Vascular injury panel. P-selectin, E-selectin, Thrombomodulin and ICAM-3 will be assessed from maternal plasma and serum using a 96 well multisport assay.**

**Salicylate concentration** 4ml whole blood obtained for serum, centrifuged at 2600 rpm for 10 minutes at 4 degrees Celsius. Serum aliquoted and stored at -80°C. Concentration of salicylate in serum used to assess compliance with aspirin.

**Placental Growth Factor 1 (PlGF-1)** levels track the placental development, peaking around 32 weeks, and then decline until delivery. 250µL of EDTA plasma is tested for PlGF-1 using the Alere Triage Meter-Pro™ point-of-care test.

#### **Sample size calculation**

Our pilot study demonstrated that 2-3 high-risk women can be recruited per week, therefore, 100 women can feasibly be recruited in 12 months. Assuming a conservative non-responsiveness rate of 20%[24], a sample size of 100 would have good estimated precision i.e. 95% Confidence Intervals from 13%-29%. The frequency of pre-eclampsia in high-risk women taking aspirin is 15%[25], as NICE also recommends Aspirin for women with more than one moderate risk factor a realistic estimate is 12%[22] (12 cases expected from the sample). We hypothesise that around 40% of aspirin non-responders will develop pre-eclampsia, compared with less than 10% of responders. A sample size of 100 women would have adequate power (a=5%; b=80%) to test this hypothesis, assuming that 15-20% of our cohort will be non-responsive to aspirin. For genetic analyses, with the envisaged number of 100 participants, we will have 80% power at 5% significance level to detect medium to large effects (OR 4-5) for variants of medium to high frequency (>10%).

#### **DESCRIPTION OF ANY DIFFICULTIES ANTICIPATED**

All methods to be used are standard and established within our laboratory, and we therefore do not expect any problems with the laboratory methods. There may be difficulties in retaining women in the study for a follow-up visit, and wherever possible this will be coordinated with other antenatal appointments, or at an agreed convenient time for the participant. Recruitment will be constantly monitored.

## **FURTHER WORK ANTICIPATED**

As we enter the era of personalised medicine, it is important to identify predictors of response to aspirin so that those not likely to benefit from standard treatment can be identified early and offered appropriate surveillance, and alternative or more intensive therapy. We anticipate that our work with aspirin non-responsiveness in pregnancy will be followed up in several ways (1) assessment of whether increasing the dose of aspirin can modify biochemical and clinical aspirin resistance, (2) exploration of the possibility of treatment with other antiplatelet agents or low molecular weight heparins. (3) investigation of genetic factors in the aspirin pharmacokinetic and pharmacodynamic pathway that correlate with platelet function and clinical outcomes. A recent systematic review of aspirin resistance in cardiovascular medicine has shown an association with a polymorphism in the GPIIIa platelet receptor gene [26]

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# EARTH

## Estimating Aspirin Resistance in High-risk women

### Participant Information Sheet

We would like to invite you to take part in our research study. Before you decide we would like to explain to you why the research is being done and what it would involve for you. A member of our team will go through the information sheet with you and answer any questions you have. This should take about 15 minutes. Feel free to talk to others about the study if you wish. (Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study). Please ask us if there is anything that is not clear.

#### **PART 1**

##### **What is the purpose of the study?**

Preeclampsia is a serious complication which occurs in the second half of pregnancy. It causes high blood pressure, loss of protein in the urine and may put the wellbeing of both mother and baby at risk. In the UK pre-eclampsia affects 1 in 20 pregnancies.

The cause of pre-eclampsia is not fully understood, but is thought to be related to the function of platelet cells in the blood, which aid clot formation. Aspirin acts on platelets to reduce the risk of pre-eclampsia in high-risk women when started in low doses in the first half of pregnancy. However, some women do not respond to aspirin adequately which could explain why some women still suffer from pre-eclampsia despite treatment with low dose aspirin.

##### **Aims**

- To Identify women at high-risk of pre-eclampsia who do not respond to aspirin.
- Assess if women who do not respond to aspirin are more likely to develop pre-eclampsia
- Investigate whether genetic make-up influences how women respond to aspirin, and their risk of pre-eclampsia.

##### **Why have I been invited?**

The EARTH study will recruit 180 women at high-risk of developing pre-eclampsia, who are prescribed Aspirin 75mg daily and attending Liverpool Women's Hospital. Your doctor will consider any risk factors you have, and if required, prescribe low-dose aspirin to reduce the risk. Low-dose Aspirin is safe for you to take in pregnancy. Because you are prescribed aspirin and you are 5 weeks to 20 weeks 6 days pregnant, you are invited to take part. If you are already taking other

medications that act against platelets, such as Clopidogrel, or thin the blood such as heparin, you cannot be included, please inform the researcher.

### **Do I have to take part?**

No. It is up to you to decide whether to join the study or not. If you agree to take part, we will ask you to sign a consent form. You do not have to answer all the questions asked by the researcher and you may stop the interview at any time, without giving a reason. This would not affect the standard of care you receive.

### **What happens to me if I take part?**

**5-20 week visit:** (will take approximately 20 minutes if you are already taking aspirin or 90 minutes if donating samples before and after the first aspirin dose, which includes 60 minutes of free time). The researcher will explain the study to you and if you decide to participate, will enroll you. This will involve signing a consent form and completing a questionnaire with the researcher. If you wish to take part in the study, the researcher will ask for your consent to donate your placenta after delivery. You will be asked a few clinical details about your pregnancy. If you have not already had an early pregnancy ultrasound scan the researcher will confirm the baby's heartbeat and gestational age with an ultrasound scan. If you have not already started taking aspirin, the researcher will take three teaspoons of blood (15ml) for platelet function tests, and DNA storage. After 60 minutes the researcher will take a further three teaspoons (15ml) of blood, and you will be asked to provide a urine sample.

If you have already started taking aspirin you can still take part in the study. The researcher will explain the study to you and if you decide to participate, will enroll you. This will involve signing a consent form and completing a questionnaire with the researcher. You will be asked a few clinical details about your pregnancy. If you have not already had an early pregnancy ultrasound scan the researcher will confirm the baby's heartbeat and gestational age with an ultrasound scan. The researcher will take three teaspoons of blood (15ml) for platelet function tests and DNA storage. You will be asked to provide a urine sample.

**20-23 week visit:** (will take approximately 25 minutes). The researcher will check that you still wish to take part. The researcher will perform an ultrasound scan to check the blood supply to your placenta. The researcher will use NICOM and arteriograph machines to accurately measure your blood pressures and assess heart function (cardiac output). To do this the researcher will ask you to wear a blood pressure cuff, they will make sure you are comfortable and ask you to rest quietly for up to 10 minutes. Having measurements taken with the NICOM and arteriography machines is not painful, and feels similar to having blood pressure measured in the usual way.

**33-35 week visit:** (will take approximately 20 minutes). 2-3 weeks prior to this visit the researcher will contact you to check that you still wish to take part. They will ask you to complete the Aspirin diary for 7 days before the appointment. At the appointment the researcher will take two and a half teaspoons of blood (12ml) for

a marker of placental function, and to check platelet function again. You will be asked to provide a urine sample. The researcher will perform an ultrasound scan to check your baby's growth, and blood flow, and measure the fluid volume around your baby. The researcher will use NICOM and arteriography machines to accurately measure your blood pressures and assess heart function (cardiac output). To do this the researcher will ask you to wear a blood pressure cuff, they will make sure you are comfortable and ask you to rest quietly for up to 10 minutes.

#### **After delivery of your baby (will not involve a research visit)**

If you have given consent to donate your placenta, a member of the research team will liaise with the midwife on delivery suite to collect and transport the placenta to the laboratory.

#### **Will I be paid expenses?**

You will not be reimbursed or paid expenses for additional visits due to taking part in the study.

#### **What will I have to do?**

If you choose to take part it is important that you can attend scheduled visits. The researcher will arrange convenient times with you.

#### **What are the alternatives for diagnosis and treatment?**

The same standard of care will be provided to all women regardless of whether they take part in the study or not.

#### **What are the risks and disadvantages?**

We do not anticipate any risks, except for the possibility of bruising at the site of the blood sample. Having measurements taken with the NICOM and arteriography machines is not painful, and feels similar to having your blood pressure measured. Taking part in the study involves potentially two additional visits to hospital however we will coordinate study visits with your usual appointments to minimise inconvenience to you.

#### **What are the possible benefits?**

This study will not benefit you in this pregnancy, but may benefit future generations of pregnant women. We would like to personalise the treatment offered to prevent pre-eclampsia and improve the health of mothers' and babies.

#### **What happens when the research study stops?**

You will receive usual care, but you will be able to contact the researchers if you have any questions.

#### **What if there is a problem?**

If problems occur in your pregnancy, your taking part in the study will not affect the standard of care you receive. Where problems occur, we may talk to you whilst you are in hospital, or by telephone. Continuing to take part is voluntary,

and we will check that you still wish to take part. Most information can be collected from hospital notes, but a short discussion with you, at a time to suit you, may be needed.

**Will my taking part in this study be kept confidential?**

Yes. Any material, which could identify you, will not be used in any reports on this study. A unique study number will be used to identify your samples and data. This unique number will ensure the confidentiality of any records we keep.

**PART 2**

**What if I don't want to carry on with the study?**

You are free to withdraw from the study at any time, without having to give a reason and this will in no way affect your future care. You can withdraw your blood samples from the study at any time before they are anonymised in the laboratory.

**Will my GP be informed of my participation in the study?**

The researchers will not inform your GP if you choose to take part in the study. With your consent, your lead maternity caregiver will be informed.

**What happens to any samples I give?**

The blood samples will be stored and analysed at the University of Liverpool, until they are used up. The urine and blood samples will be analysed for platelet function using several tests. We will examine DNA to look at genes that may be involved in how platelets work and respond to aspirin.

It is important to note that all blood samples going outside the hospital, and any notes relating to it, will be identified only by a code number. All personal details will be kept securely at the Liverpool Women's Hospital. Once the study has been completed, we will permanently anonymise your clinical details and blood samples, and it will not be possible to trace the blood sample back to you. After anonymisation, it will also not be possible for you to withdraw the blood sample.

With your consent your samples will be considered to be a gift to the University of Liverpool. In some cases, a small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that this will only be identified by a code. Approval will be sought from the ethics committee for any future studies. If you do not want her samples to be used in further research, the sample will be destroyed.

In the short-term, it is unlikely that the samples will be of any commercial value to the University or the hospital. However, it is possible that there may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient. You will not derive financial benefit from future discoveries.

**Will any genetic tests be done?**

We will investigate whether genes influence women's response to aspirin, and risk of developing pre-eclampsia. This involves DNA analysis. Any genetic information

will be anonymous and not linked to information that will identify you. Consenting to participate in DNA sampling for the study will not mean the researcher has a claim to your genetic information.

**What will happen to the results of the research study?**

The study results will be published in medical journals, your individual results will not be available, as samples and data will be anonymous.

**Who is organising and funding the research?**

The EARTH study is jointly sponsored by Liverpool Women's Hospital Research and Development Department, and the University of Liverpool. EARTH is funded by the Wellbeing of Women.

**Who has reviewed the study?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favorable opinion by the North West Research Ethics Committee. If you have a concern about any aspect of this study, you should ask to speak to the researchers, who will do their best to answer your questions. If you remain unhappy and wish to complain formally, details can be obtained from Liverpool Women's Hospital Complaints Department.

### **Further information and contact details**

More information about the study can be obtained from the Principal Investigator

Dr Kate Navaratnam  
Clinical Research Fellow  
University Department,  
Liverpool Women's Hospital  
Crown Street  
Liverpool  
L8 7SS  
Telephone: 0151 795 9567

Dr Ana Alfirevic  
Senior Lecturer  
Molecular and Clinical Pharmacology  
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Liverpool Women's Hospital Patient Advice and Liaison  
Liverpool Women's Hospital  
Crown Street  
Liverpool  
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Telephone: 0151 702 4061

## Consent Form

### Estimating Aspirin Resistance in High-risk women (EARTH)

Principal Investigator: Dr Kate Navaratnam

Please initial each box before signing the form

INITIALS

- |                                                                                                                                                                                                                                                                                                                           |                          |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| 1. I confirm that I have read and understood the information leaflet dated 01/12/14 (Version 5.0) for the above study.                                                                                                                                                                                                    | <input type="checkbox"/> |
| 2. I have had the opportunity to discuss the research and ask questions.                                                                                                                                                                                                                                                  | <input type="checkbox"/> |
| 3. I understand that my participation is voluntary and I may withdraw up until the time that my blood/urine/placental samples can still be identified.                                                                                                                                                                    | <input type="checkbox"/> |
| 4. I understand that the results of this study will not be added to my medical records                                                                                                                                                                                                                                    | <input type="checkbox"/> |
| 5. I understand that the relevant sections of my medical notes and data collected may be looked at by researchers from University of Liverpool, or regulatory authorities from the NHS Trust, where it is relevant to my taking part in this study. I give permission for these individuals to have access to my records. | <input type="checkbox"/> |
| 6. I agree to have blood samples taken for tests on genes and other factors that may be involved in determining how I respond to certain medications I take during pregnancy.                                                                                                                                             | <input type="checkbox"/> |
| 7. I agree to donate my placenta for to allow assessment of placental structure, markers of pre-eclampsia and fetal growth restriction.                                                                                                                                                                                   | <input type="checkbox"/> |
| 8. I understand that my samples will be stored anonymously, and it will not be possible to trace these samples back to me.                                                                                                                                                                                                | <input type="checkbox"/> |
| 9. I understand the samples will be gifted to University of Liverpool and used without my further consent.                                                                                                                                                                                                                | <input type="checkbox"/> |
| 10. I agree to take part in the study.                                                                                                                                                                                                                                                                                    | <input type="checkbox"/> |

.....  
Name of patient

.....  
Date

.....  
Signature

.....  
Name of researcher

.....  
Date

.....  
Signature

1 copy for the patient, 1 copy for the researcher, 1 copy for the notes

Version 5.0 01/12/14

Estimating Aspirin ResisTance in High-risk women (EARTH)

## PATIENT DRUG DIARY

Subject Number:

Chief Investigator: Dr Ana Alfirevic  
The Wolfson Centre for Personalised Medicine  
Department of Pharmacology  
University of Liverpool  
Block A: Waterhouse Buildings  
1-5 Brownlow Street  
Liverpool  
L69 3GL

Estimating Aspirin ResisTance in High-risk women (EARTH)

Version 1.1, 29<sup>th</sup> August 2014

Dear patient,

Thank you again for agreeing to take part in this research study.

Providing the requested information in your diary helps us gather important information about your medication and effectiveness of your treatment.

For purpose of this study, in between visits we need you to write the following information in your diary:

- The aspirin dose taken on each day
- Any new illnesses or symptoms
- Any other medications since the last visit

Instructions for completing the diary and examples are provided. Please call the study personnel if you have any questions about completing the diary.

**Please bring this diary with you to the next appointment you have for this study.**



## Appointments

	DATE	Start diary on:
<b>VISIT 1</b>		NA
<b>VISIT 2</b>		NA
<b>VISIT 3</b>		

## Description of study

This patient is involved in a research study called

### **Estimating Aspirin ResisTance in High-risk women (EARTH)**

The study is being coordinated by the University of Liverpool and aims to improve treatment decisions for patients prescribed aspirin therapy in pregnancy.

Pre-eclampsia is a serious multisystem disorder which occurs in the second half of pregnancy. The cause of pre-eclampsia is not fully understood, but it is thought to be related to the function of platelet cells in the blood, which aid clot formation. Aspirin acts on platelets to reduce the risk of pre-eclampsia in high-risk women when started at low doses in the first half of pregnancy. However, some women do not respond to aspirin adequately (aspirin non-responders) which could explain why some women still suffer from pre-eclampsia despite treatment with low dose aspirin.

An important part of the study is to accurately record aspirin intake and any other medications that may affect the levels of aspirin in blood.

## ASPIRIN DOSING

Please record for each day what dose of aspirin you have taken. Start your diary at least one week before your next appointment. If for any reason you have not taken your prescribed dose, please state briefly why you did not take it (e.g. dose omitted as prescribed, forgot, ill, on doctor's advice, ran out of tablets,...).

**Example:**

DAY	DATE	ASPIRIN DOSE	REASON FOR NOT TAKING ASPIRIN	Any other medication
1	Monday 30 <sup>th</sup> June	75 mg		
2	Tuesday 1 <sup>st</sup> July	--	Ran out of tablets	

DAY	DATE	ASPIRIN DOSE	REASON FOR NOT TAKING ASPIRIN	Any other medication
1				
2				
3				
4				
5				
6				
7				

Estimating Aspirin Resistance in High-risk women (EARTH)

Version 1.1, 29<sup>th</sup> August 2014

### CONTACT DETAILS

Principal Investigator: Dr Kate Navaratnam

Contact telephone number: 0151 795 9567

Contact email address: Kate.Navaratnam@liv.ac.uk

## Low dose aspirin and pregnancy: how important is aspirin resistance?

K Navaratnam,<sup>a</sup> A Alfirevic,<sup>b</sup> Z Alfirevic<sup>a</sup>

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Accepted 26 December 2015. Published Online 1 March 2016.

Antiplatelet agents are pivotal for prevention of coronary artery disease and cerebrovascular disease worldwide. Individual patient data meta-analysis indicates that low-dose aspirin causes a 10% risk reduction in pre-eclampsia for women at high individual risk. However, in the last 15 years it has emerged that a significant proportion of aspirin-treated individuals exhibit suboptimal platelet response, determined biochemically and clinically, termed 'aspirin non-responsiveness', 'aspirin resistance' and 'aspirin treatment failure'. More recently, investigation of aspirin

responsiveness has begun in pregnant women. This review explores the history and clinical relevance of 'aspirin resistance' applied to high-risk obstetric populations.

**Keywords** Activation, aspirin, platelet, pre-eclampsia, pregnancy, resistance.

**Tweetable abstract** Is 'aspirin resistance' clinically relevant in high-risk obstetrics?

Please cite this paper as: Navaratnam K, Alfirevic A, Alfirevic Z. Low dose aspirin and pregnancy: how important is aspirin resistance? BJOG 2016; DOI: 10.1111/1471-0528.13914.

### Introduction

Antiplatelet agents are pivotal in both primary and secondary prevention of coronary artery disease and cerebrovascular disease worldwide (Table 1). In high-risk pregnant women, low-dose aspirin (LDA) also confers a 10% risk reduction for pre-eclampsia and a 20% risk reduction for fetal growth restriction.<sup>8,9</sup> However, it has been postulated that a significant proportion of individuals exhibit suboptimal response to aspirin, defined biochemically as diminished suppression of platelet activation or clinically as development of thrombotic events while on treatment.<sup>10</sup> This has been referred to interchangeably, as 'aspirin non-responsiveness', 'aspirin resistance' and 'aspirin treatment failure'. Controversies remain regarding the definition, optimal means of identification, and management strategy in affected individuals.<sup>11</sup> Determination of compliance with aspirin is likely to be of central importance to discriminate non-compliance from true suboptimal response and begin to explore causal mechanisms including pharmacokinetic, pharmacodynamic and genetic factors. Recently, the concept of 'aspirin resistance' has been extended to high-risk obstetric populations where sus-

tained platelet activation despite LDA has been linked to subsequent pre-eclampsia and/or fetal growth restriction. This review explores the history and clinical relevance of 'aspirin resistance' and methods to assess platelet response to aspirin with particular focus on pregnancy.

### History of aspirin

Aspirin is one of the oldest medications still in widespread modern use. The first records of aspirin-related compounds, 'salix', derived from willow tree bark, were documented on papyrus scrolls used by Egyptian physicians in 1534 BC.<sup>12</sup> The translation of this knowledge into modern practice began in Oxford in 1758 when Reverend Edward Stone consumed, and later successfully trialed willow tree bark for relief of headaches, myalgia and fever.<sup>13</sup> In 1971 Vale, Samuelson and Bergstrom were awarded the Nobel Prize for elucidating the mechanism of action of aspirin, and clinical research investigating the antiplatelet effects of aspirin began.<sup>14,15</sup> Although aspirin was initially used as an analgesic and antipyretic, its antiplatelet effects mean it has become one of the most frequently prescribed medications worldwide, taken by more than 50 million people for prevention of cardiovascular disease alone, with approximately

**Table 1.** Clinical indications for aspirin therapy in medical and surgical specialities

Clinical area	Clinical situation	Dose	Guideline
Cardiovascular disease	Acute myocardial infarction	300 mg	NICE <sup>1</sup>
	Acute unstable angina	300 mg	
	Secondary prevention of myocardial infarction	75 mg	NICE <sup>2</sup>
	Atrial fibrillation, primary prevention of myocardial infarction	75 mg	
Cerebrovascular disease	Acute ischaemic stroke	300 mg	NICE <sup>3</sup>
	Transient ischaemic attack	300 mg	NICE <sup>4</sup>
	Secondary prevention of stroke/transient ischaemic attack	75 mg	
Peripheral arterial disease	Secondary prevention	75 mg	NICE <sup>4</sup>
High-risk Pregnancy	Antiphospholipid syndrome	75 mg from conception, combined with low-molecular-weight heparin	RCOG <sup>5</sup>
	High risk for pre-eclampsia	75 mg from 12 weeks	NICE <sup>6</sup>
	High risk for small-for-gestational-age infant	Consider 75 mg <16 weeks if at high risk for pre-eclampsia	RCOG <sup>7</sup>

40 000 tons administered annually. Aspirin's broad clinical effectiveness, cost-effectiveness and safety profile have led to its inclusion in the WHO Essential Medicines list for basic healthcare systems.<sup>16</sup>

Aspirin's most frequently reported adverse effect is gastric irritation. Enteric formulations do not appear to reduce this effect but they have been associated with diminished platelet effects and may reduce bioavailability.<sup>17</sup> A systematic review published in 2014 supports the safety of aspirin in pregnancy, particularly for women at high-risk of pre-eclampsia, finding no increase in maternal or neonatal bleeding complications.<sup>9</sup>

#### Mechanism of action of aspirin

Aspirin (acetylsalicylic acid) is a weak acid, with a white crystalline appearance in its solid state. Acetylsalicylic acid is produced by esterification of salicylic acid, catalysed by sulphuric acid, with acetic acid also generated as a by-product. Aspirin is administered orally and is readily absorbed in the upper gastrointestinal tract with most platelet effects occurring in the portal system.<sup>10</sup>

The principal pharmacological target of acetylsalicylic acid is the platelet enzyme cyclooxygenase (COX), responsible for producing prostaglandins which mediate pain and induce platelet activation. COX is a membrane-bound glycoprotein with three isoforms (COX-1, COX-2, COX-3). Acetylsalicylic acid selectively acetylates the serine residue (Ser529), altering the enzyme structure and preventing binding of arachidonic acid to the Tyr385 catalytic site. The resultant inhibition of prostaglandin H-synthase production leads to irreversible inhibition of COX-1 activity and, to a lesser degree, inhibition of COX-2 activity (Figure 1).<sup>18,19</sup> COX-1 inhibition by aspirin is rapid and sat-

urable at low doses. Peak plasma concentrations are reached within 40 minutes, and demonstrable platelet inhibition occurs within 1 hour of ingestion for non-enteric coated formulations.<sup>18</sup> In non-pregnant individuals, low-dose aspirin (0.45 mg/kg, 60–150 mg) administered once daily has been demonstrated to reduce serum thromboxane B<sub>2</sub> (TxB<sub>2</sub>, the stable serum metabolite of thromboxane A<sub>2</sub>) by a minimum of 95% within 5 days of commencing treatment.<sup>18–20</sup> It is important to note that thromboxane A<sub>2</sub> is also produced in smaller quantities via COX-independent pathways, and from non-platelet sources such as monocytes and macrophages (Figure 1). These pathways have considerable inter-connections.<sup>19</sup> Following acetylsalicylic acid exposure, COX-dependent generation of thromboxane A<sub>2</sub> (TxA<sub>2</sub>) and TxA<sub>2</sub>-induced platelet aggregation remain inhibited for the lifespan of the platelet. In the healthy non-pregnant state the average platelet lifespan is 8–9 days.

#### Platelet response to aspirin

The concept of suboptimal platelet response to aspirin, linked to recurrent cardiovascular and cerebrovascular ischaemia and cardiovascular deaths,<sup>21</sup> has become well-established in cardiovascular and stroke research over the last 20 years. Suboptimal platelet response is usually defined as a biochemical failure to inhibit platelet activation in aspirin-treated individuals, assessed in the laboratory or with point-of-care tests. Suboptimal response to aspirin has also been described clinically as recurrence of ischaemic events despite aspirin treatment at the recommended dose. However, there is currently no universally accepted definition which unifies laboratory and clinical findings. The reported prevalence ranges from 5 to 65%, varying with the assay used and the populations

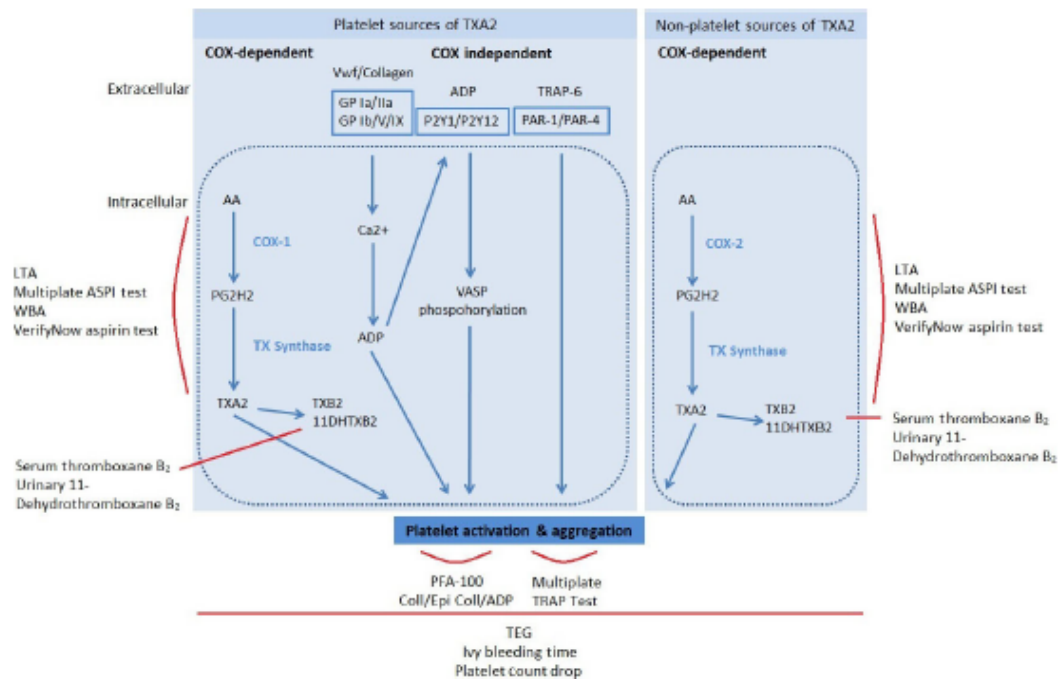


Figure 1. Platelet activation pathways and relevant assays.

studied.<sup>22–26</sup> Proposed underlying causes include drug interactions and states of enhanced platelet turnover, such as during the immediate postoperative period and in pregnancy. Genetic predisposition is also likely to be influential; however, non-compliance is widespread in cardiology populations represented in the literature and is likely to be a key determinant.<sup>27,28</sup>

### Tests of platelet response to aspirin

A range of platelet activation and function assays are available, but suffer from limited reproducibility and poor agreement between assays. With appropriate dosing and compliance, low-dose aspirin provides complete inhibition of the COX-1 pathway, the primary target of aspirin, in over 99% of individuals. Consequently, platelet function assays that target the aspirin COX pathways demonstrate lower inter-individual variability.

COX-specific assays include light transmission aggregometry (LTA) with arachidonic acid-induced platelet activation (Figure 1). The method is based on detection of light that passes through platelet-rich plasma containing aggregated platelets. VerifyNow utilises the theory of LTA but allows assessment using whole blood within a closed point-of-care

system. Additionally, the stable serum and urinary metabolites of TxA<sub>2</sub>, TxB<sub>2</sub> and 11-dehydrothromboxane B<sub>2</sub> (11-DTxB<sub>2</sub>), respectively, can be quantified (Figure 1).

In contrast, if platelet function assays rely on adenosine diphosphate (ADP), collagen or epinephrine for platelet activation, the assays are considered to assess non-COX-dependent pathways (Figure 1). Non-COX-specific assays include the PFA-100 System, a point-of-care system using citrated whole blood. It measures the time taken for platelet aggregation to occlude a micro-aperture within collagen/epinephrine or collagen/ADP-coated cartridges. Thromboelastography (TEG) measures speed and strength of clot formation as an indirect assessment of the contribution of platelet aggregation to stable clot formation. Bleeding time measurements provide an *in vivo* assessment of time taken for blood to clot in a superficial linear forearm wound. Platelet counts approximate the impact of platelet activation, aggregation, sequestration and destruction on total circulating platelets. These assays demonstrate a high degree of inter-individual variability; however, due to the multiple pathways involved in platelet activation, non-COX-specific assays may better reflect the global platelet response and provide information about *in vivo* milieu.

### Literature review

We set out to identify all biochemical tests and definitions used to define suboptimal platelet response to aspirin across all clinical applications. We searched MEDLINE, EMBASE and the Cochrane Library from 1957 to 26 February 2015, limited to humans and English language. The search terms used were 'aspirin', 'acetylsalicylic acid' appearing adjacent to 'resistance', 'non-responsiveness', 'treatment failure' and 'pseudoresistance'. All original articles were included; review articles were excluded. Additionally, we excluded articles relating to studies where participants received concomitant alternative antiplatelet agents or anticoagulants. This search yielded 492 articles after abstract and full text reviews (135) were included.

### Tests and definitions

There is now a broad range of experience with platelet activation and function testing across speciality areas: healthy individuals, cardiology, stroke, endocrinology, nephrology, rheumatology, general medicine, general surgery, vascular surgery, paediatrics and high-risk obstetrics (Supporting Information Table S1). Our review identified 13 platelet activation assays; the three most commonly used were PFA 100 collagen/epinephrine cartridges, LTA with arachidonic acid induction and PFA-100 collagen/ADP cartridges (Figure 2, expanded information in Table S1). In the examined literature, PFA-100 collagen/epinephrine cartridges were associated with 33 different cut-offs, the commonest being <165 seconds (Table S1). LTA had 19 proposed cut-offs, the commonest being a mean aggregation of  $\geq 20\%$ . PFA collagen/epinephrine cartridges had 12 different cut-offs, the commonest being <114 seconds (Table S1). The majority of studies refer to manufacturer's cut-offs, or have conducted in-house work to establish cut-offs from small numbers of predominantly male volunteers. Subsequently, there is limited applicability of these cut-offs to obstetric populations.

In total, 88 different definitions of suboptimal platelet response to aspirin have been described, the vast majority utilising only laboratory-based parameters to delineate responsiveness to aspirin. One study defined suboptimal response clinically as the occurrence of myocardial infarction in patients with stable coronary artery disease while on aspirin therapy.<sup>29</sup> Another described a combined definition with laboratory assessment of 11-DTxB2 and ischaemic cardiovascular or cerebrovascular events.<sup>26</sup> We identified 24 studies where suboptimal response to aspirin was linked to clinical outcomes: 14 in cardiology, 6 in stroke patients, 1 in nephrology and 3 in obstetrics (Supporting Information Table S2).

Obstetric studies were carried out in pregnant women at high risk of pre-eclampsia, including two prospective cohort studies, one case-control study and a dose escalation study from groups in the UK, Canada and Poland (Table S2).<sup>30–33</sup> Two studies used PFA-100 collagen/epinephrine cartridges, with diagnostic cut-offs determined from the mixed adult population.<sup>31,32</sup> One study utilised 11-DTxB2 with locally determined pregnancy-specific cut-offs, and one study used the LTA method. In these high-risk obstetric populations, suboptimal platelet response to aspirin was identified in 29–39% of participants, and was associated with increased pre-eclampsia, preterm birth and delivery of small-for-gestational-age infants.<sup>31,32</sup> Additionally, Rey et al.<sup>31</sup> assessed the impact of PFA-100 guided aspirin dose escalation and determined that women requiring escalation had a higher risk of pre-eclampsia (11/43, 25.6% versus 6/68, 8.8%;  $P = 0.03$ ).

### Non-compliance testing

To assess the clinical impact of suboptimal platelet response to aspirin, it is vital that true non-responsiveness can be distinguished from non-compliance. In cardiovascular research, patient non-compliance with medication is

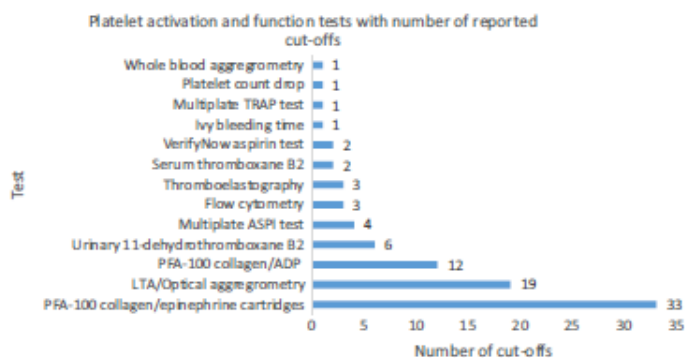


Figure 2. Platelet activation and function tests with number of reported cut-offs.

reported to be widespread at 30–50%.<sup>10</sup> The existing literature demonstrates that there is no consensus on robust assessment of compliance (Figure 3, expanded information in Supporting Information Table S3). Where researchers have endeavoured to quantify the impact of non-compliance, most have relied on qualitative measures including patient enquiries, questionnaires and drug diaries, which are likely to underestimate the true scale. In our review, 5/87 (6%) relied on assessment of serum or urinary thromboxane, both considered to be measures of platelet activation (Table S3). However, elevated  $\text{TxB}_2$  and 11-D $\text{TxB}_2$  may be secondary to non-platelet sources and therefore not a specific reflection of platelet COX inhibition by aspirin. Importantly, only 2/87 (2%) of studies measured salicylate levels, both in patients taking aspirin for cardiovascular disease prevention (Table S3). Three of four obstetric studies considered the issue of compliance and undertook verbal enquiries at study visits.<sup>31–33</sup> Biochemical assessment of aspirin metabolites has not been assessed in pregnant populations.

### Causal mechanisms for suboptimal response to aspirin with adequate compliance

Where compliance is confirmed, the remainder of individuals with persistent suboptimal suppression of platelet activation are likely to represent a heterogeneous group with interacting physiological, pharmacokinetic and pharmacodynamic factors.

In healthy adults, 10% of circulating platelet numbers are replaced with aspirin-naïve platelets daily. It has been accepted that acetylsalicylic acid irreversibly inhibits COX-1 in exposed platelets and with regular dosing, adequate global inhibition of platelet COX activity is maintained. Interestingly, recent work has demonstrated mRNA sequence coding for COX-1 within platelets, raising a possibility that genera-

tion of new COX-1, following inhibition of the native enzyme, may circumvent the antiplatelet action of aspirin.

Patients may be insufficiently treated with aspirin for a variety of reasons, including problems with total dose, inappropriate dosing intervals or dose delivery issues. Enteric coated aspirin in particular has been implicated in suboptimal suppression of platelet activation, as demonstrated by laboratory tests.<sup>17</sup> It is now clear that low-dose aspirin is as effective as high-dose aspirin for secondary prevention of ischaemic cardiovascular and cerebrovascular events.<sup>34</sup> However, there remain limitations in dose selection due to the range of low doses currently manufactured (60–150 mg). The standard low (75 mg) daily dose recommended by NICE is more than double the dose required to maintain complete COX-1 inhibition in non-pregnant individuals, although there has been no assessment of platelet effects of this dose in pregnancy.<sup>35</sup>

Once-daily dosing has become standard practice, as the effects of aspirin are dependent on the rate of platelet turnover as opposed to the half-life,<sup>36</sup> but incomplete maintenance of COX-1 suppression with once-daily doses has been described in association with increased platelet turnover in diabetes, obesity and postoperative states.<sup>37,38</sup>

Daily low doses of aspirin irreversibly inhibit COX-1 activity in platelets and impact COX-1 in megakaryocyte precursors, an effect which diminishes with accelerated platelet maturation.<sup>36</sup> This is of particular relevance for obstetric populations where platelets are continually sequestered by the placenta, balanced by an increase in platelet production and release from bone marrow, resulting in an increase in circulating immature platelets, which are pre-disposed to aggregate at lower levels of stimuli. Additionally, endothelial dysfunction in pregnancies affected by pre-eclampsia leads to pathologically increased platelet activation and destruction, which may outstrip the ability of the bone marrow to compensate, leading to thrombocytopenia.

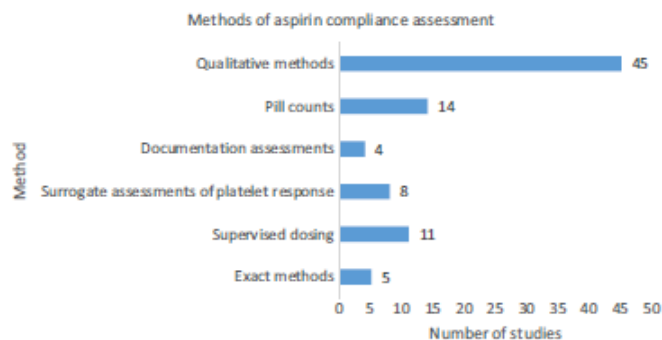


Figure 3. Methods of aspirin compliance assessment.

Circadian timing of aspirin doses may also be of significance. Recently, evening dosing has been associated with beneficial effects on ambulatory blood pressure and decreased hazard ratios of a composite of serious adverse events in high-risk pregnant populations.<sup>39,40</sup>

### Pharmacogenetics

Inter-individual variability in response to aspirin is affected by clinical, environmental and genetic factors, the latter of which have been targeted for investigation in recent years. Initial attempts to identify genetic determinants of suboptimal platelet response to aspirin in non-obstetric populations were afflicted by poor reproducibility in underpowered candidate gene studies. However, recent large studies have combined candidate gene and genome-wide association study (GWAS) approaches and have described important genetic determinants that map with platelet function.<sup>41</sup> These include loci associated with native platelet function in the following genes; PEAR1, SHH, MRVII, ADRA2A and GP6. Additionally, gene expression analysis has been used to identify >60 genes described as the 'aspirin response signature'.<sup>42</sup> The experience and recent developments in the pharmacogenetics of aspirin provide a promising start for GWAS in high-risk obstetrics.

### Implications for research in obstetrics

It is vital that true suboptimal response to aspirin is distinguished from aspirin non-compliance. We firmly believe that compliance testing should be based on detection and quantification of aspirin metabolites from maternal blood or urine.

A suboptimal response to aspirin in compliant patients does not currently have a uniform definition, nor has an adequately sensitive, specific diagnostic test emerged and this should be prioritised. Tests aligned to the COX pathway are likely to be of greatest clinical utility, particularly if point-of-care use is feasible.

Platelet activation should be measured against pregnancy-specific reference ranges, and if suboptimal response is identified, its clinical significance must be judged in light of clinically important outcomes.

Accurate and user-friendly stratification of pregnant women according to response to aspirin and compliance should encourage interventional studies of new treatments with alternative targets within platelet activation pathways or the coagulation cascade.<sup>8</sup> For genuine non-responders to standard LDA, randomised comparisons with aspirin dose escalation and low-molecular-weight heparin should be carried out. The outcomes of interest should not be restricted to platelet response to aspirin, but also include

placentally mediated adverse outcomes (pre-eclampsia, IUGR, placental abruption and stillbirths). Heterogeneity in response to aspirin due to genetic factors has not yet been investigated in pregnancy and may provide a unique, early and discriminative means of risk stratification.

The pathophysiology of pre-eclampsia remains incompletely elucidated and increased understanding of its evolution is likely to be key in guiding screening and interventional approaches. Further investigation of platelet response to LDA in high-risk pregnant women may provide fresh insight into the relatively modest risk reduction in placentally mediated disease observed with current therapy. If women with suboptimal response to aspirin experience more adverse clinical events, this may prove not only an important research area for preventative therapy but a valuable opportunity to stratify maternal and fetal surveillance in the near future. With current debates regarding the potential utility and cost-effectiveness of no test/treat all approaches to pre-eclampsia prevention, the acceptability of such approaches to women and compliance with aspirin will be key issues.<sup>43,44</sup> Additionally, recent signals of increased risk of placental abruption will need to be rigorously assessed.<sup>9</sup>

### Disclosure of interests

None declared. Completed disclosure of interests form available to view online as supporting information.

### Contribution to authorship

ZA conceived the idea. KN performed the literature searches and reviewed the papers. KN prepared the manuscript with the advice of AA. AA and ZA reviewed the final manuscript.

### Details of ethics approval

Ethical approval was not required for this review.

### Funding

Research Training Fellowship for Dr Kate Navaratnam provided by Wellbeing of Women.

### Acknowledgements

We would like to acknowledge the support of Wellbeing of Women.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Platelet activation and function tests.

**Table S2.** Clinical evaluation of aspirin resistance.

**Table S3.** Methods of aspirin compliance assessment. ■



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## GWAS Quality Control Protocol

### Sample QC

Add sex information to original fam file (i.e. 2 for females) to all samples in fam file (open up in excel, keep FID, IID and add a column of 2 for all samples). Save at text file, then use following command to create an updated .fam file:

```
plink --noweb --bfile aspirin --update-sex aspirin_gender.txt --make-bed --out aspirin_gender
```

#### 1) Removal of duplicated participant

```
plink --noweb --bfile aspirin_gender --make-bed --out aspirin_198 --remove sample342.txt
```

#### 2) Gender assessment

```
plink --noweb --bfile aspirin_198 --check-sex --out aspirin_198_sex_check
```

Produces .sexcheck file.

```
grep PROBLEM aspirin_198_sex_check.sexcheck > fail_sex_check_aspirin.txt
```

Produces a text file of samples that have failed the gender check.

#### 3) Genotyping call rate

```
plink --noweb --bfile aspirin_198 --missing --out aspirin_missing
```

Produces .imiss and .lmiss files.

#### 4) Removal of non-autosomes

Generate a list of non-autosomal SNPs by filtering the original .bim file for chromosomes 23, 24, 25 and 26. Produce a text file of these SNPs (nonautosomes.txt), to be removed from the data-set.

```
plink --noweb --bfile aspirin_198 --exclude nonautosomes.txt --make-bed --out aspirin_198_ex
```

#### 5) Heterozygosity assessment

```
plink --noweb --bfile aspirin_198_ex --het --out aspirin_198_het
```

Use the R script to produce imiss vs. het plot, record in a text file for removal.

#### 6) Cryptic relatedness assessment

```
plink --noweb --bfile aspirin_198_ex --indep-pairwise 50 5 0.2 --out aspirin_198_ex_thin
```

```
plink --noweb --bfile aspirin_198_ex --extract aspirin_198_ex_thin.prune.in --genome --out aspirin_198_ex_thin.genome
```

Use the R script to produce an IBD histogram. To output and view a file with outliers use:  
awk '\$10 >=0.1875 (Duley et al.)' aspirin\_198\_ex\_thin.genome.genome >  
aspirin\_ibd\_outliers  
(check F\_MISS values in the .imiss file to decide which sample in each pair to remove).

### Merging with HapMap3

Open aspirin\_198\_ex bim file. Keep all rs IDs, (delete top two rows and other columns). Create text file, snplist\_aspirin.txt. Use the HapMap3 .bed, .bim and .fam files to run the commands in this section.

```
plink --noweb --bfile Hapmap3 --extract snplist_aspirin.txt --make-bed --out Hapmap3_aspirin
```

Open Hapmap3\_aspirin bim file in Excel and remove all columns but keep the rs IDs, save as a text file, snplist\_Hapmap3.txt.

```
plink --noweb --bfile aspirin_198_ex --extract snplist_Hapmap3.txt --make-bed --out aspirin_Hapmap3
```

```
plink --noweb --bfile Hapmap3_aspirin --bmerge aspirin_Hapmap3.bed aspirin_Hapmap3.bim aspirin_Hapmap3.fam --make-bed --out Hapmap3_aspirin_merged1
```

If a missnp file has been outputted this indicates strands need flipping. Therefore use:

```
plink --noweb --bfile Hapmap3_aspirin --flip Hapmap3_aspirin_merged1.missnp --make-bed --out Hapmap3_aspirin_flipped
```

If this works, then continue with merging the datasets.

```
plink --noweb --bfile Hapmap3_aspirin_flipped --bmerge aspirin_Hapmap3.bed aspirin_Hapmap3.bim aspirin_Hapmap3.fam --make-bed --out Hapmap3_aspirin_merged2
```

If that still does not work, exclude the missnp file from the previous flip attempt and use:

```
plink --noweb --bfile Hapmap3_aspirin_flipped --exclude Hapmap3_aspirin_merged2.missnp --make-bed --out Hapmap3_aspirin_flipped_excl  
(Note: after this step the .missnp file should be excluded from the aspirin_Hapmap3 file).
```

```
plink --noweb --bfile aspirin_Hapmap3 --exclude Hapmap3_aspirin_merged2.missnp --make-bed --out aspirin_Hapmap3_excl
```

Use --bmerge to merge this file with Hapmap3\_aspirin\_flipped\_excl.

```
plink --noweb --bfile Hapmap3_aspirin_flipped_excl --bmerge aspirin_Hapmap3.bed aspirin_Hapmap3.bim aspirin_Hapmap3.fam --make-bed --out Hapmap3_aspirin_merged_final
```

To ensure the same number of SNPs in Hapmap3\_aspirin\_flipped and Hapmap3\_aspirin\_merged\_final:

```
plink --noweb --bfile Hapmap3_aspirin_merged_final --exclude  
Hapmap3_aspirin_merged2.missnp --make-bed --out  
Hapmap3_aspirin_merged_final_excl
```

## 7) Ethnicity assessment

```
plink --noweb --bfile Hapmap3_aspirin_merged_final_excl --indep-pairwise 50 5 0.2 --out  
Hapmap3_aspirin_merged_thin_excl
```

```
plink --noweb --bfile Hapmap3_aspirin_merged_final_excl --extract  
Hapmap3_aspirin_merged_thin.prune.in --genome --out  
Hapmap3_aspirin_merged_thin_MDS_excl
```

```
plink --noweb --bfile Hapmap3_aspirin_merged_final_excl --cluster --mds-plot 10 --out  
Hapmap3_aspirin_merged_MDS10_excl --read-genome  
Hapmap3_aspirin_merged_thin_MDS_excl.genome
```

Process output .mds file:

- Open the MDS10.mds file in excel to add ethnicities for patient samples. Use file Hapmap3\_populationID\_502\_CEU\_CHB\_JPT\_YRI.txt for Hapmap3 ethnicity information.
- Sort file by IID column, Z to A.
- Excel file should now contain: FID, IID, Population, Data label, Sol, C1, C2 to C10
- Population column:
  - order the Hapmap3 ethnicities in the same order (IID column, Z to A) as the output file
  - copy and paste the ethnicities into the population column from Hapmap3
  - EARTH study samples are left blank

Produce a PCA plot in SPSS:

File > open > data (select file). Chart builder > scatterplot > drag on: y-axis (C1), x-axis (C2), colours (population) > point labels > select the column called data labels

Identify outliers, and write to a text file or spreadsheet (ethnicity outliers.xlsx).

## 8) Removal of samples outliers

Create a text file, sampleexclusionssexIBDethnicity.txt, containing all previously identified outliers. Exclude the outliers from the original .fam file

```
plink --noweb --bfile aspirin_198 --make-bed --out aspirin_sampleqc --remove  
sampleexclusionssexIBDethnicity.txt
```

## 9) SNP QC

```
plink --noweb --bfile aspirin_sampleqc --pheno asp_pheno.txt --pheno-name asp_resp --  
geno 0.05 --hwe 0.000001 --maf 0.001 --make-bed --out aspirin_finalqcpheno
```

## GWAS Statistical analysis protocol

### Prior to analysis

Open the aspirin\_finalqcpheno.fam:

- Delete column 3 to 6
- Add column headers: FID, IID, C1, C2, BMI
- Add in phenotype information (-9 for exclusion or missing values), write BMI values for included samples.
- Save as text file asp\_covar.txt

Open aspirin\_finalqcpheno.fam:

- Delete column 3 to 6
- Add column headers: FID, IID, asp\_resp
- Add in phenotype information (-9 for exclusion or missing values)
- Save as text file asp\_pheno.txt

### Results analysis

```
plink --noweb --bfile aspirin_finalqcpheno --logistic --pheno asp_pheno.txt --pheno-name  
asp_resp --covar asp_covar.txt --covar-name C1,C2,BMI --out aspirinresponse_bmipheno -  
-hide-covar
```

Produces a .assoc.logistic file.

```
plink --noweb --bfile aspirin_finalqcpheno --logistic --pheno asp_pheno.txt --pheno-name  
asp_resp --covar asp_covar.txt --covar-name C1,C2 --out aspirinresponsepheno --hide-  
covar
```

Produces a .assoc.logistic file.